Ethephon-induced shoot production in two cultivars of gentian cultured *in vitro*: Anatomical, morphological and physiological aspects associated with endogenous balance of auxin and cytokinin

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Dedication

This thesis is dedicated to my parents for being strong and supportive and to my love Mohsen.

Abstract

For gentian as an export-orientated ornamental crop in NZ, the research within this thesis was motivated by the industry goal of producing highly branched propagules, in order to make high quality pot plants. As part of the *in vitro* propagation system, application of ethephon as a plant growth regulator (PGR), increased the formation of shoots from nodal explants of the cultivar 'Little Pinkie' but not in 'Showtime Diva'. While only two shoots were expected to arise from a metamer of gentian, a maximum of 20 shoots developed from explants of 'Little Pinkie', at 40 $mg.L^{-1}$ ethephon. Therefore, the research undertaken within this thesis focussed on investigating the mechanism by which shoot formation in explants of gentian was influenced. Main objectives were to investigate the origin of high shoot numbers, the effect of ethephon on shoot number and the underlying mechanisms behind the ethephon's effect. As a results of these studies it was proposed that a possible underlying mechanism was the effect of ethephon on auxin/cytokinin balance.

Both light and scanning electron microscopy were utilised to identify the type of buds that shoots originated from in both cultivars of gentian. Shoots arising at the node of 'Little Pinkie' typically had a combination of axillary, co-lateral and adventitious origins. Number of adventitious shoots increased in the presence of ethephon as well as with PAT inhibitor 1-n-Naphthylphthalamic acid (NPA) or PGR (cytokinin). Therefore, it was suggested that morphological mechanism for stimulation of shoot formation was similar for all types of buds. However, 'Showtime Diva' did not produce multiple shoots beyond those derived from pre-existing axillary buds, irrespective of the presence or absence of ethephon as a stimulator for shoot formation.

A range of experiments using laser ethylene detector ETD-300 carried out to quantify ethylene concentration in the headspace of culture vessels released from ethephon in the medium. Ethylene was supplied either as gaseous ethylene, at a concentration of 60 $nL.L^{-1}$ or released from ethephon incorporated into the media, but only direct absorption of ethephon from the medium was effective to increase shoot formation.

The unique characteristic of meristematic activity at nodes of 'Little Pinkie', was likely derived from cells differing in their sensitivity to the endogenous balance of phytohormones and, in particular, a low ratio of auxin: cytokinin. Therefore, low ratio of auxin: cytokinin was the hypothesized mechanism for increased shoot formation following the application of ethephon, NPA or cytokinin. Increased shoot formation in 'Little Pinkie' as a result of either

ethephon or NPA applied through the culture medium, provided evidence to support the hypothesised inhibitory effect of ethephon on polar auxin transport (PAT). However, while basipetal transport of radiolabelled auxin (^{14}C-IAA) was inhibited by NPA, ethephon unexpectedly did not affect it. Rather than the direct inhibition on PAT, ethylene is suggested to have reduced auxin synthesis and/or changed its metabolism. In doing so, the reduction in endogenous concentration of auxin by ethephon or NPA, as well as any increase in endogenous content of cytokinin, could be responsible for the hypothesised reduction in the ratio of auxin: cytokinin and increased shoot formation.

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Last, but by no means least, thanks to my parents for raising me, loving me unconditionally and supporting me. I may not have had the best of toys or gone to the best of schools but none of that matters. Because I had the best parents in the world and sweetest brothers and sisters that I love so much. Mom and dad, you don't know English so, I write some words here for you in our Persian language, the beautiful language that you first taught me to express my feelings with:

تقدیم به کسانی که مرا با عشق به این دنیا آوردند،

از خواسته‌های خود گذشتند تا من به خواسته‌هایم برسم،

برایم با عشق کتاب و دفتر میخریدند، تا بیاموزم آنچه را که فکر میکردند خود نمیدانند.

پدر و مادر عزیزم، پس از این همه سال، و حالا که برای آموختن بیشتر به این سر دنیا آمده ام،

اعتراف می‌کنم هنوز نه به اندازه مادر صبر آموخته ام، و نه به اندازه پدر مقاومت در برابر مشکلات.

با این وجود، رویا پردازی را از تو پدر و پشتکار را از تو مادرم در خود دارم و نهایت تلاشم را می‌کنم تا مایه افتخار و سر بلندیتان باشم.

به خاطر هر آنچه در زندگی برایم انجام داده‌اید مقابلتان سر تعظیم فرود می‌آورم.

دوستتان دارم و

قلبم همیشه با شماست...

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List of Symbols and Abbreviations

ACC	1-Aminocyclopropane-1-carboxylic acid
$AgNO_3$	Silver nitrate
AVG	Aminoethoxyvinylglycine
BA	N6-benzyladenine
BAP	2-hydroxybenxylamino purine
Bp	Bud primordia
C	Cortex
$CoCl_2$	Cobalt chloride
CPAS	3-cyclopropyl-1-enyl-propanoic acid sodium
CRD	Completely Randomised Design
C_{Tm}	Concentration of ethylene at the time of measurement
Dpm	disintegrations per minute
ETD-300	ethylene detector
Ethephon	[2-chloroethyl] phosphoric acid
FW	Fresh weight
GA3	Gibberellic acid
$g.L^{-1}$	gram per litre
GC	Gas chromatography
HPLC	High pressure liquid chromatography
RP-HPLC	Reverse phase high pressure liquid chromatography
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
kPa	Kilopascal

$L.h^{-1}$	Liter per hour
LS	Linsmaier and Skoog vitamins
LSD	Least significant difference
$mg.L^{-1}$	milligram per litre
mL	Millilitre
Mm	Millimetre
mM	Millimolar
µM	Micromolar
MS	Murashige Skoog salts
NAA	1-naphthaleneacetic acid
$nL.h^{-1}$	nanoliter per hour
$nL.L^{-1}$	nanoliter per liter
NPA	1-n-Naphthylphthalamic acid
NZ	New Zealand
ODS	Octadecyl silica
P	Pith
Ph	Phloem
PAT	Polar auxin transport
PATI	Polar auxin transport inhibitors
PBZ	Paclobutrazol
PFR	The NZ Institute for Plant & Food Research Ltd.
PGRs	Plant growth regulators
Ppb	part per billion
PPFD	Photosynthetic photon flux density

Pptv	part per trillion volume
Ps	Primary stem
REML	Restricted Maximum Likelihood
R_L	Rate of leakage
R_{MR}	rate of release from medium
RP-HPLC	Reverse phase high pressure liquid chromatography
SAM	S-adenosylmethionine
SEM	Scanning electron microscopy
SLs	Strigolactones
Ss	Secondary shoots
Ssvc	Secondary shoot vascular cylinder
STS	Silver thiosulfate
TDZ	Thidiazuron
TEA	trimethylamine
TIBA	2,3,5 triiodobenzoic acid
Vc	Vascular cylinder
$\mu mol.m^2.s^{-1}$	Micro molar per square meter per second
°C	Degree centigrade
^{14}C-IAA	2-^{14}C-indole-3-acetic acid;
1-MCP	1-methylcyclopropene
1º	Primary
2º	Secondary
3º	Tertiary
2,4-D	2,4-dichlorophenoxyacetic acid

List of publications

- Keshavarzi, M., K. Funnell, D. Woolley, and J. Heyes. 2015a. Explant position influences in vitro flowering of 'Little Pinkie' gentian, p. 111-118. In: III International Conference on Quality Management in Supply Chains of Ornamentals 1131.
- Keshavarzi, M., K.A. Funnell, J.A. Heyes, and W. D.J. 2015b. Gentian 'Little Pinkie' and the production of explants as influenced by ethephon and growth duration *in vitro*. 6th International Symposium on Production and Establishment of Micropropagated Plants, San remo, April 19-24, 2015. Submitted to: Acta Horticulturae.
- Keshavarzi, M., K.A. Funnell, J.A. Heyes, and D.J. Woolley. 2014a. Ethephon and secondary shoot induction in Gentian (*Gentiana* spp.) hybrids *in vitro*. Sci. Hortic. 179:170-173.
- Keshavarzi, M., D. Woolley, J. Heyes, and K. Funnell. 2014b. Sources of morphological non-uniformity in gentian propagated *in vitro* without plant growth regulators, p. 157-164. In: XXIX International Horticultural Congress on Horticulture: Sustaining Lives, Livelihoods and Landscapes (IHC2014): 1113.

Chapter 1. General introduction and background literature

1.1. Introduction

1.1.1. The crop and market opportunity

Gentians (primarily *Gentiana triflora*, *G. scabra*, and hybrids of the two) are used as a new export-orientated ornamental crop in New Zealand (NZ). Historically gentians have been used as medicinal plants (Bartlett, 1975) and, in Japan, are among the most important plants for the cut flower and potted plant industries. Hybrids of both *G. triflora* and *G. scabra* species have been cultivated commercially in Japan for over 50 years, and are developing in NZ's export flower market (Doi et al., 2011; Takahashi et al., 2012). The great distance of NZ from markets and high transportation costs has driven future investment toward the production/sale of high value plant propagules as a strategy to support growth of this industry (Funnell et al., 2013). The value of exports of live plants has increased 124% during a recent twenty year period, whereas for cut flowers it was approximately 84% (Statistics New Zealand and Plant and Food Research, 2013). Hence, the NZ Institute for Plant & Food Research Ltd (PFR) has for example invested in developing gentians for the potted plant market, and the research within this thesis has been planned as part of the larger research programme focussed on propagules which make high quality pot plants.

Within the context of the current thesis, the quality of a pot plant is assessed as being "high" by displaying multiple shoots, carrying multiple buds or flowers, which are uniform in flowering time and high yielding (i.e. high proportion of crop flowering simultaneously) at the time of selling in the market. While varying in different markets and plant species, a high quality flowering pot plant typically has an overall height of the plant plus container no more than 1.6 times the plant diameter measured at the top, with a good display of flowers on multiple shoots which usually covers the diameter of the plant at the top (Figure 1-1) (Sachs et al., 1976). Several gentian cultivars suitable for use as potted plants have been developed in Japan, generally requiring the use of growth retardants (Mishiba et al., 2006) for control of plant form. In NZ, 'Little Pinkie' was bred and recently introduced within European and North American markets as a cultivar with characteristics suitable for potted plants (Morgan, 2015). As discussed in detail below 'Little Pinkie' was selected as the primary focus of this research because of its enhanced shoot formation when exposed to ethephon, a response therefore that was of interest.

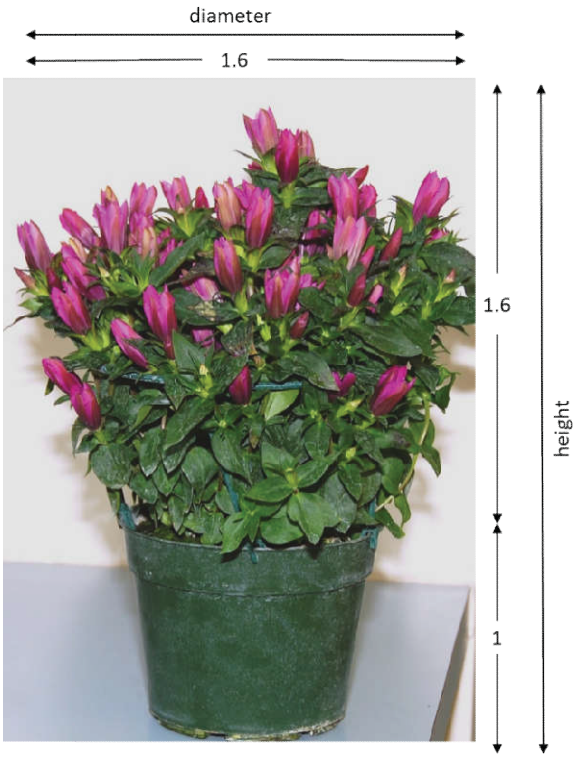


Figure 1-1: A high quality pot plant of gentian with overall height of the plant plus container (here 2.6) no more than 1.6 times the plant diameter measured at the top, with a good display of flowers on multiple shoots (Sachs et al., 1976). The plant illustrated here is the gentian cultivar 'Little Pinkie'.

In order to develop new genotypes of gentian for the pot market, technical research challenges include breeding, propagation, production techniques and post-harvest physiology which is a long process. This long process sometimes results in a genotype such as 'Showtime Diva' that has most of the desirable characteristics such as favourable colour of flowers, tolerance to disease, etc. but with low number of shoots. Hence, as explored within the current thesis, developing an understanding of the underlying physiology that contributes to shoot formation was considered important.

As a part of the over-arching research programme on developing cultivars for the pot plant market, there was also a need to develop a propagation system which ideally could generate, a high number of propagules with multiple shoots which produce high quality pot plants (Figure 1-2). *In vitro* methods of propagation of gentian in the tissue culture lab of PFR, utilizing various plant growth regulators (PGRs), have provided a system to raise a uniform population of true-to-type propagules of gentian (Morgan et al., 1997). However, changing and improving protocols in order to optimise the plant characteristics demanded (i.e. good display with multiple buds or flowers, uniform in flowering time and high yielding) was required. As part of this broad subject area, the research undertaken within this thesis

focussed on investigating the effect of PGRs on the shoot formation of gentian plants; in particular ‘Little Pinkie’.

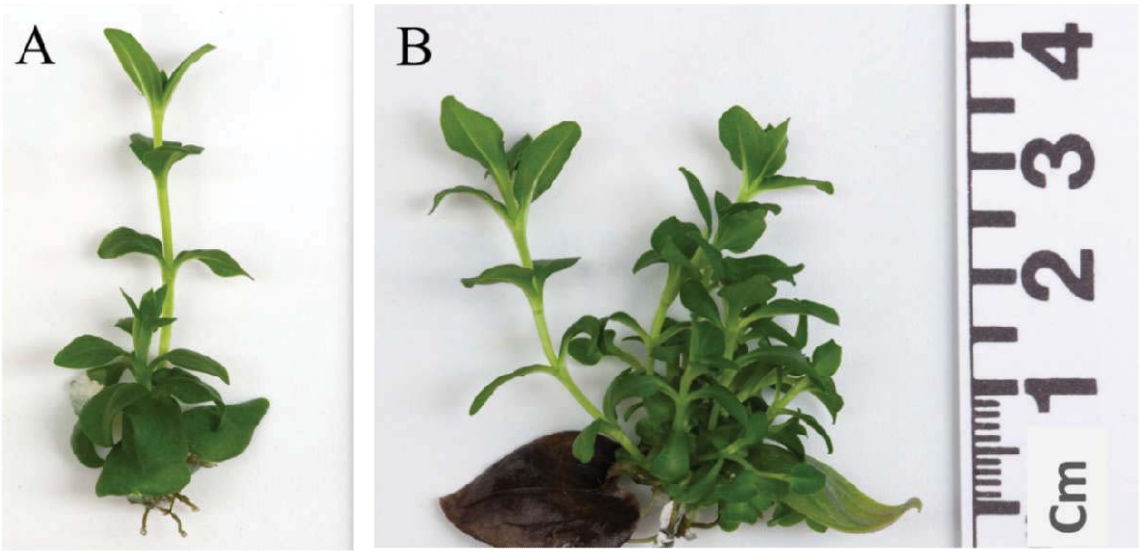


Figure 1-2: Plantlets of gentian ‘Little Pinkie’ with **(A)** low number of shoots and **(B)** high number of shoots developed *in vitro* to be used as propagules for pot plant production. As a result of increased shoot formation the plantlet depicted in (B) is considered of higher quality to be grown as a potted plant.

1.1.2. Taxonomy, botany, morphology and propagation

In the family Gentianaceae, the genus *Gentiana* L. (Gentian) has been reported to be composed of over 360 species of perennial or annual herbs (Struwe, 2002). If including *Gentianella* (Glenny, 2003), gentians are endemic to Europe, North and South America, northwest Africa, Australia, and NZ (Struwe, 2002). The mountains of south-western China and north-eastern Myanmar contain the highest concentration of species (Ho, 2001). In NZ the Gentianaceae is represented by at least 24 species of *Gentianella* found on both the main islands, in addition to the five species that are restricted to the Chatham or Sub-Antarctic Islands (Allan, 1961).

The above-ground stems (i.e. shoots) of gentian plants (focussing here on *Gentiana triflora*, G. *scabra*, and hybrids of the two) are quadrangular shaped and are typically with high number of shoots (Ho, 2001; Struwe, 2002). Along the shoots leaves are positioned opposite in a metameric structure which consists of two phytomers (each phytomer comprise an attached single-leaf, axillary bud, and node, together with the internode just below it; Figure 1-3B). Hence, each metamer includes two nodes and a pair of leaves on the primary shoot which, for application within this thesis, was referred to as the node position (White, 1979;

Funnell et al., 2015). Within this thesis each metamer was potentially utilised as an explant to produce rooted plantlets after being previously sub-cultured *in vitro* for 4 to 5 weeks.

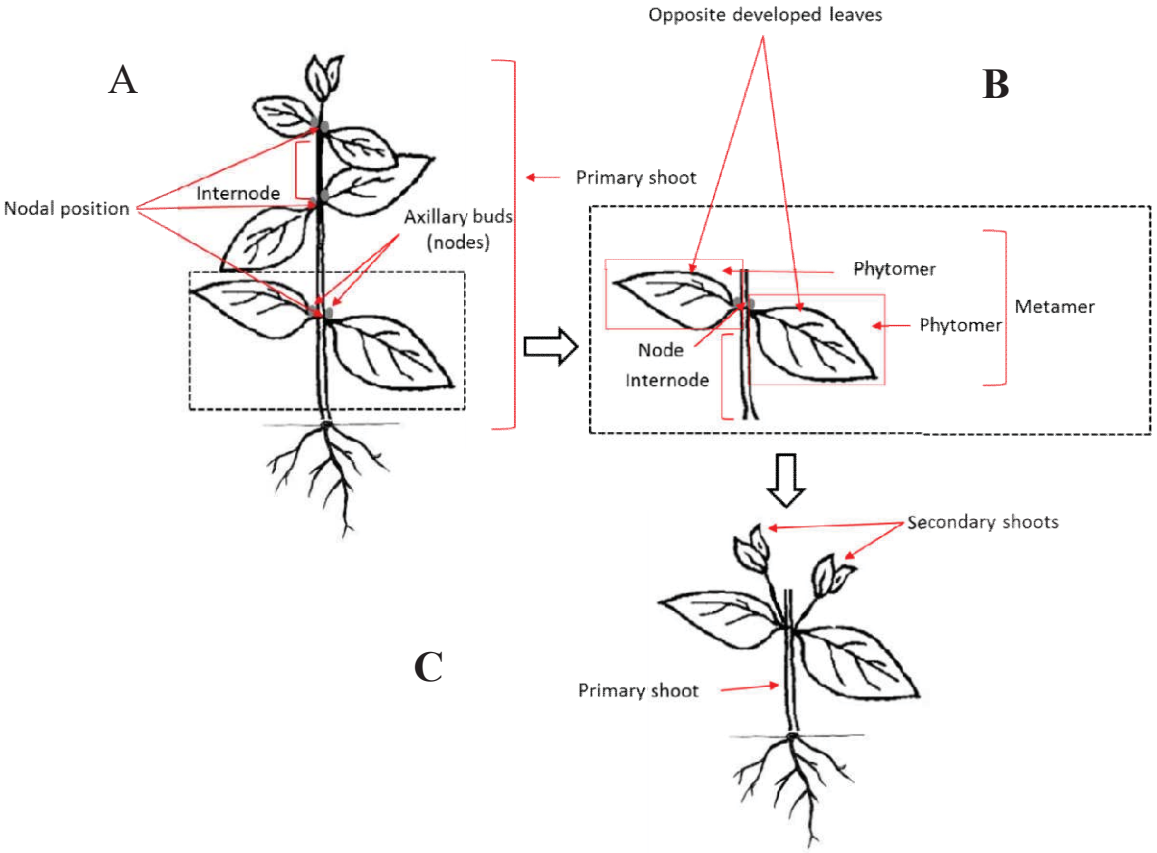


Figure 1-3: Schematic diagram, illustrating the morphological characteristics of **(A)** a gentian plant having opposite leaves, with each repeating unit comprising **(B)** two leaves together with two axillary buds (arising from the two nodes) make a metamer (dashed box). A metamer consisted of two phytomer, which each included a single leaf and axillary bud. In this thesis the term metamer was also used to refer to the nodal positions along the primary shoot, relative to the tip. **(C)** Secondary shoots grow from axillary buds at the node position on a primary shoot.

Leaves are simple with entire margins and an interpetiolar sheath (Ho, 2001; Struwe, 2002). Leaf venation is usually characterized by a few pairs of secondary veins diverging close to the base of the leaf (Ho, 2001; Struwe, 2002). Depending upon species, flowering times range from spring to autumn. The trumpet-shaped bisexual flowers are attached to the leaf axils towards the ends/apices of stems that arise from an underground crown. Although gentian species are famous for their blue colour, there are other colours present in the various species such as red, white, cream, yellow, and pink (Bartlett, 1975; Kohlein, 1991; Eason et al., 2004).

Historically, as reported by Jomori et al., (1995) seeds and cuttings are propagation methods used for various genotypes of gentians. In commercial cultivation however, most shoots

arising from gentians in the field arise from overwintering, pre-formed crown buds (Samarakoon, 2012). As a heterotic plant, gentians exhibit segregation following seed cultivation, and most of the key varieties are interspecific or intraspecific hybrids (Doi et al., 2010). *In vitro* propagation has been developed as part of the commercial cultivation system for clonal propagation of gentian and *Gentianella* species and hybrids (Yamada et al., 1991; Ivana et al., 1997; Morgan et al., 1997). Rapid multiplication of plants, uniformity of performance among the planting material, less inherent disease, requirement of a limited number of mother plants and, non-dependence on environmental conditions, are considered as benefits of *in vitro* propagation methods.

Similar to any *in vitro* system focussed on clonal propagation, a successful and high performance *in vitro* propagation system for gentian depends on making a high frequency of shoot formation from explants (Figure 1-2). These shoots are used as the source of explants *in vitro*. With an explant growing *in vitro,* shoot formation is a result of initiation and development of axillary (located at the leaf axils on the primary shoot; Figure 1-3A) and/or possibly adventitious buds (those which develop from places other than a shoot apical meristem; Evert (2006) in plants. Since bud initiation is the first stage of shoot formation and developing new shoots, visualising the microscopic features of explants was likely to assist in identifying the origin and type of buds (axillary and/or adventitious), in addition to determining their arrangement in the node. For this thesis, comparative morphological studies of natural shoot formation and increased shoot formation of cultivars with different shoot formation response to ethephon as a shoot formation stimulator as introduced in Section 1.1.3, was also considered as a potentially useful strategy (Cosic et al., 2015; Funnell et al., 2015). Different cultivars with various shoot formation responses to ethephon could be used as a valuable research tool to study the possibility of genetic dependent differences which might affect the response.

PGRs are historically regarded as one of the major plant-related factors which alone, or together with other factors, influence different stages of cell differentiation toward the initiation of adventitious/axillary buds and their outgrowth, as explained in greater detail within Section 1.2 (Thimann and Skoog, 1934; Gaspar et al., 1996; Mason et al., 2014). External application of chemical factors, especially PGRs in the culture medium, is one of the environmental factors which has commonly been used to influence shoot formation of various plant species cultured *in vitro*, including gentian (Sharma et al., 1993; Gaspar et al.,

1996; Hosokawa et al., 1996; Zhang & Leung, 2002; Pumisutapon et al., 2011; Rademacher, 2015).

1.1.3. Observations leading to the current research

From preliminary data and anecdotal information, number of shoots which developed from nodal explants of gentian 'Little Pinkie' (Figure 1-3B) cultured *in vitro* increased following the application of ethephon ([2-chloroethyl] phosphoric acid) in the medium (Funnell, pers. comm.). Ethephon decomposes to the PGR ethylene, phosphate and chloride ions (Yang, 1969; Goudey et al., 1987a; Biddington, 1992; Marin et al., 2006). Hence, it was hypothesised that, the increase in the number of shoots was attributed to ethylene. This hypothesis is supported by evidence from other plant species: for example, ethylene has been shown to be involved in adventitious bud/shoot formation from bulb-scale explants of *Lilium* × *hybrida* (lily) (van Aartrijk et al., 1985a). Also, the application of ethephon modified the architecture of seedlings of *Petunia* x *hybrida* (petunia) by increasing lateral shoots, as a result of the outgrowth of lateral buds (Haver et al., 2002). In gentian, as detailed in Appendix 13, the number of shoots per explant of 'Little Pinkie' increased from 2 shoots in the absence of ethephon to 10 shoots with ethephon added (Figure 1-4). However, other cultivars of gentian showed no response or less increase in shoot formation as a result of ethephon application *in vitro.* For instance, application of ethephon has been reported to increase the number of crown shoots (arising from the underground stem) of the cultivar 'Showtime Diva' cultured *in vivo* up to 1.5 as compared with 0.5 shoots per stem for control (Samarakoon, 2012; Samarakoon et al., 2015). Also, number of secondary shoots (individual shoots arising from axillary buds within nodes on the stem (Figure 1-3B) of this cultivar cultured *in vitro* has been reported to slightly increase in media treated with ethephon as compared to ethephon-free medium (Wang, 2014). Hence, investigations over the mechanism by which ethephon influences shoot formation in gentian, became the subject of this research.

Figure 1-4 Explants of gentian 'Little Pinkie' cultured *in vitro* for 8 weeks in culture medium in **(A)** presence of ethephon at 10 mg.L^{-1} and **(B)** absence of ethephon. Explants developed in the presence of ethephon produced more shoots but shorter shoots, than explants in the absence of ethephon.

1.2. Control of shoot formation in explants propagated *in vitro*

In vitro propagation of plants can take many forms. In gentian, plants can be regenerated *in vitro* via organogenesis, whereby roots and shoots develop from the callus produced from cultured tissue in the medium (Yamada et al., 1991; Zhang and Leung, 2002). While quantities of the constituent components of the medium used by others propagating gentian *in vitro* vary between researchers (Morgan et al., 1997; Morgan et al., 2003; Morgan, 2004), a base medium typically consists of macro & micro Murashige (MS) salts (Murashige and Skoog, 1962), LS vitamins (Linsmaier and Skoog, 1965), agar and sucrose. Depending upon the morphogenetic stage of growth to be influenced, various quantities of external chemical factors or PGRs can also be included in the medium (Gaspar et al., 1996; Pumisutapon et al., 2011). While many other factors might be also involved in controlling shoot formation, Section 1.2 is focused on only introducing the effect of key PGRs which might be involved.

1.2.1. Auxin

Auxin, which was the first major PGRs to be discovered, is one of the main constituent components provided through the culture medium influencing growth and development of plants cultured *in vitro*. The most well-known and researched member of the auxin family is indole-3-acetic acid (IAA) which, together with 4-chloroindole-3-acetic acid, phenylacetic acid and indole-3-butyric acid (IBA) are natural endogenous forms of auxin synthesised by plants (isolated and identified by Kögl and others in 1934 as reviewed by Luthen (2015). Synthetic auxins include 1-naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid

(2,4-D), and many others (Simon and Petrášek, 2011). Transport and distribution of auxin within the plant are key factors influencing development of plant organs including shoots (Friml, 2003). Hence, for the current research it was considered necessary to investigate the transport of auxin in an explant of gentian cultured *in vitro*, in relation -to shoot formation.

1.2.1.1. Auxin transport and apical dominance concepts

Auxin translocation in the plant is from apices of shoots to apices of roots (i.e. basipetal), via the phloem for long distances (Thimann and Skoog, 1934). For short-distance transport, there is a unique system of active polar auxin transport (PAT) directly from cell to cell (Friml, 2003). The flow of auxin molecules through the neighbouring cells is driven by carriers (membrane transport proteins) on the plasma membrane of cells. This short-distance transport exhibits some morphogenetic properties. Carriers are mostly located on the basipetal (i.e., root-ward) side of the cell, so auxin is flowing from cell to cell toward the roots (Rubery and Sheldrake, 1974; Friml, 2003; Petrasek et al., 2006).

"Apical dominance" is one of the most important principles of shoot organization based upon auxin distribution; the mechanism of which has been a matter of hot debate for more than 100 years and includes:

- The nutritive theory was the first theory for apical dominance, in which the apical bud was suggested to be a strong sink for nutrients in the plant and consequently inhibiting the growth of axillary buds below, by limiting the availability of nutrients (Loeb, 1918). However, this theory might be of lower importance in an *in vitro* culture system in which nutrients are provided through the culture medium, and probably more available to the lower buds as compared with the apical bud.
- The first hormonal-involved theory called the "direct theory", indicated that auxin produced by the apical bud is transported downwards and directly inhibits the development of axillary bud growth (Thimann and Skoog, 1934). Later researchers opposed the direct theory because they couldn't identify any radio-labelled auxin applied on the decapitated stump in axillary buds (Hall and Hillman, 1975). As a term "apical dominance" then was later suggested to be replaced by "Correlative inhibition", which was a more general term used for the dominance (Martin, 1987).
- Recent theories about apical dominance are based on results that indicated the ability of axillary buds to export auxin to the basipetal mainstream of auxin transport in the stem, as a factor affecting its growth potential after decapitation (Leyser, 2009). One of these

theories has suggested cytokinins as growth promoters (refer to section 1.2.2) and strigolactones (SLs) as inhibitors (refer to section 1.2.3) to be probable candidates for the second messengers capable of acropetal transport involved in controlling bud outgrowth (Leyser, 2005). Ethylene and abscisic acid as other candidates for the second messenger (i.e. "second messenger" theory) have been investigated too (Chatfield et al., 2001).

- Another theory named "canalization" suggested that the apical bud has the ability of producing auxin to make the canals operate at their full capacity. These canals are made through narrow files of cells which have high auxin transport activity in the PAT system. Any decline in this capacity by decapitation or applying transport inhibitors allows an axillary bud to export its own auxin and initiate growth (Leyser, 2005; McSteen and Leyser, 2005; Leyser, 2009).
- Polar auxin transport generates a pattern of auxin gradients throughout the plant (Friml, 2003) which could be inhibited by quercetin (a flavonol) and genistein as naturally-occurring polar auxin transport inhibitors (PATI), or by 1-n-Naphthylphthalamic acid (NPA) and 2,3,5 triiodobenzoic acid (TIBA) which are synthetic inhibitors of auxin efflux (Taiz and Zeiger, 2002).

Given the importance of auxin transport and gradient inside the plant on bud outgrowth and development to become a shoot, PAT was investigated in the current thesis for examining the effect on shoot formation in gentian. Therefore, PATI were considered for potential application in the culture medium.

1.2.1.2. Auxin interaction with other plant growth regulators

Phytohormones, including auxin, do not affect plants through a linear pathway and, therefore, the final outcome arising from the application of an individual phytohormone is the result of an interconnection in hormonal pathways through a complex network of interactions between phytohormones (Burg and Burg, 1966a; Christianson and Warnick, 1983; Yang and Hoffman, 1984; Vanstraelen and Benkova, 2012). Interaction of auxin with cytokinin, represented as the ratio between them (auxin: cytokinin), is considered as one of the important factors influencing the regeneration response towards a specific *in vitro* morphogenic process (Christianson and Warnick, 1983). For instance, exposing callus cultures to a high auxin: cytokinin ratio (high concentration of auxin: low concentration of cytokinin) resulted in root formation, whereas a low ratio of these PGRs promoted shoot development (Skoog and Miller, 1957). Cytokinin is regarded therefore as being responsible

for bud outgrowth while auxin inhibits its release. Hence, any decrease in auxin supply results in either increasing the synthesis or the export of cytokinins from roots to shoots (Coenen and Lomax, 1997; Turnbull et al., 1997). Cytokinin may be synthesized in roots (Bangerth, 1994) or in the stem (Tanaka et al., 2006). The existence of synergetic, antagonistic and additive interactions with auxin and cytokinin have been demonstrated in reports indicating a cross talk between these two phytohormones (Coenen and Lomax, 1998). At the level of gene activity it has recently also been suggested that auxin is required for expression of genes involved in the biosynthesis of strigolactones (Foo et al., 2005; Johnson et al., 2006) and may act upstream of strigolactones in controlling shoot formation (Foo et al., 2005). Other studies suggested that shoot formation inhibition may result from reducing the basipetal flow of auxin transport from the dormant lateral buds by strigolactones (Crawford et al., 2010). With regard to ethylene, auxin can either inhibit the formation or induce the synthesis of ethylene, depending on the concentration (Crawford et al., 2010; McManus, 2012). Given the *in vitro* system provided a useful tool for exogenous application of PGRs, modification of endogenous phytohormones was hypothesized to be possible toward making the influence on shoot formation in gentian in current research.

1.2.2. Cytokinin

Cytokinins are another type of PGR included within the culture medium because of their potential influence on growth and development of plants cultured *in vitro*. Cytokinins are a class of phytohormones involved in root and shoot apical dominance, axillary bud growth, and many other developmental processes in plants through promoting cell division, growth and differentiation (Kieber, 2002). Kinetin, zeatin, and 6-benzylaminopurine, are adenine-type cytokinins. Phenylurea-type cytokinins are represented by diphenylurea and thidiazuron (TDZ). Adenine-type cytokinins fall into two distinct groups, the isopentenyl ones such as zeatin, and the aromatic ones like 2-hydroxybenxylamino purine (BAP). Most adenine-type cytokinins are produced in plant roots whereas phenylurea-type cytokinins are synthetic. Cambium and other actively dividing tissues also synthesize cytokinins (Chen et al., 1985; Mok and Mok, 2001). For the purpose of inducing multiple shoots *in vitro*, various types of cytokinins have been applied through the culture medium by previous researchers at various concentrations depending on the plant species and the tissue used as an explant (Seelye et al., 1994; Hosokawa et al., 1996; Sudha et al., 1998; Huang et al., 2000). BAP, as an adenine-type, and TDZ, as a phenylurea-type, have been the most commonly used cytokinins *in vitro*. Using callus of *Gentiana scabra*, TDZ at concentrations between 0.5 and 1 $mg.L^{-1}$ were more

efficient (80 to 100% calli produced shoots) in shoot regeneration compared to BAP (2 to 5.6% calli produced shoots) applied at a similar concentration range (Jomori et al., 1995). However, in another study, out of various types of cytokinin on 5 cultivars of *Gentiana* leaf explants, BAP was the most effective type of cytokinin on percentage of somatic embryogenesis (Fiuk and Rybczynski, 2008). Hence, TDZ and BAP were selected as comparative sources of cytokinin to apply in the current study.

Aerial parts of the plant have the capacity to synthesize their own cytokinin, independent of long-range transport from the root system (Nordström et al., 2004). Therefore, in an *in vitro* system, aerial parts of the explant should be capable of synthesising cytokinin required for the growth and development of the explant, even if no cytokinin would be provided through the culture medium. Hence, this possibility was investigated in this research by application of PGR-free medium on explants, as compared with the culture medium including cytokinin.

Cytokinins might contribute to the outgrowth of dormant buds which are under apical dominance induced by auxins (Shimizu-Sato and Mori, 2001). Application of cytokinin to non-growing buds results in the up-regulation and polarization of auxin efflux carrier proteins (PIN1), which suggests that cytokinin might contribute to the activation of dormant buds through the stimulation of auxin export from buds (Kalousek et al., 2010a). Cytokinins have a highly synergistic effect with auxins, and the ratios of these two groups of phytohormones affect most major growth periods during a plant's lifetime (Ferguson and Beveridge, 2009). In rapidly growing axillary buds, the ratio of cytokinin and IAA was high at the basal and upper nodes whereas the ratio was low in the slow growing axillary buds at nodes in the middle of the stem (Emery et al., 1998). Therefore it can be concluded that bud outgrowth only occurs when buds accumulate more cytokinin than auxin. This conclusion was used to hypothesise that the effect of ethephon on increasing axillary bud outgrowth was related to changing the auxin: cytokinin ratio through encouraging cytokinin biosynthesis, or movement alone or together with an inhibition in the transport and/or metabolism of auxin. While endogenous quantification of phytohormones (especially auxin and cytokinin) in the explants treated with ethephon would provide the information to investigate the hypothesis more directly, it would be a resource intensive process and was not the focus of the current thesis. Alternatively, within an *in vitro* system the application of various concentrations of PGRs in the culture medium could provide an opportunity to investigate the proportional relationship between these two PGRs and their effect on shoot formation.

1.2.3. Strigolactones

Strigolactones (SLs) have been recently defined as novel phytohormones, wherein they or their derivatives act as long-distance factors which suppress lateral shoot formation (Gomez-Roldan et al., 2008). It has been suggested that apically derived auxin can inhibit lateral bud outgrowth via strigolactone production (Brewer et al., 2009). Strigolactones reduce auxin transport and decrease PIN1 (auxin efflux carrier proteins) on the basal membranes of xylem parenchymal cells (Crawford et al., 2010) Thus, strigolactones might prevent canalization from the axillary bud, by reducing the efficiency of auxin transport in the main stem, and enhance the competition between buds for their common auxin sink in the stem (Prusinkiewicz et al., 2009).

Auxin is also required for the expression of genes responsible for the biosynthesis of strigolactones (Foo et al., 2005). Further to the interaction between auxin and strigolactones, there might also be an interaction between strigolactones and cytokinins, as decapitation resulted in a high reduction in the expression of RMS1 and RMS5 (genes responsible for strigolactone synthesis) below the treatment site (Ferguson and Beveridge, 2009). While strigolactones may influence shoot formation in explants as well as other PGRs such as auxin and cytokinins, in the absence of much data on gentians with regard to understanding ethylene's significant control over shoot formation in 'Little Pinkie', examination of the influence of auxin and cytokinin was prioritised for investigation within the current research.

1.2.4. Ethylene

Ethylene is a gaseous phytohormone contributing to several controlling aspects of plants including growth, development and reproduction (McManus, 2012). In an *in vitro* system, ethylene is not always added to the culture medium on purpose but, as a gaseous phytohormone, is synthesised in plant tissue as a result of changes in developmental stage, and external factors such as mechanical wounding, environmental stress, and certain chemicals including auxin and other regulators. Ethylene can accumulate together with other gases in poorly ventilated culture vessels and affects (most commonly negatively) growth and development of plants (Chi and Pua, 1989; Kieber et al., 1993; Pua and Chi, 1993; Pua & Tiong-Chew Koh., 1999; Thomann et al., 2009). However, as detailed below various compounds have been utilised to either produce ethylene or inhibit its effect on plants cultured *in vitro*.

1.2.4.1. Ethylene biosynthesis in the plant

Ethylene can be produced from all parts of higher plants including leaves, stems and roots. (Yang and Hoffman, 1984). Ethylene production in plants is affected by internal factors such as developmental stage, and external factors such as mechanical wounding, environmental stress, and certain chemicals including auxin and other regulators. Ethylene biosynthesis pathways have been determined and the enzymes involved have been described. Ethylene is biosynthesized in plants from the amino acid methionine, and can be induced by endogenous or exogenous ethylene in climacteric systems. Synthesis of ACC, which is a precursor for ethylene biosynthesis, increases with high levels of auxins, especially indole acetic acid (IAA) and cytokinins, while abscisic acid inhibits ethylene production (McManus, 2012). Since in the current research an *in vitro* culture system was to be used with exogenous ethylene provided in the form of ethephon in the medium, as well as other chemicals and PGRs, this complex of interacting factors was considered likely to affect the biosynthesis of ethylene and, therefore, worth exploring.

1.2.4.2. Ethylene donors and inhibitors

1-Aminocyclopropane-1-carboxylic acid (ACC) and ([2-chloroethyl] phosphoric acid (ethephon) are ethylene precursors used as an exogenous source of ethylene to study the effect on plants (Huxter et al., 1981; Sethi et al., 1990). Likewise, the effect of ethylene can be inhibited by the application of ethylene inhibitors either at the level of perception or synthesis. In plants, ethylene is perceived by a group of membrane-located proteins (Rubery and Sheldrake, 1974; Friml, 2003; Petrasek et al., 2006). Inhibitors of ethylene perception include compounds that have a similar shape to ethylene, but do not elicit the ethylene response. One example of an ethylene perception inhibitor is 1-methylcyclopropene (1-MCP) which binds irreversibly to ethylene receptors (Blankenship and Dole, 2003). This chemical has been used to investigate the physiological and biochemical bases of ethylene mediated responses in fruits and vegetables (Watkins, 2006). Aminoethoxyvinylglycine (AVG) and cobalt chloride ($CoCl_2$) are inhibitors of ethylene biosynthesis and silver nitrate ($AgNO_3$) is an ethylene antagonist (Pua and Chi, 1993; Mohiuddin et al., 1997). Given that in the current thesis ethephon as an ethylene donor was to be used, then application of ethylene inhibitors would seem to be a useful research strategy to explore.

While ethylene has been reported as the most probable chemical resulting from the decomposition of ethephon in the medium to influence shoot formation, other chemicals

arising from the decomposition of ethephon (e.g. phosphate or chloride ion) could also be involved. Hence, the possibility of ethylene's effect on shoot formation by using gaseous ethylene on explants of gentian cultured *in vitro* also needed to be investigated.

1.2.4.3. Ethylene on development of various plants *in vitro*

Both negative and positive effects of ethylene on the development of plants cultured *in vitro* have been reported by previous researchers. Most studies have reported adverse effects of ethylene accumulation following the growth of explants in a sealed system *in vitro* in several plant species (Chi and Pua, 1989; Kieber et al., 1993; Pua and Chi, 1993; Pua & Tiong-Chew Koh., 1999; Thomann et al., 2009). Following the use of exogenous ethylene, elongation of primary roots of *Arabidopsis* was inhibited (Thomann et al., 2009), as well as a dwarfing effect and decreased cell size (Kieber et al., 1993). In mangosteen leaf explants, ethylene was not effective in influencing the percentage of the buds forming shoots, but delayed their emergence (Jin Goh et al., 1997). In five commercial cultivars of cucumber, ethylene inhibited *in vitro* shoot induction, but application of ethylene inhibitors enhanced it (Mohiuddin et al., 1997). However, in a study on *Brassica oleracea* hypocotyl cultures, both negative and positive effects of ethylene were reported (Sethi et al., 1990). Callus proliferation was increased in the presence of ethylene, but shoot initiation was increased in the absence of ethylene (Sethi et al., 1990). Also, while ethylene made an initial limitation of growth in some plants (Rodrigues-Pousada et al., 1993), in some other species such as *Nicotiana tabacum*, *Rumex palustris*, *Oryza sativa* and *Triticum sativum*, growth stimulation has been reported (Métraux and Kende, 1983; Rijnders et al., 1997; Suge et al., 1997; Pierik et al., 2004).

Ethylene also has been reported to affect plant cell differentiation *in vitro* and, consequently, enhance the production and regeneration of embryogenic callus in *Zea mays* (Vain & Flament, 1989), adventitious bud formation on bulb scale explants of *Lilium speciosum* (van Aartrijk and Blom-Barnhoorn, 1983; van Aartrijk and Blom-Barnhoorn, 1984; van Aartrijk et al., 1985a; van Aartrijk et al., 1985b), and shoot regeneration in shoot culture of *Rosa hybrida* L. (Kevers et al., 1992; Pua and Chi, 1993). Ethylene promoted lateral bud growth and shoot proliferation in rose (Kevers et al., 1992), lavandin (Panizza et al., 1993), eastern white cedar (Nour and Thorpe, 1994), petunia (Dimasi-Theriou et al., 1993) and peach (Dimasi-Theriou and Economou, 1995). In lavandin cultures, the N6-benzyladenine (BA)-induced shoot proliferation was mediated by ethylene, and ethylene could replace BA in inducing shoot

proliferation. In eastern white cedar, besides causing bud break, ethylene also promoted axillary shoot elongation (Nour and Thorpe, 1994), suggesting a role for ethylene in the control of apical dominance, possibly by suppressing the auxin effect. Ethylene promoted shoot formation in *Digitalis obscura* tissues, but only in the presence of IAA and kinetin in the medium (Perez-Bermudez et al., 1985). According to the experiences reported within the tissue culture lab prior to the commencement of this thesis, application of ethephon *in vitro* increased shoot formation in gentian (refer to Section 1.1.3). Current study then was undertaken to relate the ethylene released from the ethephon within the culture medium, with the number of shoots produced by the explants of gentian 'Little Pinkie' cultured *in vitro*.

In those studies where ethylene accumulation produced adverse effects on morphogenesis of several plant species, supplementation of the culture medium with inhibitors blocking ethylene production or action has been used to protect *in vitro*-cultured tissue from toxic effects of accumulated ethylene (Magdalita et al., 1997) and stimulate their growth and development (Purnhauser et al., 1987; Chi and Pua, 1989; Pua and Chi, 1993; Mohiuddin et al., 1997; Pua & Tiong-Chew Koh., 1999). For example, the presence of an ethylene inhibitor has been reported to be mandatory for *in vitro* shoot regeneration from *Brassica campestris* (Chi and Pua, 1989), *Passiflora edulis* (Faria and Segura, 1997a) and in *Triticum aestivum* L. and *Nicotiana plumbaginifolia* callus cultures (Purnhauser et al., 1987). Given that in the current thesis ethephon as an ethylene donor is to be used, then ethylene inhibitors would seem to be a useful research strategy to explore whether the effect of ethephon was related to ethylene. Besides methods comprising amendments to the growing medium, an alternative strategy would be to manipulate the gaseous conditions within the vessel headspace by modifying ventilation and gas exchange (Lai et al., 1998; Zobayed et al., 1999). The positive effects noted by manipulation of the gaseous atmosphere of the vessel is mainly due to ethylene and CO_2 exchange with the open air (Trevisan and Mendes, 2005). In a study with tobacco callus in which ethylene was added, or CO_2 removed from the culture vessels, and with ethephon and ACC added to the medium, it was found that ethylene had two contrary effects on shoot initiation. Ethylene inhibited the process early in culture but speeded up primordia formation later in culture (Huxter et al., 1981). Removal of ethylene and CO_2 from the headspace of the sealed cultures of *Pinus radiata* resulted in suppression of shoot bud differentiation under shoot-forming conditions (Kumar et al., 1987; Kumar and Thorpe, 1991). A synergistic effect of ethylene and CO_2 in enhancing adventitious shoot bud regeneration and shoot elongation was also observed in cultures of *Thuja occidentalis* (Nour

and Thorpe, 1994). In the current research focussed on the increased shoot formation as a result of ethephon application, the culture vessels utilized had the ability for gas exchange, and, therefore, potentially could influence the effect of ethylene produced from ethephon on plants under investigation.

Considering the potential adverse effects of ethylene on morphogenesis stated in the previous paragraph, it is concluded that enhancement of the regeneration of adventitious buds and/or shoots by ethylene appears to be determined by different factors in different culture systems. In most of the studies presenting adverse effects of ethylene on plants, sealed vessels were utilized and quantification of ethylene concentration involved sealing the culture vessel in order to accumulate a concentration of ethylene detectable by gas chromatography equipment. It was likely therefore that a comparatively high concentration of ethylene accumulated in those sealed vessels in which inhibition of growth was noted. In contrast, in those studies reporting the encouraging effect of ethephon on the growth, vessels with the ability of gas exchange were used, which possibly resulted in accumulation of comparatively lower concentrations of ethylene. Hence, while various studies differed in a lot of other factors such as species, cultivar, tissue type, culture system and the presence of other PGRs (Biddington, 1992; Kumar et al., 1998; Dias et al., 2009; Trujillo-Moya & Carmina, 2012), it was considered likely that the difference in the concentration of ethylene accumulated in their various vessels could also be an influential factor. Few studies have reported an actual concentration of ethylene in culture vessels with gas exchange operating and containing explants. Nowadays, advances in technology have provided an opportunity to monitor ethylene in real time, without incubation periods, and to measure it at low concentrations down to 300 pptv (1 pptv = 1 part per trillion volume = $1{:}10^{12}$) and time resolution of 5 s using commercial laser based ethylene detectors such as ETD-300 (Sensor Sense B.V., Nijmegen, the Netherlands) (Woltering et al., 1988; Cristescu et al., 2008; Clarke et al., 2009; van den Dungen et al., 2011; Forni et al., 2012). Therefore, measuring the concentration of ethylene in vessels with the ability of gas exchange, by using the ETD system, was considered for potential utilisation in the current study.

While decomposition of ethephon to ethylene and other by products has been the subject of research in the past, little information could be found specifically on the ethephon-ethylene relationship in an *in vitro* system. Ethephon, which is stable in aqueous solution below pH 4, decomposes to ethylene, phosphate and chloride ions as the pH is raised above this (Yang, 1969; Goudey et al., 1987a; Biddington, 1992; Marin et al., 2006). The reaction is catalysed

by a hydroxyl ion. The half-life of ethephon at pH 6 is about 96 hours; at pH 7 under the same conditions about 80% conversion to ethylene occurs over the same time (Domir and Foy, 1978). However, in an *in vitro* system using culture medium at a pH of 5.7 (Morgan et al., 1997) within vessels designed to provide gas exchange, specific details quantifying the ethephon-ethylene relationship and concentrations were not available which was considered to be a useful strategy to research within this thesis.

1.2.4.4. Ethylene interaction with other plant growth regulators

At all stages of plant development, as explored within the following sections, ethylene has been reported to interact with other phytohormones such as auxin, cytokinin, and strigolactones (Lieberman, 1979; Yu and Yang, 1979; Riov et al., 1990; Biddington, 1992; Kapulnik et al., 2011).

– ***Ethylene and auxin***

While auxin has been reported to stimulate ethylene biosynthesis (Yu and Yang, 1979), many auxin effects are also mediated by ethylene (Burg and Burg, 1966a; Yang and Hoffman, 1984). For instance, the reason for noting the reduction in the endogenous concentration of auxin in leaf discs of *Citrus sinensis*, following the application of ethylene, was suggested to be through its conjugation with IAA and formation of indole-3-acetylaspartic acid and/or decarboxylation (Sagee & Goren, 1990). Similarly, providing a high concentration of ethephon and ethylene in habituated cultures of *N. tabacum* reduced the growth due to reduction in endogenous auxin, while NAA was effective in compensating for the reduction (Biddington, 1992).

The inhibitory effect of ethylene on PAT is another mechanism suggested for the ethylene effect on endogenous concentration of auxin (Goldsmith, 1977). In a series of experiments investigating adventitious bud formation on bulb scale explants of *Lilium speciosum*, ethylene was hypothesised to enhance bud formation by an inhibitory effect on PAT (van Aartrijk and Blom-Barnhoorn, 1983; van Aartrijk and Blom-Barnhoorn, 1984; van Aartrijk et al., 1985a; van Aartrijk et al., 1985b). Although the effect of adding NAA improved bud formation more than ethylene, it was suggested that ethylene increased tissue sensitivity to NAA. AVG, as an ethylene inhibitor, reduced bud formation, whereas ACC as an ethylene precursor and TIBA as an auxin polar transport inhibitor, both reversed the effect and enhanced bud formation. Accumulation of intracellular auxin as a result of inhibition of PAT by ethylene or TIBA was

suggested to be responsible for promoting bud formation in bulb scale explants (van Aartrijk et al., 1985a), but data substantiating this hypothesis was not presented. Nevertheless, there has not been any report on the effect of ethylene on auxin concentration and/or movement in explants of gentian cultured *in vitro* and, therefore, this was considered for exploration in this thesis. Quantifying and comparing the auxin concentration in ethylene-treated and non-treated samples was a possible research strategy that could test the inhibitory effect of ethylene on PAT.

– ***Ethylene and cytokinins***

Although ethylene has mostly been reported to affect shoot formation via its interaction with auxin, application of cytokinin to non-growing buds resulted in the up regulation and polarization of PIN1 (auxin carriers on the basal membranes of xylem parenchymal cells), which led to the suggestion that cytokinin might contribute to the activation of dormant buds through the stimulation of auxin export from buds (Kalousek et al., 2010a). Also, cytokinin had been suggested to enhance ethylene production by IAA by an undetermined mechanism, which was only marginally related to suppression of IAA conjugation (Lieberman, 1979). However, while there are several reports of ethylene biosynthesis being influenced by other PGRs (Lieberman, 1979; Sińska, 1989; Riov et al., 1990), there is little information on the effect of applying exogenous ethylene on the production of other endogenous phytohormones, especially *in vitro*. In a study on young petunia seedlings, ethephon as an ethylene releasing compound decreased the auxin: cytokinin ratio as a result of ethylene's effect on IAA concentration within the shoot and, therefore, increased the number and length of secondary shoots of seedlings which were under increased apical dominance (Haver and Schuch, 2001). This change resulted in an increase in the percentage bud break, formation of secondary shoots, and suppression of internode elongation. Similarly therefore, in the current research it was hypothesized that ethylene released from ethephon will change the auxin: cytokinin ratio in the explant through the inhibition of PAT which, alone or together with any effect on cytokinins, will result in increased shoot formation. This hypothesis then was to be explored in this thesis. While at the outset of this thesis quantification of endogenous phytohormones such as auxin and/or cytokinins, as affected by ethephon/ethylene, was considered as a potential research strategy to explore the relationship with auxin: cytokinin ratio, altering the concentration of PGRs within the *in vitro* culture medium to achieve the same outcome was considered an easier to achieve strategy to initially test the hypothesis.

– ***Ethylene and strigolactones***

As a comparatively recently discovered plant growth regulator, data on the interaction between ethylene and strigolactones is understandably limited (Kapulnik et al., 2011). However, a crosstalk between auxin, cytokinin and strigolactones in root formation has been suggested (Vanstraelen and Benkova, 2012). Analysis of the effect of hormonal treatments on root hair elongation in PGR-signalling mutants of *Arabidopsis* has led to the hypothesis that strigolactones and ethylene regulate root hair elongation via a common regulatory pathway, in which ethylene is epistatic to strigolactones, wherein the effect of strigolactones on root hair elongation requires ethylene synthesis. Auxin signalling was not necessary, but enhanced root hair response to strigolactones, implying that the strigolactone and auxin hormonal pathways are connected for regulation of root hair elongation. The ethylene pathway requirement for the root hair response to strigolactones was used to hypothesise that ethylene forms a cross-talk junction between the strigolactone and auxin pathways (Kapulnik et al., 2011; Koltai, 2011). Based on previous reports indicating the regulatory effect of ethylene on auxin biosynthesis and transport in roots (Růžička et al., 2007), it was then suggested that ethylene is part of the mechanism by which strigolactones regulate the auxin transport capacity in their control over root architecture (Koltai, 2011). It would be interesting to see whether this crosstalk is also involved in other functions of strigolactones involving auxin, such as shoot formation and particularly shoot formation within 'Little Pinkie'. While it was not the focus of current research, to the best of the author's knowledge, nothing has as yet been reported on the possible cross-talk between strigolactones and ethylene in shoot formation in gentian. As noted within Section 1.2.3 however, within the current thesis the interaction of other phytohormones was prioritised.

– ***Ethylene and Polyamines***

Polyamines, which are not classified as PGR but act as endogenous plant growth regulators, have been reported to be effective in the control of organogenesis and embryogenesis *in vitro* (Burtin et al., 1990). They have been suggested as being involved in the control of reprogramming the cell in new patterns of development (Meijer and Simmonds, 1988). The endogenous concentration of polyamines and ethylene tend to be related via their inhibitory effect on each other's biosynthesis (Galston and Kaur-Sawhney, 1987). Hence, although polyamines were not the focus of current research, interpretation of the effect of ethylene during *in vitro* culture needs to be cognisant of possible polyamine effects and vice versa.

1.2.5. Research hypothesis and objectives

As explored within the preceding sections, phytohormones are hypothesised to be key influencing factors involved in the underlying mechanism for the increasing effect of ethephon on shoot formation in explants of gentian 'Little Pinkie' cultured *in vitro*. As illustrated in summary form within Figure 1-5, it was recognised that the effect of phytohormones like ethephon/ethylene released from ethephon is not likely to be through a linear pathway, but results from a complex network of interactions potentially between a number of phytohormones (Vanstraelen and Benkova, 2012). Those phytohormones which interact with ethylene and affect shoot formation have been previously reported to include auxin, cytokinin and strigolactones.

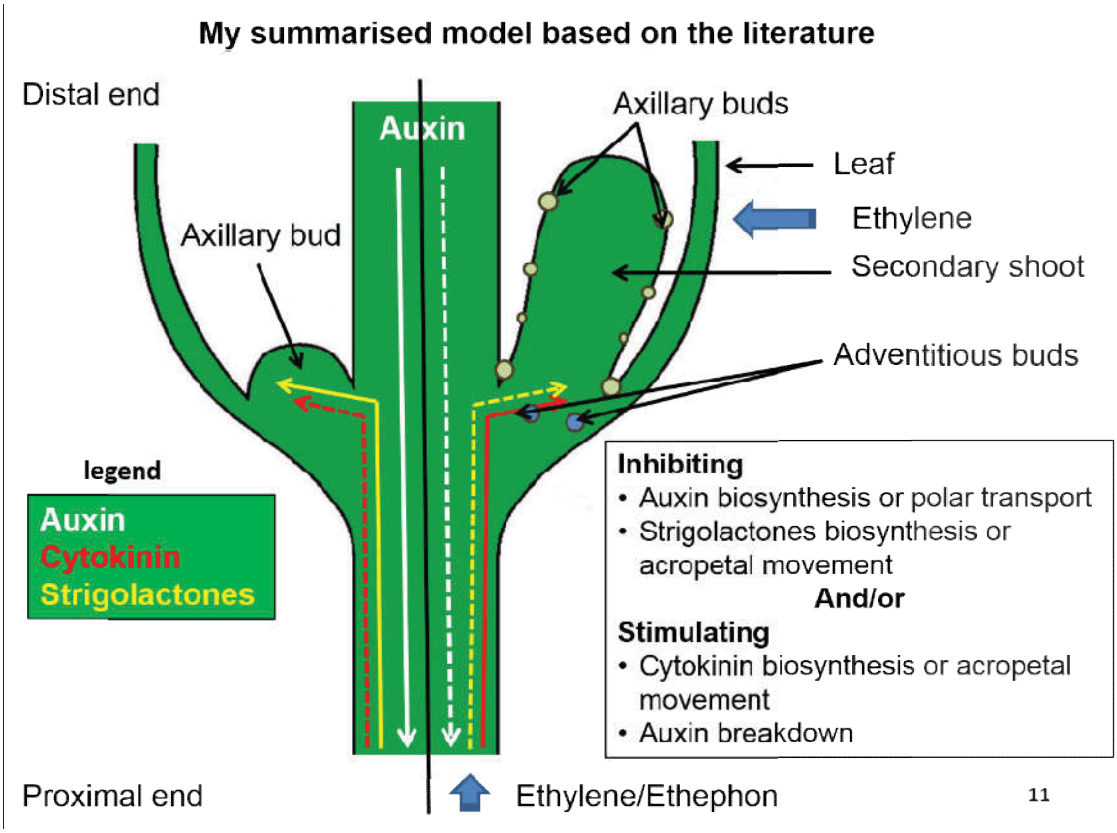


Figure 1-5: A diagram representing the proposed model of the effect of ethephon on endogenous the hormonal relationship and bud outgrowth in an explant of Gentian 'Little Pinkie' cultured *in vitro*. The black line divides the explant in two; non stimulated (right side) and stimulated (left side), by ethephon. White arrows represent the flow of auxin, yellow arrows strigolactones and red arrows cytokinin. Solid arrows show active movement of the phytohormones whereas dashed arrows represent inactive or no involvement of the phytohormones.

At the outset of this thesis the hypothesis is that during shoot formation, axillary buds are formed in the axil of each leaf, each with the potential to develop into shoots. In an intact plant, as discussed in Section 1.2.1, auxin is synthesized within the apical bud at the tip, flows basipetaly from tip to the root, and plays a central but indirect role in inhibition of

axillary bud outgrowth (Figure 1-5). Acropetal movement of root-derived strigolactones, together with basipetal movement of auxin, restricts the axillary buds to send their own synthesized auxin to the main stream of PAT and, therefore, buds remain suppressed. Auxin might inhibit the biosynthesis of cytokinins, which are growth promoters in axillary buds. Furthermore, root derived cytokinins may not be able to transfer acropetally, due to the saturation of the stem with downward auxin flow. The effect of a high auxin to cytokinin ratio, in addition with the effect of strigolactones, inhibits axillary buds from out-growth. It might also inhibit differentiation of cells toward making adventitious buds.

Any depletion in the auxin flow, for instance following decapitation, may cause an increase in the biosynthesis of cytokinins as a secondary messenger in buds or, its transference from the roots to axillary buds (Coenen and Lomax, 1997; Turnbull et al., 1997). Similarly, ethylene released from the application of ethephon has been reported to have an inhibitory effect on PAT. Production of strigolactones, as another potential secondary messenger, decreases in roots as a result of a decrease in the auxin flow. Concentration of strigolactones may therefore decrease in axillary buds. Under such a situation there seems to be more space than before in the main stream in the stem and therefore axillary buds would be able to send more auxin to the stream. The absence or lower concentration of strigolactones, as a branching inhibitor, together with a lower ratio of auxin to cytokinin, may cause axillary bud out-growth or differentiation of cells to make new adventitious buds. Therefore, similarly, within this thesis the increased shoot formation in gentian ‘Little Pinkie’ as a result of ethephon application *in vitro*, was hypothesised to be due to changing endogenous balance of phytohormones. While it was recognised phytohormones such as strigolactones may also be involved in shoot formation, research was centred on auxin: cytokinin ratio.

Once a plant has been divided to produce nodal explants for propagation *in vitro*, although it has been separated from roots and tips as two main locations for biosynthesis of phytohormones, it is recognised that it still contains some pre-existing amount of phytohormones which are available to be involved in *in vitro* morphogenesis. Hence, it is hypothesized that any auxin which has accumulated in axillary buds starts to move out to be connected to the main stream in the stem, and axillary buds start their outgrowth. Accumulation of auxin within the proximal end of the nodal explant would probably help organogenesis towards the formation of roots. After growing the bud and developing the roots, the explant would be expected to have a similar hormonal condition as that of the initial intact donor plant. The apex of the newly developed shoot and roots of each explant

would then become the potential locations of hormonal biosynthesis. As a result, subsequent axillary buds below the shoot apex will be prevented from outgrowth due to correlative inhibition (refer to Section 1.2.1). It was also possible to change the morphogenesis of the *in vitro* explant by changing the endogenous content of phytohormones via exogenous application of PGRs. In order to investigate the effect of ethephon on shoot formation, and the mechanism involved, the research strategy was to explore the interaction of ethylene with other PGRs. Therefore, modification in auxin: cytokinin ratio as one of the possible mechanisms by which ethephon increased shoot formation in explants, was the main hypothesis for the underlying mechanism of this research.

Application of various concentrations of PGRs in the culture medium were viewed as providing an opportunity to investigate the relationship between these PGRs (ethylene, auxin and cytokinin) and their effect on shoot formation. Additionally, experiments utilising PAT inhibitors and cytokinins, together with examining the resulting macroscopic and microscopic observations on the morphology of the explants, was prioritised so as to offer further understanding of the mechanism of ethephon's effect on shoot formation. Cultivars of gentian with different shoot formation response to ethephon applied *in vitro* were also considered as useful tools to study the mechanism of ethephon on shoot formation. It was hypothesised that the effect of ethephon on increasing bud outgrowth in gentian was related to lowering the auxin: cytokinin ratio through an inhibition in the transport and/or metabolism of auxin alone or together with encouraging cytokinin biosynthesis. Therefore, in order to test the hypothesis for the current thesis, the main research objectives were to:

- identify the origin and type of shoots arising from the node position of an explant of gentian cultured *in vitro* with high number of shoots as a result of treatment with ethephon (Chapter 3),
- quantify the concentration of ethylene released from ethephon into the headspace of culture vessels (Chapter 4),
- identify in what form and by what process do explants of gentian respond to ethephon (through direct absorption from the culture medium and/or decomposition to ethylene within the headspace) (Chapter 5),
- identify the involvement of ethylene or chemicals arising from the decomposition of ethephon (e.g. phosphate or chloride ion) on shoot formation by using gaseous ethylene on explants of gentian cultured *in vitro* (Chapter 5),

- identify the critical stage and duration of exposure to ethylene for the explants to increase their shoot formation (Chapter 6),
- investigation of the effect of ethephon on lowering auxin: cytokinin ratio through:
    - comparing auxin transport in ethylene-treated and non-treated samples as well as samples treated with PAT inhibitors (Chapter 6),
    - comparing ethephon with cytokinins in culture medium on stimulation or increase in shoot formation (Chapter 7).

Chapter 2. Factors influencing morphology of gentians *in vitro*

A: Non-uniformity in vegetative growth of gentians *in vitro*; sources and control strategies

2.1. Introduction

Some of the information reported in this chapter has been presented at international symposia (IHC2014 (Australia)), and published in the resulting Acta Horticulturae (refer to Appendices 14 & 16).

Using plant material which is uniform (similar overall size and number of shoots produced) in growth and development is considered to be important when planning and conducting experiments. In being uniform, this should decrease the experimental error and thereby assist in determining significant treatment effects (i.e. those aimed at increasing shoot formation). Knowledge of the sources of non-uniformity is also considered important in contributing towards the development of a rapid and efficient method for clonal propagation of highly plants with high number of shoots for the pot market; hence is also of commercial horticultural significance.

Preliminary experiences of the NZ Institute for Plant & Food Research Ltd (PFR) using explants of two cultivars of gentian; 'Little Pinkie' and 'Showtime Diva', indicated that when grown *in vitro* they differed in their natural frequency of shoots produced. Anecdotally, plantlets which developed from explants of 'Showtime Diva' were generally similar to each other (i.e. uniform) in terms of their overall size and number of shoots produced (shoot formation; Funnell, pers. comm.). In contrast, not all individual explants of 'Little Pinkie' grew equally when clonally propagated *in vitro* (i.e. non-uniform). In particular they were not visually uniform in height nor in the number of shoots (i.e. individual shoots arising from leaf axils on the main stem of the explant; Chapter 1, Figure 1-2) or tertiary shoots (i.e. individual shoots arising from those shoots arising from leaf axils on original explant). However, these differences between cultivars were anecdotal, with no data to substantiate them. The experiments presented here, therefore, were planned to both quantitatively describe the growth and development of explants, and also to investigate the potential sources of variation noted previously.

As explored within the following sections, the extent and variation of vegetative growth achieved by explants could be influenced by environmental and/or plant-related factors.

Environmental factors such as light intensity, photoperiod, temperature, pH of the culture medium, concentration of nutrient and inclusion of plant growth regulators (PGR) in the medium, etc., either individually or as a result of interactions, have been reported to influence the vegetative growth in various crops cultured *in vitro* (Kristiansen et al., 1999; Yang et al., 1999; Seabrook, 2005; Yen et al., 2008; Wang et al., 2009b). Any of these factors could therefore result in non-uniformity among the plantlets used as plant material for the experiments. However, vegetative growth could also be affected by various plant-related factors such as the physiological age of the tissue that explants originated from, correlative inhibition imposed by the tip and other organs, and the distance of the explants from the tip; with each of these potentially being influenced by factors such as the endogenous content of various phytohormones, nutrition and carbohydrate content within explants (Tanimoto and Harada, 1981; Dickens and Van Staden, 1988; Zhang and Leung, 2002; Campos and Kerbauy, 2004).

As noted for other crops, the distance of the explant from the tip (Volkeart et al., 1990; Maneesh & Pathak, 2001; Nguyen Thi & Kozai, 2007; Shekafandeh & Khosh- khui, 2008) could be interpreted to reflect tissue age or physiological maturity of the explants and could probably affect the vegetative growth. In *Limonium* for example the number of vegetative shoots was greatest in cuttings taken from top-half of stem tissue (less mature) as compared with the bottom-half which was more mature (Funnell et al., 2003). In another study on roses however, there was a variation in variables representing vegetative growth among buds taken from the same position on the plant (Marcelis-Van Acker, 1994). Hence, it was worthy of consideration in experiments presented in this thesis, to investigate the effect of explant position on vegetative growth. Furthermore, although for other crops investigations on the number and length of shoots, leaf area and, relative growth rate were affected by the position (Barrick and Sanderson, 1973; Volkeart et al., 1990; Maneesh & Pathak, 2001; Nguyen Thi & Kozai, 2007; Shekafandeh & Khosh- khui, 2008), to the best of the author's knowledge, there was no similar report on gentian. Experiment 1 therefore investigated the effect of position of explants on growth variables of two cultivars of gentian used in the current thesis.

Experiment 2 benefited from the findings of Experiment 1 on the effect of position which explants were taken from, along the original donor shoot, on growth variables. Building upon these results it was hypothesised that extending the duration of growth (up to 8 weeks) would probably provide those shoots of short length (i.e. as observed in Experiment 1) with enough time to develop adequately and reach the average length of other shoots. As a result,

uniformity and number of shoots (Figure 2-1) were expected to increase. The second experiment presented in this chapter (Experiment 2) therefore, was designed based on the findings of Experiment 1 to test this hypothesis. In Experiment 2, microscopy was also utilized to observe the node position more closely and study the morphological source of non-uniformity.

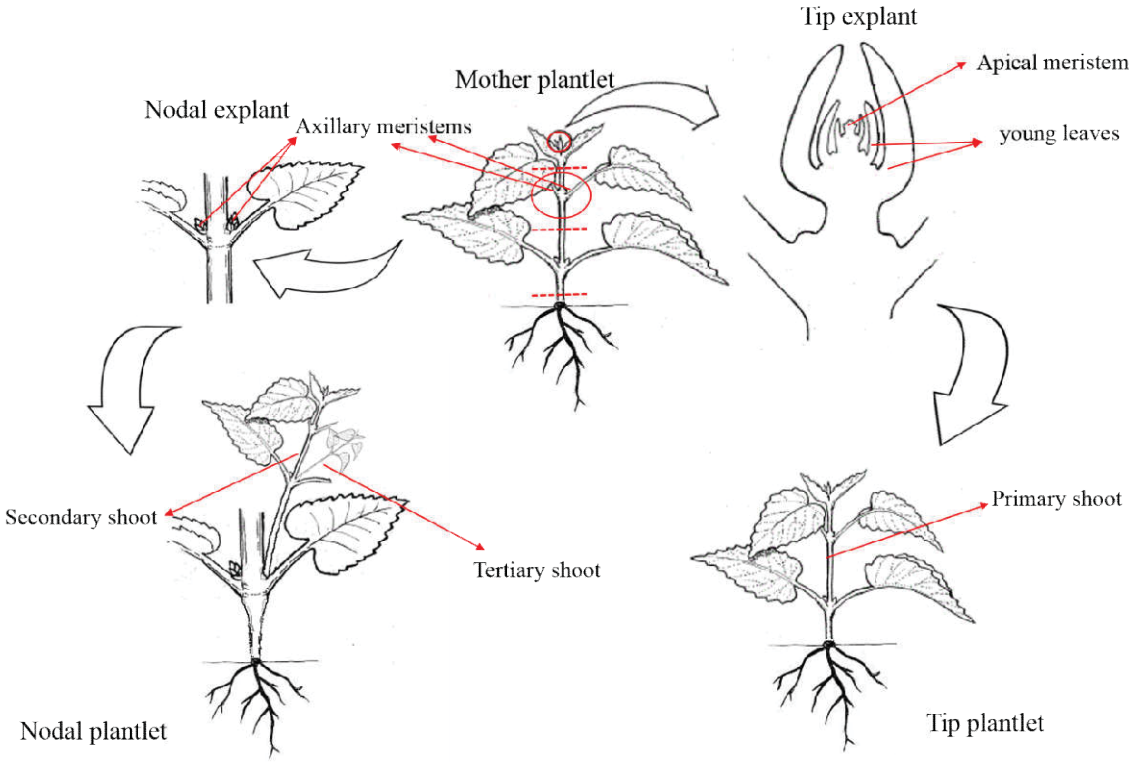


Figure 2-1: Schematic diagram representing different types of shoots (primary, secondary or tertiary) arising from the meristems in explants (tip or nodal) taken from different parts of the donor plant, and the resulting plantlets derived. Adapted from (National Gardening Association, 2001).

As part of the general protocol used within the tissue culture lab of PFR, the culture medium used for sub-culturing explants of gentian was a full strength MS (Murashige and Skoog, 1962) medium, including a range of plant growth regulators (PGR; (Morgan et al., 1997). Accumulation of PGRs and nutrients in their tissue as a result of being exposed to them through several cycles of *in vitro* growth beside other possible influential factors, might affect vegetative growth of the explants (Wingler, 2011; Kumar et al., 2012). Therefore, PGRs were removed from the culture medium in order to minimise the possibility of interacting with the treatment/s in the current experiments as had been previously utilized for gentian by other researchers (Morgan, 2004). Also, concentration of MS macro-salts in the medium was modified from full strength to half strength for these experiments.

As also would be used in subsequent chapters in this thesis, shoots which arose from the node could be identified either as primary (1º), secondary (2º) or tertiary (3º), depending on which part of the donor shoot was used as the source of explant (Figure 2-1), and the duration of growth. If tip explant was used as the explant (i.e. tip explant), it comprised a pre-formed primary shoot with multiple axillary bud primordia, unexpanded internodes, and an apical meristem. The apical meristem of the tip explant would continue to develop and extend the primary shoot so as to produce the main stem with axillary buds forming 2º shoots if they arose. In contrast, a nodal explant comprised a single node/metamer, expanded internode and two developed leaves. Shoots arising from nodal explants were in the first instance 2º shoots arising from axillary buds located at the leaf axils on the main stem (detailed in Chapter 3). Individual shoots which arose from the leaf axils of these 2º shoots then would be identified as 3º shoots. As explored within the experiments of current chapter, tip and nodal explants differed in their composite structure, especially number of nodes. Therefore, explants differed in the bud primordia they contain. It was possible that the vegetative growth arising would differ between these two sources of explants (Nguyen Thi & Kozai, 2007). For the cultivars used in the current research therefore, investigations on the positional effect of explants on vegetative growth and uniformity were considered necessary to assist with selecting the most uniform plant material for future experiments in this thesis.

As used in this thesis, the number of shoots (1º, 2º, and 3º) were considered as vegetative growth variables, which quantitatively described the extent of shoot formation. In addition it would appear reasonable to assume that at any point in time the greater the number of shoots on each explant, the more new explants which can be used for propagation; this reflecting a commercial horticultural outcome for this research. As only explants with at least 10 mm internode length are considered usable for propagation (Funnell, pers. comm.), it was considered worthy of investigation that the source of explants (nodal position) might influence the number of new usable explants to be derived after a period of culture *in vitro*. Total weight was also considered useful as a variable to quantitatively describe the size (Funnell et al., 1998) of plantlets derived from explants. As plantlets frequently differed in both the number and length of shoots, total weight was considered to be a useful continuous variable to describe treatment effects.

In an effort to explore growth variables that might more readily quantitatively describe the positional effects encountered in Experiment 1, in Experiment 2 data of additional growth variables such as the length of shoots was planned to be recorded. Additionally,

multiplication rate was calculated as a more representative variable to explain propagation efficiency, so as to reflect differences in the duration of growth. To achieve this, the length of shoots was categorized as either long (i.e. more than 10 mm, carrying well-developed leaves and internodes), medium (5-10 mm), or short (i.e. less than 5 mm). The number of explants which produced flowers instead of vegetative shoots were also recorded.

Within this chapter the broad goal was, therefore, to determine the sources of variation in the vegetative growth response of two cultivars of gentian utilized in the preceding chapters of this thesis. Two experiments were carried out to investigate the hypothesis that there would be a positional effect on the growth response of the explants derived from explants of gentian.

The specific objective for Experiment 1 was, therefore, to:

- quantify and compare the influence of position from which explants of 'Little Pinkie' or 'Showtime Diva' were derived, on the variables describing their growth.

Hence, as a part of developing an understanding of the *in vitro* growth of gentian and a rapid and efficient propagation method for the rest of the thesis, using 'Little Pinkie' the specific objective for Experiment 2 was to:

- quantify and compare growth of explants taken from different positions along the donor shoot and the resulting uniformity,
- quantify the growth of explants after increasing durations *in vitro* on uniformity and,
- identify the microscopic sources of non-uniformity in explants from different positions.

2.2. Materials and methods:

Plantlets of the gentian cultivars 'Little Pinkie' and 'Showtime Diva' which previously had been grown and sub-cultured *in vitro* for several four-week growth cycles were used as donor plantlets to take explants. Explants had been previously sub-cultured using a medium containing a range of plant growth regulators (PGR) in line with the general protocol for gentians within the tissue culture lab (Morgan et al., 1997). Due to anecdotal concerns over PGRs negatively influencing growth, the base medium for both experiments was PGR-free, comprising a modified MS (Murashige and Skoog, 1962) medium, with MS macro-salts at half strength, MS micro-nutrient salts, B5 vitamins, 7.5 $g.L^{-1}$ agar (Davis) and 3% (87.6 mM) sucrose. The pH of the medium was adjusted to 5.7 with either 0.1 N NaOH or 0.1 N HCl,

prior to autoclaving at 121°C and 103 kPa for 15 minutes. Culture vessels were disposable 290 ml (80 mm base diameter × 60 mm deep) tubs with snap-on lids, manufactured by blow moulding of a general purpose styrene and K-resin plastic (Alto Packaging, Hamilton, New Zealand). Each culture vessel contained 50 mL of medium. Explants were cultured at 25 ± 1 °C, at a photosynthetic photon flux density (PPFD) of 30 ± 5 $\mu mol.m^2.s^{-1}$ and 16 h photoperiod, provided by cool-white fluorescent tubes.

2.2.1. Experiment 1

Plantlets (derived from a mixed population of tip or nodal explants; Figure 2.1) were selected for uniformity of shoot length and number of nodes on the shoot (typically ~ 40 mm and comprised of two developed and visible nodes/metamer; Figure 2-2). Shoots were cut into single-node explants as well as the unexpanded tip explant, each 10 mm in length. Dependent upon the position along the shoot from which the explants were derived, they formed the treatments which were identified and described as either;

- Tip, which consisted of the apex and undeveloped leaves and nodes located at the distal end of the shoot,
- Base, or original explant which was the closest node to the roots at the proximal end of the plantlet (roots and the callus at the bottom of the stem were removed before inserting into the medium), or
- Node, which comprised a mixture of nodes (two), between the tip and base positions, been pooled to get adequate numbers of subsamples and replicates (Figure 2-2)

The explants were placed into the medium with the nodes just above the surface of the medium.

Figure 2-2: Plantlets of gentian (either 'Little Pinkie' or 'Showtime Diva') raised from either **(A)** tip explant or **(B)** nodal explant, used to create the treatment positions (Tip, Node and Base). Red dashed lines indicate the positions of cutting to create each treatment.

The experiment comprised a factorial arrangement of treatments of cultivar ('Little Pinkie' or 'Showtime Diva') and position which explants were taken from (Tip, Node or Base). In this preliminary experiment donor plantlets, which themselves were derived either from tip or nodal origin; Figure 2.1, were pooled. Each treatment comprised six culture vessels as replicates. Each culture vessel utilised eight explants, all from the same position, as subsamples. After a four week period of growth and establishment as plantlets, the variables recorded per plantlet were: the number of shoots (1º and/or 2º and 3º), number of usable explants (i.e. with at least 10 mm internode length) and, total fresh weight of the plantlet.

2.2.2. Experiment 2

Plantlets of gentian 'Little Pinkie' of similar pre-history as described in Section 2.1 were used as the source material. The base medium for the current experiment (i.e. PGR-free), and the growth conditions for the explants, were the same as previously described (refer to Section 2.1).

Donor plantlets were derived exclusively from nodal explants (Figure 2-1) in the growth cycle prior to being used in the current experiment. The most uniform shoots in terms of length and number of nodes were selected and cut to give three positions as treatments. Unlike in Experiment 1 where Tip, Node and Base positions were used as treatments, in the current experiment explants from the Base were excluded. Explants from the Tip were used as well as explants from the Node, which were categorised according to their distance from

the tip to; Node 1 (first node below the tip) and Node 2 (second node below the tip; Figure 2-2).

The experiment was conducted as a factorial design comprising two factors: position which explants were taken from; Tip, Node 1 or Node 2 and culture duration (5 or 8 weeks). Each individual vessel contained eight explants as sub-samples, all from the same position. There were six culture vessels as individual replicates for each treatment. After both 5 and 8 weeks growth *in vitro*, length of shoots categorized as either long (i.e. more than 10 mm, carrying well-developed leaves and internodes), medium (5-10 mm), or short (i.e. less than 5 mm), number of shoots per explants, along with total plant fresh weight and number of usable explants (i.e. potential propagules for propagation) were recorded (refer to Section 2.2.1). In addition, the number of flowering explants was recorded and expressed as a percentage from the total number of explants per replicate vessel. To explain propagation efficiency, the multiplication rate, was calculated as the total number of usable explants achieved when every single explant potentially produce on average 4 usable explants over the duration of culture.

Data for both experiments (1 and 2) were analysed as a 3 × 2 factorial design using the General Linear Model in Genstat (Genstat, version 17, 2014, VSNi Ltd, Hemel Hempstead, UK) or Minitab (Minitab version 16.1.1, Minitab Inc., State college, Pennsylvania, USA). Mean separation was done using Fisher's protected least significant test at $P \leq 0.05$ in Genstat. Box and whisker plots (Sigma Plot version 10, Systat Software Inc., San Jose, CA, USA) were used to quantitatively describe the distribution of the data for growth variables. The 80% spread was calculated based on the number of each variable between the 10th and 90th percentiles. Boundaries of the box indicate 50% of the spread (i.e. between 25th and 75th percentiles).

2.3. Results and discussion

The following sections present the results and discussion for both Experiment 1 and 2 together. The results presented on 'Little Pinkie' first and then subsequently compared with 'Showtime Diva'.

2.3.1. Position of the explant

After four or five weeks of growth, explants derived from all positions grew to become rooted plantlets. Growth variables for explants of 'Little Pinkie' in Experiments 1 & 2 were

as listed below, and then compared with growth variables of 'Showtime Diva' in Section 2.3.2.

2.3.1.1. Number of shoots (1º, 2º or 3º)

As the distance of the position that explants of 'Little Pinkie' were taken from along the donor shoot, increased from the tip toward the base, the number of 1º and/or 2º shoots (refer to Figure 2-1) increased in both experiments (Table 2-1 & Table 2-2). In Experiment 1, the number of 1º (only arised from tip explants) and/or 2º (arised from the nodal and basal explants) shoots developed by explants derived from the Base position increased by 4 times as compared with explants from the Tip. Similarly, in Experiment 2 explants of Node 2 developed on average three shoots, which was respectively ~1.5 and 3 times more than the number of shoots developed from Node 1 and tip positions. Within the 4 week period of assessment in experiment 1, explants of 'Little Pinkie' did not develop 3º shoots from explants taken from the Base nor the Tip position (Table 2-1). Only explants taken from the Node position produced 3º shoots which may possibly have been due to removing the apical bud, as the source of high correlative inhibition (Snow, 1937; Hall and Hillman, 1975). In comparison, explants from the Node positions produced up to two 3º shoots per explant (Figure 2-3C). Explants of 'Little Pinkie' taken from the Base position, in comparison with the explants taken from other positions, produced multiple shoots (2º) all arised from the node while there was only two shoots expected to grow from two axillary buds. Identification of shoot's origin was almost impossible due to their multiple number as well as small size of the node. Microscopic observations were needed to identify the origin (refer to Chapter 3). Shoots arised from the node (2º) were short and not developed enough to permit 3º shoots (arised from axillary buds on the 2º shoots) to emerge within the duration of the experiment. Although shoots arised from the Tip explants were longer than the shoots from Node and Base explants, correlative inhibition by the apical bud probably prevented axillary buds on the leaf axil to grow and develop 3º shoots (Samarakoon, 2012); therefore explants from the Tip position mostly produced a single shoot which was primary shoot (1º) due to being developed from the extension of apical meristem.

Table 2–1: Experiment 1; influence of different positions from which explants were derived, on the growth of gentian cultivar 'Little Pinkie' and 'Showtime Diva', after 4 weeks of culture *in vitro*.

Position	Fresh weight (mg)	No. of shoots (1º and/or 2º)	No. of 3 º shoot	No. of usable explants [y]
'Little Pinkie'				
Tip	165.6 b [Z]	1.2 a	0.0 a	2.4 a
Node	292.4 c	2.6 b	0.7 c	2.0 a
Base	270.6 c	4.8 c	0.0 a	2.0 a
'Showtime Diva'				
Tip	81.1 a	1.2 ab	0.45 bc	4.8 b
Node	89.9 a	1.3 ab	0.75 bc	4.8 b
Base	97.4 a	1.3 ab	0.41 b	4.0 b
P value				
Position	≤0.001	≤0.001	≤0.001	NS
Cultivar	≤0.001	≤0.001	≤0.001	≤0.001
Cultivar*Position	≤0.01	≤0.01	NS	NS

[Z] Groupings for mean separation were based on Fisher's unprotected least significant difference test. For the same variable, means followed by different letters in each column were significantly different at $P \leq 0.05$, n = 6 vessels each containing 8 explants.

[y] explants harvested with at least 10 mm internode length.

Table 2–2: Experiment 2; influence of different positions from which explants of gentian 'Little Pinkie' were sourced from the donor shoot, on the growth variables, after 5 weeks of culture *in vitro*.

Position	Fresh weight (mg)	No. Shoots (1º and/or 2º)	No. short shoots (<5 mm)	No. medium shoots (5-10 mm)	No. long shoots (>10 mm)	Maximum length of shoots (mm)	No. usable explants
Tip	160 c	1.00 b	0.04 b	0.25 a	0.79 a	25.41 a	4.04 a
Node 1	220 b	1.70 b	0.16 b	0.72 a	1.00 a	27.58 a	4.83 a
Node 2	290 a	3.36 a	1.90 a	0.58 a	0.77 a	31.18 a	4.95 a

[Z] Groupings for mean separation were based on Fisher's unprotected least significant difference test. Means followed by different letters within each column were significantly different at $P < 0.05$, n = 6 vessels each containing 8 explants.

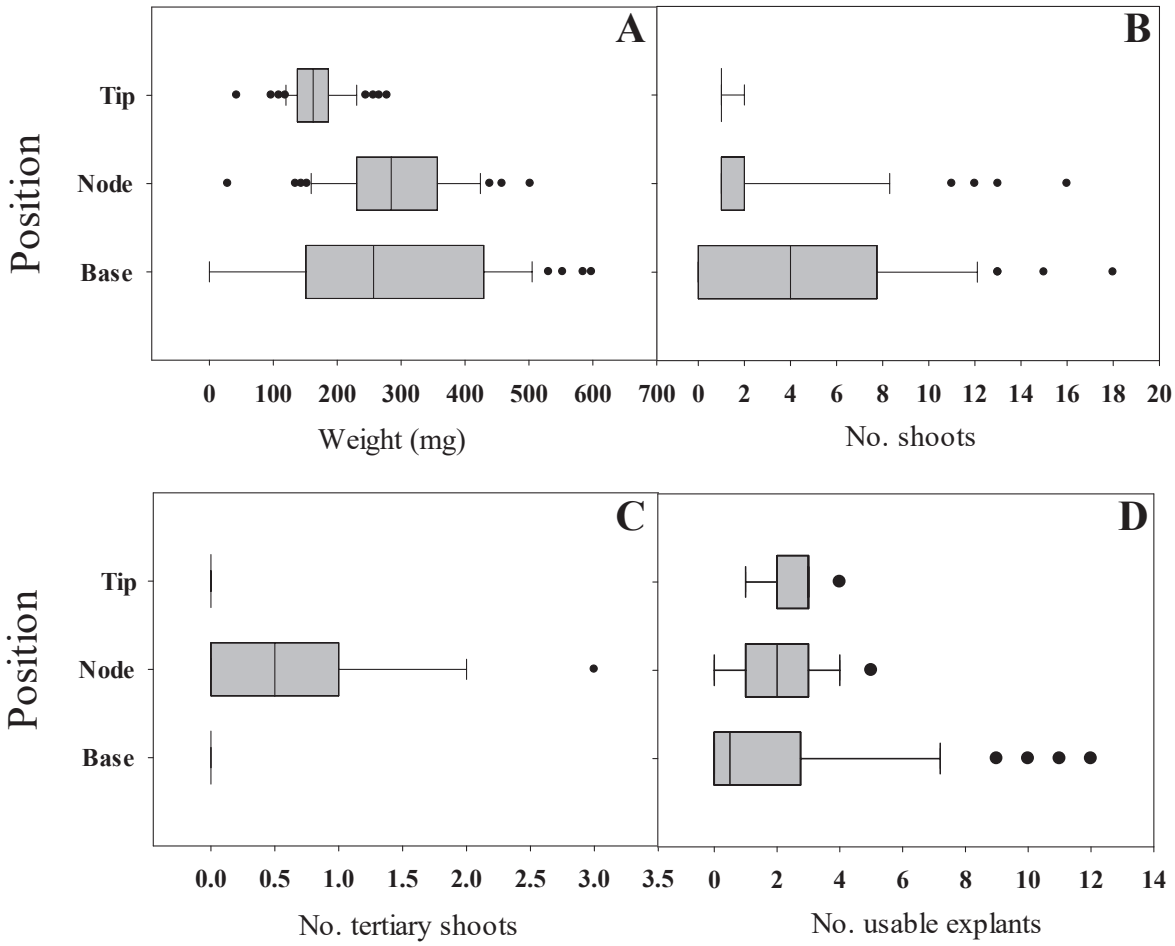


Figure 2-3: Experiment 1; variability of growth of gentian **'Little Pinkie'** explants taken from various positions (Tip, Node, and Base) after four weeks; **(A)** total fresh weight (mg), **(B)** number of shoots (1º and/or 2º) per explant **(C)**, number of tertiary shoots (3º) per explant **(D)** and number of usable explants. Vertical line in the centre of the box indicates median value. Boundaries of box indicate 25th and 75th percentiles; whiskers present the range between 10th and 90th percentiles, individuals beyond these boundaries are represented as stars, n = 6 vessels each containing 8 explants.

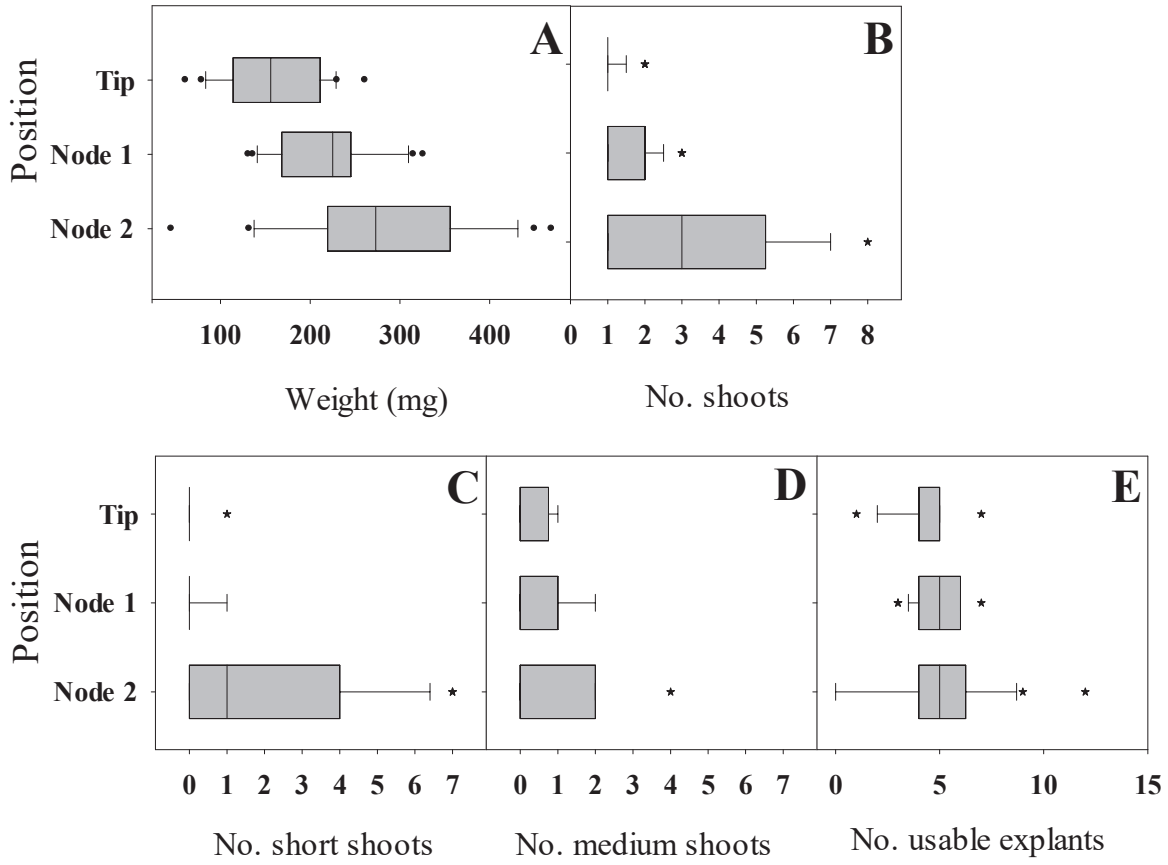


Figure 2-4: Experiment 2; variability of growth of gentian **'Little Pinkie'** explants taken from different positions (Tip, Node 1, and Node 2) after five weeks; **(A)** total fresh weight (mg), **(B)** number of shoots per explant, **(C)** number of short shoots, **(D)** number of medium shoots, and **(E)** number of usable explants. Vertical line in the centre of the box indicates median value. Boundaries of box indicate 25th and 75th percentiles; whiskers present the range between 10th and 90th percentiles, individuals beyond these boundaries are represented as stars, n = 6 vessels each containing 8 explants.

In both experiments, as the position of the explant along the donor stem increased from the tip, the number of 1º and/or 2º shoots increased, but the non-uniformity among the explants of each position in their number of shoots also increased (Figure 2-3B & 2-4B). As such, explants from the Tip position produced plantlets which were the most uniform in terms of the number of shoots (Table 2-1& Table 2-2) (Figure 2-3). In Experiment 1, in explants of 'Little Pinkie', while averaging 4.8 shoots, almost 50% of explants from the Base position produced up to eight shoots (i.e. 2º shoots), evident as the boundaries of box plots within Figure 2-3B. There were some individual explants taken from the Base position which were capable of producing up to 18 shoots. Explants of the Base position were of more distance from the tip (source of auxin) as compared with the explants of the Node position and therefore hypothesized to differ in their endogenous hormonal balance (ratio of auxin: cytokinin) and sensitivity of their cells in response to endogenous phytohormones/PGRs. Ratio of auxin: cytokinin was hypothesised to decrease and sensitivity of cells increase as the

distance of the position of the explant increased from the tip along the donor shoot. While further investigations were required to support the hypothesis, the similarity of the increase in number of shoots in explants taken from the Base position, to that achieved following treatment with ethephon, could be a potentially useful comparative research tool in discovering the mechanism of ethephon's effect. In comparison with the explants taken from the Base position, explants from the Node position produced 2.6 shoots (2º), and those from the Tip position mostly (81.25%) produced a single 1º shoot, which resulted from the extension of the apex (Figure 2-3B). However, in this experiment it was recognised that nodal explants included a range of 'nodal positions' not just one (Figure 2-2), potentially confounding results of differences in physiological age and, therefore, posing a question for further examination (refer to Figure 2-2). In Experiment 2, explants taken from the tip produced not more than a single shoot (Table 2-2), (Figure 2-4B) while almost 50% of explants from Node 1 and Node 2 produced anywhere up to five shoots, all of which were 2º (boundaries of box plots, Figure 2-4B). Almost 25% of explants taken from Node 2 (upper whisker, Figure 2-4B) were capable of producing up to eight 2º shoots which would probably explain the non-uniformity observed in the number of 1º and/or 2º shoots developed from the plantlets taken from the Node position as it had been pooled within Experiment 1 to get adequate numbers of subsamples and replicates. Despite the decrease in the uniformity from tip to the base, overall uniformity among the explants in the number of 1º and/or 2º shoots improved by 61% more in Experiment 2 as compared with Experiment 1 (Figures 2-4B). This improvement in uniformity in the number of 1º and/or 2º shoots was considered as likely due to the longer duration of culture, as well as removing the basal explants as a source of plant material in Experiment 2.

The existence of a physiological gradient of increased shoot formation from tip to the base was also reported within *in vitro* shoots of another cultivar of gentian (Zhang and Leung, 2002). In contrast, in another study using explants of *Arachis* sp. which can be considered similar to gentian in terms of their opposite leaves and nodes/metamer, shoot regeneration ability reported to significantly decrease from tip to base (Gagliardi et al., 2002). However, in the study on *Arachis* sp. the maximum number of shoots which was produced from each phytomer, regardless of the position that explants originated from, did not exceed one shoot, whereas in the current study there were some individual explants (metamer) taken from the Node 2 position of 'Little Pinkie' which were capable of producing up to 7 shoots (Figure 2-4B). Most plant species normally produce an axillary bud at each phytomer which potentially

develop into shoots (Evert, 2006). In gentian, leaves (i.e. each phytomer) are positioned on the shoot in pairs opposite each other, making a metamer consisting of two nodes, each with an axillary bud (Chapter 1; Figure 1-3) which could potentially grow to a 2º shoot and, therefore, two 2º shoots are expected to develop from the leaf axils within each metamer (i.e. explant from nodal positions). However, in addition to two shoots which were probably of 2º origin, there were extra shoots which considered to be most likely adventitious in origin (Samarakoon et al., 2013), and thereby highlighting the likely source of the morphological non-uniformity in their number and level of development. However, in order to confirm the type of buds and meristematic activity at the node, further microscopic studies considered to be done, presented in following chapter.

2.3.1.2. Plant fresh weight

For the growth period of four or five weeks, fresh weight of the explants of 'Little Pinkie' taken from the Base position was 1.6 times more than the explants from the tip in both experiments ($P \leq 0.001$; Table 2-1 & Table 2-2). In addition, the variation in total fresh weight of the explants of 'Little Pinkie' in both experiments increased as the distance of the position increased from the Tip toward the Base position (Figure 2-3A & Figure 2-4A). For instance, in Experiment 1 50% of the explants from the Tip position (i.e. boundaries of box plots, Figure 2-3A) produced respectively 61% and 85.5% less fresh weight than the explants from the Node and Base positions. In Experiment 2, total fresh weight of 50% of plantlets derived from Node 1 and Node 2 were respectively, 40% and 80% greater than those derived from the Tip (i.e. boundaries of box plots, Figure 2-4A). The increasing trend in fresh weight of the explants as well as their non-uniformity from tip to the base was probably related to the increasing trend in number of 2º and 3º shoots detailed in the preceding section (Table 2-1). Given that non-uniformity among the explants taken from the Base position was extensive, within this thesis, in order to get enough uniform plant material for future experiments, then the basal position would not be recommended for inclusion in a propagation system. Also within a commercial horticultural scenario, explants from Tip and Node positions would be more preferable, in order to obtain high quality nodal explants for propagation.

2.3.1.3. Length of shoots

In addition to other growth variables, for 'Little Pinkie' length of shoots was also different between treatments in both experiments (Figure 2-5). Shoots arising from explants derived from the Base position were visually small; typically short, carrying small leaves. Explants

from Node 1 and 2 typically produced one long shoot (more than 10 mm), and between one and two shoots of medium length (5-10 mm; Table 2-2) as well as some additional medium-length shoots (Figure 2-4D). Explants taken from Node 2 produced significantly more number of short shoots (less than 5 mm), than the explants of Tip and Node 1 positions (Table 2-2).

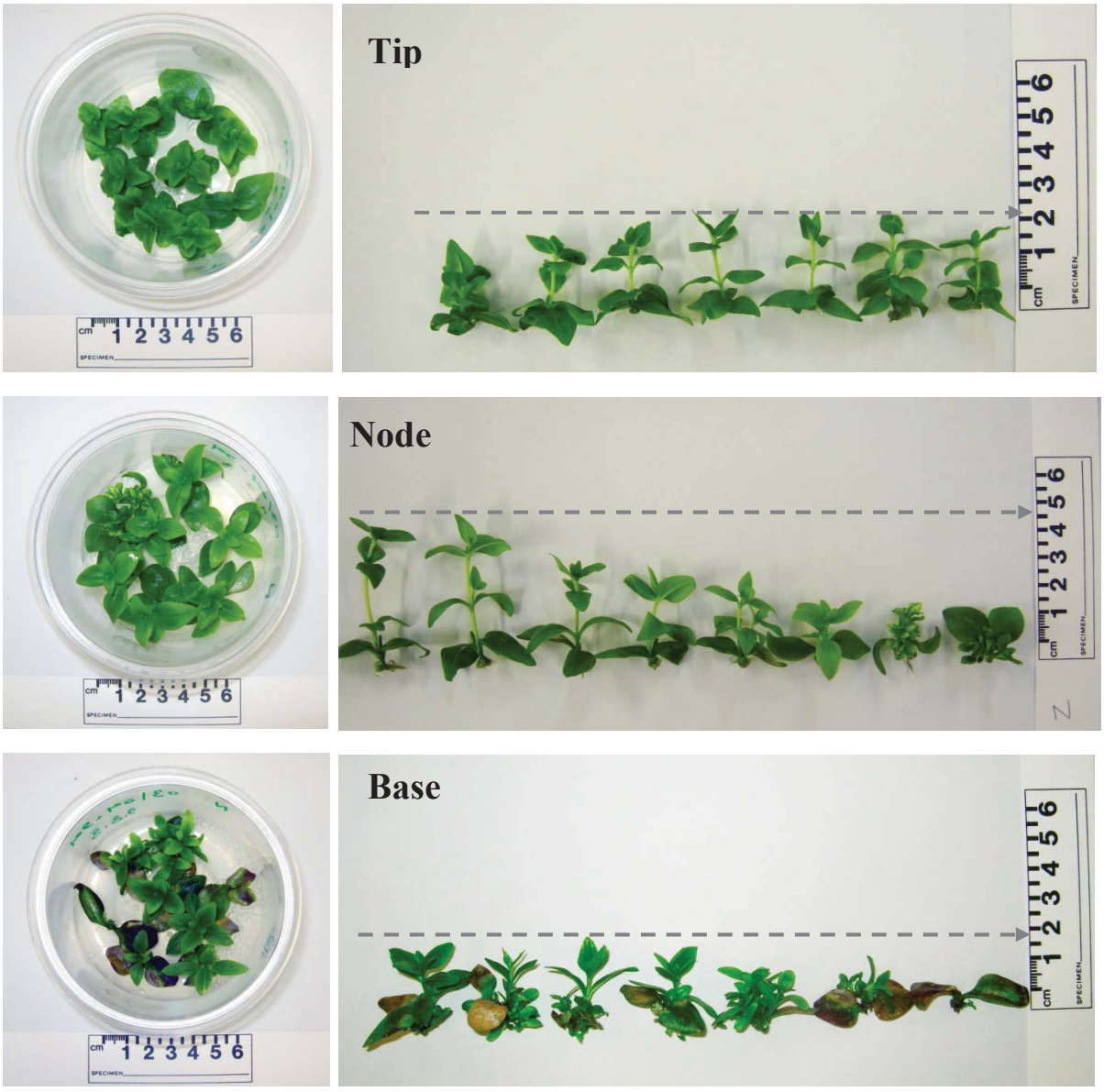


Figure 2-5: Aerial and side view of typical replicate sample of plantlets of gentian cultivar 'Little Pinkie' taken as explants from three positions (Tip, Node and Base) on the donor shoot (refer to Figure 2.2), after 4 weeks of growth *in vitro*. Arrows illustrate the maximum length of plantlets in each treatment.

Results achieved here were in agreement with that using single node cuttings of coffee plants *in vitro*; axillary shoots were shorter as the cutting position they were taken from along the donor shoot was further away from the tip toward the base (Nguyen Thi & Kozai, 2007). The decreasing trend of shoot length with the increase in shoot number in explants taken from Tip

to the Base, could simply have been due to the competition among shoots in their response to plant growth regulators and other components of the culture medium such as nutrients, if included (Kim et al., 1997). Also, differences in the number and length of axillary shoots arising from explants taken from different positions may be attributed to the difference in the physiological age of the explant; with those closer to the apex of the donor shoot being younger in age with cells probably less differentiated (Marcelis-Van Acker, 1994). In such conditions, different regenerative responses are to be expected according to the degree of cell differentiation and capacity for regeneration (Le Bris et al., 1998; Santana-Buzzy, 2005). Explants taken from the position closer to the tip produced less number of shoots probably because their cells were less differentiated than the explants taken from the positions close to the base. Expressed similarly therefore, the final size of an organ is often correlated with the number of divisions of meristematic cells, that probably increases as the distance of the position that explants were taken from increases from the tip (Vidal et al., 2003). As the initial number of cells and, therefore, the size of buds in leaf axils might differ at the start of the experiment, perhaps it might also reflect such a variability in the number and length of shoots that develop from them (Xu et al., 2015). Alternatively, different positions could be different in either the endogenous hormonal content or sensitivity of their cells to hormones (Trewavas, 1983; Emery et al., 1998). In a series of articles by Trewavas in the 1980's (Trewavas, 1981a; b; 1982; 1983), it was suggested that the capacity of cells to respond to a growth substance may not be fixed or cells may differ in their sensitivity to the growth substance. Additional interactions between the growth substance and its receptor also may contribute to differences in responsiveness between cells (Firn, 1986). Firn demonstrated factors which could contribute to sensitivity changes, which included: the growth substance, the receptor for the growth substance and, the affinity of the receptor for the growth substance. These factors can affect the magnitude of the evoked growth response in different positions as well as between different cultivars, which is related to the number of receptors occupied at any time. Each of the potential hypotheses raised potentially warrant further investigation, but has not been the focus of this thesis.

Despite the visually apparent decreasing trend in the length of shoots arising from the explants originating from the tip toward the base, shoots of different heights were evident within each explant within a treatment. This natural variation in growth behaviour limited the ability to use height as a variable describing growth differences between treatments. Therefore, while a treatment effect was visibly clear, it was best statistically determined using

other variables such as number of shoots, weight and, number of usable explants, as described in next section.

2.3.1.4. Number of usable explants for propagation

Consistent with established protocols of the laboratory for *in vitro* multiplication of gentian, and as illustrated in Figure 2-2, developed shoots which were able to be harvested and used as explants potentially included multiple nodal positions (i.e. 2 or 3 nodes). Since in Experiment 1 there was a significant increase in the number of shoots produced by explants from the Base position of 'Little Pinkie' compared to other positions (Figure 2-3A), more usable explants were expected from the Base position. However, when recorded after 4 weeks growth, not all 2º shoots from this position were developed enough to generate usable explants. A well-developed explant should at least have 10 mm of internode length to facilitate inserting the explant into the *in vitro* culture medium. Explants which originated from the Base position produced shoots which were shorter in total length, with smaller leaves than the explants harvested from the Tip and Node positions. Therefore, in spite of the increase in the number of shoots arising from the Base, the total number of usable explants was the same from all positions ($P > 0.1$; Table 2-1). In Experiment 2, while the number of useable explants did not differ between positions ($P > 0.1$; Table 2-2), the uniformity in the number of usable explants declined as the distance of the explant along the original shoot increased from the tip (Figure 2-4E). Some explants derived from Node 2, at the greatest distance from the tip, produced up to 12 usable explants, whereas others were not developed enough to achieve any. In spite of the increase in the number of shoots arising from Node 2, the total number of usable explants was the same for plantlets derived from all positions ($P > 0.1$; Table 2-2). This lack of difference which, as discussed previously, presumably resulted from correlative inhibition within the plant system under investigation, was observed in both Experiment 1 and 2. Within Experiment 2, in which explants of base position were excluded from the source of plant material, and the duration of culture was extended from 4 to 5 weeks, the total number of usable explants increased by 1.7 times when compared with Experiment 1 (Table 2-2 vs Table 2-1).

2.3.2. Cultivar difference

Data recorded from the Experiment 1 confirmed previous visual observations on the difference between cultivars of gentian in their growth (Table 2-1). Explants of 'Little Pinkie'

produced 2.5 times more fresh weight, 2.25 times more number of 1º and/or 2º shoots, compared to the explants of 'Showtime Diva' ($P \leq 0.001$; Table 2-1). While there was an increasing trend in the fresh weight and number of 1º and/or 2º shoots of the explants of 'Little Pinkie', as the distance of their position along the shoot increased from the tip, there was no difference between positions for 'Showtime Diva' (Table 2-1; Figure 2-3 and 2-6). Also, explants of 'Showtime Diva' produced up to a maximum of 3 shoots per explant, with the range of variation being much smaller than with 'Little Pinkie', which produced up to a maximum of 18 shoots (Figure 2-3B vs 2-6B). Explants of 'Showtime Diva' taken from various positions did not differ in the number of 3º shoots produced (Table 2-1; Figure 2-3C vs 2-6C). For explants of 'Showtime Diva' therefore, there was not an obvious difference between treatments in their overall appearance, whereas for 'Little Pinkie' there was a significant difference (Figure 2-7 & Figure 2-5).

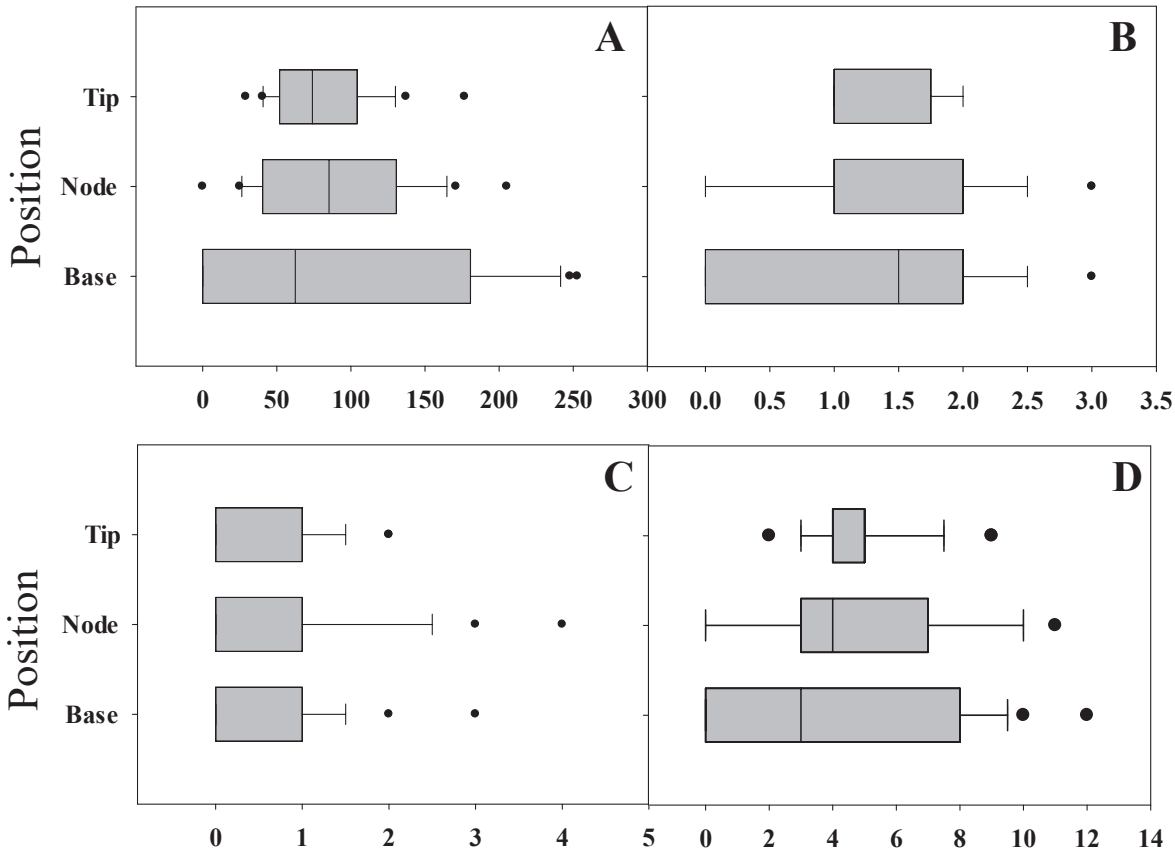


Figure 2-6: Box plots of, **(A)** total plant fresh weight, **(B)** number of shoots per explant, **(C)** number of 3º shoots and, **(D)** number of usable explants, of the gentian cultivar **'Showtime Diva'** taken from tip (Tip), node (Node) and base (Base) positions on the donor shoot (refer to Figure 2.2). Vertical line in the centre of the box indicates median value. Boundaries of box indicate the 25th and 75th percentiles (i.e. 50% spread between); whiskers present the 10th and 90th percentiles (i.e. 80% spread between); individuals beyond these boundaries are represented as stars, n = 6 vessels each containing 8 explants.

The number of harvestable explants produced by the explants of 'Showtime Diva' was approximately 2 times more compared to that of 'Little Pinkie' ($P \leq 0.001$; Table 2-1). In both cultivars, as the position of the donor explants was closer to the base, variation in resulting production of usable explants (i.e. after 4 weeks culture) increased. With 'Little Pinkie' 50% of the explants taken from the Tip position produced between 2 and 3 usable explants compared to the Node and Base positions, which produced a wider range of 1 and 3, or 0 and 3, usable explants, respectively (i.e. boundaries of box plots, Figure 2-3D). With 'Showtime Diva' however 4 and 5 harvestable explants could be taken from 50% of the Tip explants, compared to 3 and 7 explants from the Node, and 0 and 8 explants from the Base (Figure 2-3D vs 2-6D).

The cultivar difference in shoot proliferation noted in this current study may explain why Zhang and Leung (2002) only reported a slight effect of explant position on subsequent formation of shoots with the cultivar 'Akita Blue'. Their results matched the current results with 'Showtime Diva', but differed from the significant effect of position on the number of shoots developed using 'Little Pinkie'. Also, in opposition to findings in the literature for other plant species reporting more shoots per explant when explants were derived from positions closer to the tip (Barrick and Sanderson, 1973), the current results support the hypothesis that any positional effect is species dependent.

Figure 2-7: Aerial view of plantlets of gentian 'Showtime Diva' taken as explants from three positions (Tip, Node and Base; refer to Figure 2.2), after 4 weeks of growth *in vitro*.

2.3.3. Duration of culture

By extending the duration of culture from four weeks in Experiment 1 to five, uniformity among resulting plantlets improved in Experiment 2 (Figure 2-8), and total number of usable explants increased by 1.7 times (Table 2-2 vs Table 2-1). The improvement in uniformity could also be related to excluding the basal explants as a source of donor plant material in

Experiment 2 as compared to Experiment 1. After 8 weeks however, despite a longer duration of culture, not only did growth variables nor uniformity not significantly improve compared to 5 weeks (Table 2-3; Figures 2-9), but also the number of usable explants reduced by 30% less (Table 2-3). The decrease in the number of usable explants resulted from flowering of some of the explants (observed as mostly associated with explants derived from the Tip position (Figure 2-8), Hence, multiplication rate was considered to be a more representative variable to explain propagation efficiency, which was improved by 17% after increasing the duration of culture from 4 to 5 weeks. After 8 weeks growth however, the multiplication rate declined by 66% compared to after 5 weeks (Table 2-4). As a salient summary therefore, as evident by the boundaries of the box plots in Figures 2-9B&C, explants which grew for 5 weeks produced the most uniform population of plantlets, in terms of the number of shoots and usable explants, as compared with either 4 or 8 weeks growth.

Table 2–3: The influence of duration of culture *in vitro* on the growth of gentian 'Little Pinkie' taken from various positions along the donor shoot (Tip, Node 1 and Node 2) after 5 or 8 weeks.

Growth duration (Week)	Fresh weight (mg)	No. Shoots (1º and/or 2º)	No. short shoots (<5 mm)	No. medium shoots (5-10 mm)	No. long shoots (>10 mm)	Maximum length of shoots	No. usable explants
5 weeks	221.1 a	2.0 a	0.7 a	0.5 a	0.9 a	25.2 a	4.6 a
8 weeks	251.7 a	2.1 a	0.9 a	0.6 a	0.8 a	30.1 a	3.1 b

[z] Groupings for mean separation were based on Fisher's unprotected least significant difference test. Means followed by different letters within each column were significantly different at $P < 0.05$, n = 6 vessels each containing 8 explants.

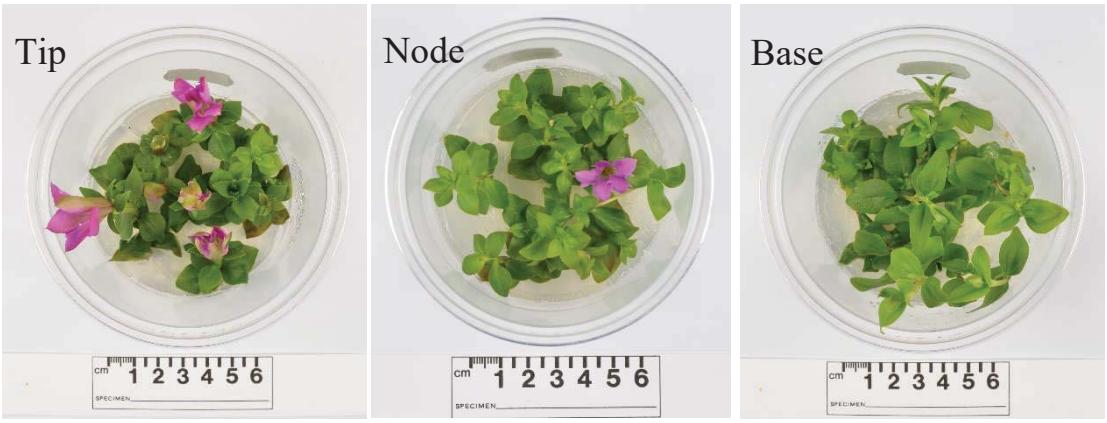


Figure 2-8: Aerial view of flowering plantlets of gentian 'Little Pinkie' grown from three positions (Tip, Node and Base; refer to Figure 2.2), after 4 weeks of growth *in vitro.* Flowering mostly happened with explants taken from either Tip or Nodal positions, not on the shoots which arose from the Base position.

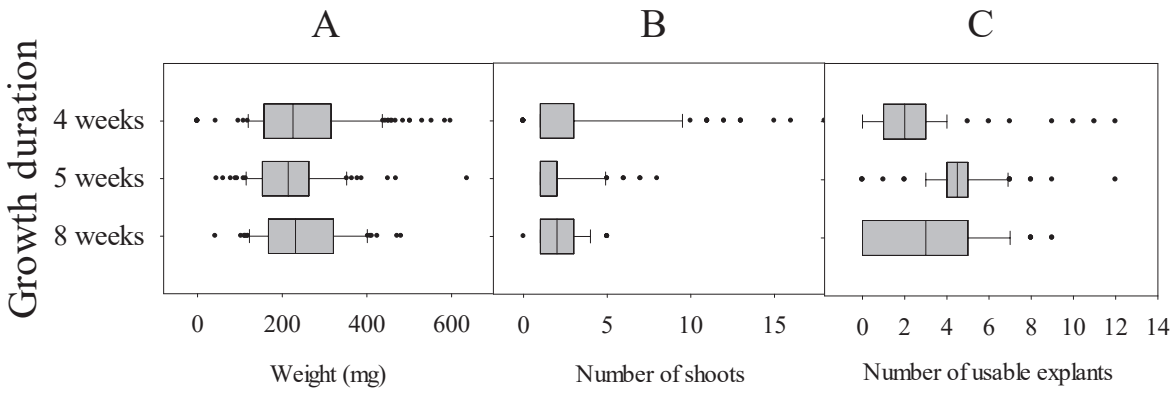


Figure 2-9: Growth of gentian 'Little Pinkie' explants after 4, 5 or 8 weeks; **(A)** fresh weight, **(B)** number of shoots, **(C)** number of usable explants. Vertical line in the centre of the box indicates median value. Boundaries of box indicate 25th and 75th percentiles; whiskers present the range between 10th and 90th percentiles, individuals beyond these boundaries are represented as stars, n = 6 vessels each containing 8 explants.

Table 2–4: The influence of duration of culture *in vitro* on the growth of gentian 'Little Pinkie' taken from explants of a range of all positons.

Growth duration (Week)	Multiplication rate (%)	Flowering (%)
4 weeks	75	0
5 weeks	92	0
8 weeks	26	30

It had been previously hypothesised that; by extending the duration of culture from 4 to 8 weeks the number of usable explants taken from the less developed shoots would increase. The results achieved in the current experiment however (Table 2-4), did not support the hypothesis, due to flowering after 8 weeks. Also, extending the duration of culture from 5 to 8 weeks, did not result in a reduction in the non-uniformity (Figure 2-9). This was interpreted as indicating that 5 weeks of culture was probably optimum for the explants taken from various positions to reach to their maximum possible growth, albeit it is acknowledged that other limiting factors such as correlative inhibition among the shoots and/or declining water and nutrition content of the medium, gaseous condition (including CO_2 content of the atmosphere in the headspace of vessels) in an *in vitro* system, could also be influential (Snow, 1937; Nour and Thorpe, 1994; Takahashi et al., 2012).

For the current thesis, although explants from the Tip position gave more uniform plantlets with less shoot formation, which might seem to be ideal to assess the effect of treatments in

future experiments, the logistics of producing an adequate number of explants solely from this position were considered too limiting for the purposes of future experiments. Also, for durations of culture for more than 5 weeks there was a high risk of explants becoming floral and being removed from the propagation system. Therefore, explants from the nodal position were utilised to maximise the number of propagules to be derived per donor plantlet. Also, to optimise growth of nodal explants, a duration of culture of 5 weeks was recommended above 4 and 8 weeks for *in vitro* propagation of 'Little Pinkie'. As general experimental protocols arising from both Experiment 1 and 2, increasing the number of replications, ensuring an even distribution of the same number of explants from each position within each experimental unit (i.e. culture vessel), or keeping each position in separate vessels as an experimental block, should be considered for future experiments to minimise and/or account for variation likely to be present.

Assuming that for commercial propagation it is important to get uniform explants that subsequently produce a uniform number of usable explants when grown themselves, then explants from Node 2, which were quite variable in the number of usable explants subsequently derived, would not be regarded as being most suitable for propagation. On the other hand, almost one in every four explant taken from Node 2 (25% of the population at the upper whisker, Figure 2-6B) was capable of producing up to eight shoots/explant. Within a commercial horticultural scenario, a propagule with more number of shoots per explant is more likely to produce a high-quality pot plant (Sachs et al., 1976). Modification of the protocol for commercial propagation is likely to be required, should all positions be used, requiring grading of explants on the basis of their shoot production at the point of de-flask for supply to pot-plant forcers. Upper nodes like Node 1, which would give many propagules, could be used for further subculture *in vitro*; but from the same original plantlet lower nodes could also be used to generate material with high number of shoots (25% in the case of Node 2) targeted to be used for production of potted plants. While it is recognised these are interpretations of the performance of propagules from the current experiments, further research is recommended to target stimulation and development of non-developed primordia/shoots, arising from the Base and Node 2, so as to achieve more developed shoots in the shortest possible timeframe.

2.4. Conclusions

There was a strong positional effect on the growth response of explants of gentian 'Little Pinkie' clonally propagated *in vitro*. However, the effect of position on the growth variables of another cultivar ('Showtime Diva') was not significant, being indicative of the cultivar dependency of these responses in gentian. With 'Little Pinkie' it is likely that, differences between positions was probably due to diverse physiological conditions prevailing in the explant used (e.g. endogenous levels of plant growth regulators) and different physiological stages of development at different positions.

Due to the wide variation in growth response (number of 1º, 2º or 3º) shoots, number of usable explants from these shoots, and fresh weight) of 'Little Pinkie', explants from the Base position might not be suitable to be the source of uniform plant material for experiments within this thesis. By excluding the Base position from the source of plant material and keeping explants in culture for 5 weeks, which was recommended above 4 or 8 weeks for *in vitro* propagation of 'Little Pinkie', the uniformity was improved. Extending the duration of culture to 8 weeks was not recommended as it resulted in a decline in the multiplication rate because of flowering. From the results of both experiments, the commercial implication was to modify the protocol of the propagation system from one of mass propagation to selective propagation.

B: Early flowering; causes and control

2.5. Introduction

While this section is presenting the initial experimental chapter within the thesis, it should not be interpreted as being one of the first experiments conducted in a chronological series of experiments. In fact the problematic situation that was addressed in the experiments in this chapter emerged and developed while conducting the research reported in subsequent chapters of this thesis. As a consequence, while the content of the current chapter is not targeted at the central core of the research question explored within the thesis (i.e. determining the mechanism of ethephon's effect on shoot formation), it contains details of investigations which benefited the entire research program, and is therefore presented here.

A high percentage of flowering in explants of 'Little Pinkie' (more than 50% in some subculture populations) became a substantial problem during subsequent multiplications, it

was not an issue in 'Showtime Diva' (data not presented). From Experiment 2 in Chapter 2, the number of flowering explants increased by 30% simply by extending the duration of culture from 5 to 8 weeks and, as a result, multiplication rate decreased (refer to Section 2.3.3). Hence, a culture duration of 5 weeks was utilized for *in vitro* propagation of 'Little Pinkie' through the rest of the research work (i.e. Chapters 2 through 7). However, despite removing the flowering explants from the population and keeping time intervals between subsequent sub-cultures to no longer than 5 weeks, some explants derived from this propagation system flowered. Thus, a duration of culture longer than 5 weeks did not seem to be the only factor that controls flowering *in vitro*. Prior to this research early flowering had been also reported anecdotally to occur *in vivo* after being de-flasked, which would affect the uniformity in flowering time. Factors both *in vitro* and *in vivo* might affect the phase change from vegetative to flowering (Dickens and Van Staden, 1988; Roldan et al., 1999; Funnell, 2008; Da Silva et al., 20014) at any stage of the pot plant production system and, therefore, affect uniformity in time to flower. Section B of this chapter therefore presents a series of experiments carried out to investigate the incidence of early flowering observed in gentian and identify influential factors and strategies to control it.

Initiation of flowering in an *in vitro* system designed for clonal propagation is typically unwelcome, due to the reduction in efficiency of multiplication *in vitro* and/or production *in vivo*. Given the determinate shoot growth habit of gentian, initiation of flowering *in vitro* might be expected to affect the vegetative growth of the apical bud and, if so, the efficiency of the micro propagation system. Within a system designed for propagation, flowering imposes an end to vegetative growth of the explant and, therefore, is unwanted. Flowering is associated with alteration in the endogenous hormonal balance (Tanimoto et al., 1985; De Melo Ferreira et al., 2006), consumes nutrient resources and results in a reduction in the efficiency of the propagation system by reducing the multiplication rate (refer to Section 2.3.3). In terms of the current research programme, flowering would change the number of usable explants, and potentially interfere with the results of experiments designed to assess other treatment effects. Within the current thesis therefore, keeping explants in active vegetative growth was a prerequisite to undertake experiments focussed on shoot formation, typically a vegetative growth phenomenon. It was hypothesised that factors affecting *in vitro* flowering might also affect the uniformity in time to flower *in vivo*, once de-flasked. Hence, as noted previously within this chapter, flowering progressively emerged as a problematic situation to be addressed as the thesis progressed. As such however, it was considered a

critical objective for the whole research programme to minimise the percentage of explants which flowered *in vitro*.

In a commercial production system of ornamental plants, the duration to flower, timing of flowering and its uniformity are often of particular focus (Funnell, 2008; Chen et al., 2010). As a newly developed cultivar of gentian suited to potted plant production, the research programme initially set out to determine the duration to flowering of *in vitro* propagated plants of 'Little Pinkie' growing *in vivo* subsequent to de-flasking. As perennial herbs, gentians are reported to require several growth cycles before flowering occurs (Ohkawa, 1983), so time to flower following propagation *in vitro* was initially quantitatively determined in the first experiment of this section (1), with this data being provided by staff within Plant & Food Research for integration into the current thesis.

Flowering in ornamental plants is a critical developmental stage which, depending on a plant's age involves their transition from a vegetative (producing leaves) to reproductive phase (producing flowers), as a result of favourable conditions/signals (Fekih et al., 2016). Some plants are not capable of producing flowers, even under favourable conditions, if they are in early stages of their life cycle and, therefore, remain in a phase known as the juvenile vegetative phase. The juvenile vegetative phase is then followed by the adult vegetative phase, in which plants can respond to flower induction signals and enter the reproduction phase (Matsoukas, 2014; Sgamma et al., 2014). Various species differ in length of the juvenile phase, from a period of a few days, to periods longer than 20 years (Matsoukas, 2014). Gentian has been reported to have a long (1 to 3 years) juvenile phase from seed, but once reached to the stage of producing flowers during two subsequent years, it would most likely flower in the next year (Rose et al., 1998; Nakatsuka et al., 2009). Therefore, it seems that once flowering has been established within the plant it will be continued unless strategies would be used to revert the plant to the juvenile phase (i.e. re-juvenation) (Hackett, 1985).

The effect of position that explants were originated from along the donor shoot, on early flowering, was investigated in Experiment 2. Anecdotal observations while sub culturing, as well as the results of previous experiments (refer to Section 2.3) indicated more frequency of flowering in explants derived from the Tip (apex and undeveloped leaves and nodes located at the distal end of the shoot) or Nodal (a mixture of nodes (two), between the tip and base positions) positions of 'Little Pinkie' compared to the Base (original explant which was the closest node to the roots at the proximal end of the plantlet) position. However, data were

needed to statistically support this observation. Explants derived from the lower nodes, as compared to the upper nodes, produced a multiple number of shoots which, as evident via SEM (Figure 3-5), were likely originated from adventitious bud primordia. While SEM has not been done on the explants of the Base position, by the evidence of development of multiple shoots (Figure 3-7B), probably multiple bud primordia also existed at the node of Basal explants. Shoots arising from adventitious buds, were considered to probably be less developed (i.e. smaller leaves, narrower stem with fewer nodes) as compared to the shoots arising from preformed axillary buds. It seemed plausible therefore that shoots arising from adventitious buds were potentially juvenile and, therefore, in their vegetative phase, compared to the shoots from pre-existing axillary buds (Samarakoon et al., 2013). Therefore, using shoots arising from adventitious buds for clonal propagation, was hypothesized to decrease the possibility of early flowering in the resulting plantlets and, therefore, decrease the frequency of flowering within the propagation system. Hence, within this chapter an experiment was conducted using rejuvenated plant material (raised from only the Base position), to study the effect of various positions on flowering frequency.

For explants cultured *in vitro*, the culture medium was a source of PGRs and/or nutrients, and would be one of the environmental factors which could contribute towards changing explants from vegetative to floral phase, while internal factors such as age and endogenous hormonal balance, are also considered influential (Koornneef et al., 1998; Da Silva et al., 2014). Nutrient composition of the *in vitro* medium, high phosphorus and low nitrogen, has been reported to encourage flowering in *Dendrobium* cultured *in vitro* (Tee et al., 2008). Application of various PGRs such as 6-benzyladenine (BA), N6-isopentenyladenine (2- iP), TDZ, paclobutrazol (PBZ or PP333), GA_3 or combinations of other PGRs and nutrients, have also induced flowering within a variety of plant species cultured *in vitro* (De Melo Ferreira et al., 2006; Tee et al., 2008; Te-chato et al., 2009; Wang et al., 2009a). For application within the current thesis therefore, it was hypothesised that *in vitro* flowering of gentian explants could also be potentially affected by PGRs and/or nutrients in the medium and, therefore, could be possibly reduced by removing PGRs and/or nutrients from the medium, or changing their quantity within the medium. However, it is acknowledged that the experiments did not attempt to include and investigate all components of the medium. Only the effect of removing PGRs from the medium and decreasing the concentration of MS salts was investigated within this thesis. In doing so, considering the confounding effect of PGRs versus strength of MS salts, the comparative trial (Experiment 3) was designed merely to investigate the possibility

of decreasing the number of flowers produced by the explants cultured in a modified medium (PGR-free and with MS salts at half strength) as compared with the medium which had been commonly used within the tissue culture lab of PFR for propagation of gentian.

As reported by previous investigators, phase change from vegetative to reproductive in a variety of plant species would start following encountering environmental changes such as temperature and/or photoperiod (Campos and Kerbauy, 2004; Vaz et al., 2004; Singh et al., 2006; Shou et al., 2008; Chen et al., 2010). Irrespective of whether grown *in vivo* or *in vitro*, information on flowering control by environmental factors in gentian is very limited, and the effect of artificial modifications of shading/lighting and temperature control has been reported to have no predictable effects on flowering (Nakatsuka et al., 2009). In one of the few publications on environmental physiology of gentian (not juvenile), plants did not produce flowering shoots in the second growth cycle *in vivo* if they did not receive temperatures below 16 ºC during winter (Samarakoon, 2012). In contrast, within the current thesis donor plantlets and explants were grown and sub-cultured continuously *in vitro* at 25 ± 1 °C and, therefore, flowering was not expected to occur. Photoperiod corresponding to the native habitat of parental lines of the cultivars (*Gentiana triflora* and *G. scabra*) used in this research has been reported to be 14h to 17h in June (i.e. summer) and a minimum of 7h to 10h in December (i.e. winter) (Lammi, 2005; Samarakoon, 2012). It seemed reasonable to assume that the photoperiodic requirements of the cultivars utilized had been inherited from their parental lines and, therefore, the aforementioned range of photoperiod were used as long (16h) and short (8h) photoperiods for application within the current research. Average air temperatures in Japan, where cultivation of gentian has been commercially done, ranges from 22.5 ºC to 13.7 ºC in summer and -1.4 ºC to -7 ºC in winter (Samarakoon, 2012). However, as practised as part of the standard protocols of sub-culturing within the lab, an air temperature of 25 ± 1 °C and a 16 h photoperiod (i.e. long days) corresponding to summer-time conditions in natural habitats and commercial production sites was applied. At the time commencement of this thesis, donor plantlets and explants had been sub-cultured for multiple growth cycles (at least 3 to 4 years). Such differences between the natural environmental conditions and those applied *in vitro* was, therefore, hypothesised to be another possible factor responsible for the progressive increase observed in the frequency of flowering.

It is possible that for the explants cultured *in vitro* under continuous growth, there was a need for a period of dormancy (Shimizu-Sato and Mori, 2001) which would normally occur during winter in the natural annual growth cycle of a plant. Application of 2-3 weeks short

photoperiod (8h) in winter, could make plants dormant which then could be broken by cold temperature at 2°C; being a temperature noted as sufficient to break seed dormancy in gentian (Morgan et al., 1997). Winter dormancy possibly provided an opportunity to stop/slow down the active vegetative growth of the plants, and perhaps re-set the endogenous condition which control biological activities within the plants in the next growth season/cycle. Donor plant material initially taken from the field to initiate the *in vitro* population of explants, had already gone through natural dormancy and were in their active growth season, while they were physiologically capable of flowering. Consequent exposure of the explants to conditions that provoke continuous vegetative growth *in vitro*, without any dormancy period, was hypothesised to result in flowering *in vitro*. If so, as explored within the current thesis, application of cold temperature (2°C) and short photoperiod (8h) would provide the condition for both induction (both endo-dormancy and eco-dormancy) and breakage of dormancy; Faust et al, 1997) on donor plantlets of gentian cultured *in vitro*. As a result, frequency of flowering on explants would be expected to decrease. Provided that dormancy occurred, then there was also a need to investigate the effective duration of dormancy. In the current research therefore, a comparative trial (Experiment 4) was conducted to investigate the possibility of decreasing the number of flowers produced by the explants exposed to dormancy as compared with the explants without dormancy. Temperature and photoperiod, applied as a confounded treatment within the current research (i.e. combination of cold temperature of 2ºC and short photoperiod of 8h) to compare with sustaining the conditions used during cultivation within the lab (i.e. temperature of 25 ± 1°C and 16 h photoperiod), in order to initially test the hypothesis that explants of 'Little Pinkie; been under frequent sub-cultures require a dormancy period to inhibit flowering and retain them in their vegetative condition.

In the current section therefore, Experiment 1 was carried out to quantify the time to flower *in vivo* subsequent to de-flasking, as well as to statistically substantiate the anecdotal observations of early flowering *in vivo*. Also, in order to find a practical strategy to decrease early flowering *in vitro*, the effect of influential factors such as position of the explant, constituents of the culture medium, cold temperature and photoperiod on early flowering of gentian cultured *in vitro* were investigated in Experiments 2 to 4. With the overall aim of optimising current protocols for vegetative propagation of 'Little Pinkie', a series of experiments (1 to 4) were conducted with the objectives to:

- quantify the time needed for rooted plantlets from being de-flasked to the date of appearance of the first coloured flower bud when grown *in vivo* (Experiment 1),
- quantify the frequency of flowering in explants cultured *in vitro* originating from various positions along the donor shoot (Experiment 2),
- quantify frequency of flowering in explants originating from rejuvenated shoots cultured *in vitro* arising from the bases as compared with non-rejuvenated explants arising from nodal explants (Experiment 2),
- quantify and compare the frequency of flowering in explants cultured *in vitro* in a modified medium (PGR-free and MS salts at half strength) as compared with the commonly used medium in the tissue culture lab (Experiment 3),
- quantify and compare the frequency of flowering in explants cultured *in vitro* following exposure to a dormancy (induction/breaking) treatment as compared with the explants without such exposure (Experiment 4),
- quantify the effective duration of dormancy (induction/breaking) treatment to prevent early flowering *in vitro* (Experiment 4).

Results of all experiments (1 to 4) are presented and discussed at the end of this section.

2.6. Materials and methods:

2.6.1. Experiment 1

Time to flower in vivo

Plant material originated from plantlets which had been previously grown and sub-cultured in a five-weekly cycle *in vitro* (refer to Section 2.2), with plant growth regulators (PGR) included within the medium to stimulate shoot proliferation or rooting as required (Morgan et al., 1997). Over a four-week period of establishment, rooted propagules were de-flasked into a bark-based growing medium in 60-cell trays (45 ml cell volume) and placed in a fog tent with basal heating (23 ± 2°C for 1 week), before transfer to an open mist bench for a further week in a heated greenhouse (16°C minimum, vented at 24°C). Plants in plug trays were subsequently transferred to an open bench in the greenhouse with capillary irrigation for a further 2 weeks of growth before being potted into 15 cm diameter pots in the same greenhouse environment. The growing medium used comprised A-grade bark fines (50%), bark fibre (30%), and pumice 7 mm (20%) supplemented with 1 $kg.m^{-3}$ serpentine super, 1.5 $kg.m^{-3}$ dolomite, 2 $kg.m^{-3}$ 8–9 month Osmocote® (16N–3.5P–10K, Everris International,

Geldermalsen, The Netherlands), and 1 kg.m^{-3} 3–4 month Osmocote® (15N–4.8P–10.8 K). Throughout the experiment, irrigation was delivered by micro-tubes on a drained capillary matting bench for 10 min three to five times each day, depending on plant requirements. Despite multiple dates at which plants were de-flasked, daily air temperature typically ranged between 15°C and 24°C, from July until the end of the experiment in November. During the progression of natural photoperiod throughout July to November, in order to eliminate any potential effect of photoperiod on flowering, a long day photoperiod regime (2 h night break lighting at 4.6 µmol.m^{-2}.s^{-1}from 2300 to 0100 HR; Samarakoon et al., 2015) was applied to plants after being de-flasked in the greenhouse.

Treatments comprised plantlets which were de-flasked on each of five dates at approximate 4-week intervals, i.e. 8 July, 5 August, 9 September, 7 October or 3 November. Plants were monitored weekly and, for individual plants, the date of first coloured flower bud, and for each date of de-flask the number of saleable pot plants (i.e. three flowers open) was recorded. Within each date of de-flask, the experiment was arranged as a Completely Randomised Design, utilising 40 single-plant replicates.

2.6.2. Experiment 2

Position of the explant on flowering

Plant material originated from plantlets which had been previously taken from either Node or Base positions, grown separately and repeatedly sub-cultured in a five-weekly cycle *in vitro* (refer to Section 2.2), with plant growth regulators (PGR) included within the medium to stimulate shoot proliferation or rooting as required (Morgan et al., 1997). Plantlets which had been exclusively grown from only the Base position in their previous growth cycle were considered as having been rejuvenated, whereas plantlets grown from the Node position were considered as non-rejuvenated for the current experiment. Two experiments were carried out in this section on the effect of position on flowering. Different plant material (rejuvenated or non-rejuvenated) were used in these two experiments. In the first experiment, all plant material were non-rejuvenated. However, in second experiment, rejuvenated plant material were used to be compared with the non-rejuvenated plant material in first experiment, for the effect of their position on flowering. In both experiments plantlets were selected for uniformity in length and number of nodes on their donor shoots. Donor shoots of uniform length with three expanded internodes were selected and cut into explants, each 10 mm in length as either: Tip (apex and undeveloped leaves and nodes), Node 1 (first node below the

tip) or Node 2 (the second node below the tip; Figure 2-11) for the first experiment. In the second experiment, explants were taken from only Tip and Node 1 positions (the most likely positions to produce flowering explants) on the rejuvenated shoots, allowing comparison with Tip and Node 1 positions of the non-rejuvenated explants in first experiment. Explants of each position were cultured in separated culture vessels containing 50 mL of medium (PGR-free) (refer to Section 2.2).

Data for the number of explants which flowered *in vitro* as well as the number of usable explants (minimum internode length of 10 mm) derived from each explant, were recorded after a 5-week period of culture and percentage of flowering explants per vessel was calculated. First experiment of this section was conducted in a completely randomised design, utilising positions as treatments (Tip, Node 1 or Node 2), and each of the six culture vessels as individual replicates, each containing eight explants as subsamples. Statistical analysis to compare both experiments was done using a 2 × 2 factorial arrangement of treatments: age (rejuvenated and non-rejuvenated) and position (Tip and Node 1). ANOVA for the first experiment and General Linear Model for the contrast between first and second experiment was done in Genstat (Genstat, version 17, 2014, VSNi Ltd, Hemel Hempstead, UK) and Fisher's unprotected least significant test at $P \leq 0.05$ was applied to undertake mean comparisons.

2.6.3. Experiment 3

In vitro medium on flowering

The effect of composition of the *in vitro* medium on flowering in the explants was investigated utilising two media, i.e. either:

- common medium – being that utilised historically as part of the sub-culturing protocol within the tissue culture lab of PFR, comprising MS (Murashige and Skoog, 1962) macro-salts at full strength, B5 vitamins as well as a combination of PGRs (0.05 $mg.L^{-1}$ Indole Butyric Acid (IBA), 0.3 $mg.L^{-1}$ BA and, 0.1 $mg.L^{-1}$ GA_3) (refer to Section 2.2), or
- modified medium - a PGR-free medium comprising MS macro-salts at half strength and LS (Linsmaier and Skoog, 1965) vitamins.

Both media included MS micro-nutrient salts (Murashige and Skoog, 1962), 7.5 $g.L^{-1}$ agar (Davis) and 3% (87.6 mM) sucrose.

Plant material for this experiment had been initially sub-cultured from the main population of 'Little Pinkie' in the tissue culture lab of PFR which had been previously cultured in a five-weekly cycle *in vitro* (refer to Section 2.2), with plant growth regulators (PGR) included within the medium to stimulate shoot proliferation or rooting as required (Morgan et al., 1997) for at least two years. Therefore, to minimise the potential influence of any residual PGRs, and to create a PGR-free treatment (i.e. modified medium), explants were sub-cultured using the modified medium for at least 3 growth cycles before the current experiment (Figure 8-1). Shoots were selected and cut into explants, as either: Tip, Node 1, Node 2 or Base (Chapter 2, Figure 2-2).

A factorial combination of treatments was applied, comprising position (Tip, Node 1, Node 2 or Base) and medium (common medium or modified medium). There were five culture vessels each as individual replicates, with each including eight explants taken from all positions as sub-samples, which were distributed evenly between vessels. After 5 weeks culture under the standard *in vitro* conditions (refer to Section 2.2), data were recorded for the number of flowering explants. Statistical analysis utilised the General Linear Model in Genstat (Genstat, version 17, 2014, VSNi Ltd, Hemel Hempstead, UK) and Fisher's unprotected least significant test at $P \leq 0.05$ was applied to conduct mean comparisons.

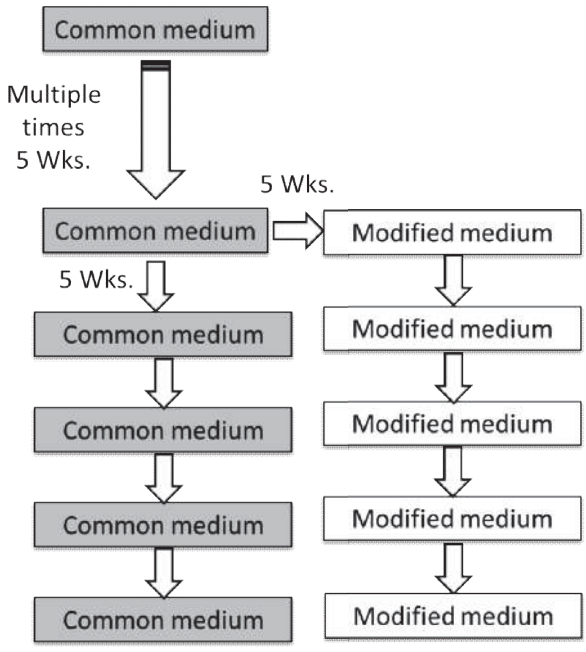


Figure 2-10: Schematic diagram illustrating the prehistory of *in vitro* medium(either common medium or modified medium) on which explants were sub-cultured on 5-weekly cycles before being utilised as plant material for the comparative medium trial (Experiment 3). Arrows represent 5 weeks duration of growth before plantlets were sub-cultured to a new medium.

2.6.4. Experiment 4

Temperature and photoperiod on flowering

As illustrated in Figure 2-10, when originally initiated into an *in vitro* system the donor plant material taken from the field had previously passed through natural dormancy, were in their active growth season, and physiologically capable of flowering. Consequently explants had been exposed to continuous *in vitro* vegetative growth without any dormancy period using the protocol described earlier (refer to Section 2.2). Plantlets grown *in vitro* then were used in order to create treatments for the current experiment.

A factorial combination of treatments was applied, comprising position of the explants (Tip, Node 1, Node 2 or Base; Chapter 2, Figure 2-2) and duration of exposure to cold temperature (2°C ± 1 °C; for either 0, 5, 7, 9 or 12 weeks) as delivered within an incubator (SK650, Skope Industries Ltd., NZ.), and 8h photoperiod at a PPFD of 30 ± 5 μmol.m^{-2}.s^{-1}, provided by cool-white fluorescent tubes. There were five culture vessels as individual replicates for each treatment combination, each including eight explants taken from all positions as sub-samples, with positions distributed evenly between vessels. In order to make the treatment combinations, five replicates of culture vessels were randomly selected, taken out of the incubator periodically after 0, 5, 7, 9 or 12 weeks, and introduced into fresh *in vitro* medium (PGR-free) and grown under the standard environmental conditions (refer to Section 2.2) for 5 weeks before evaluation which has been previously noted to be effective on flowering decrease to some extent (Figure 2-11). After 5 weeks of culture, data were recorded for the number of flowering explants and percentage of flowering explants from the total number of explants was calculated.

Data were analysed using the General Linear Model in Genstat (Genstat, version 17, 2014, VSNi Ltd, Hemel Hempstead, UK) and Fisher's unprotected least significant test at $P \leq 0.05$ was applied to undertake mean comparisons. In some cases, Square root-transformation was applied to the data in order to make the variability more equal between replicates and between explants within each replicate.

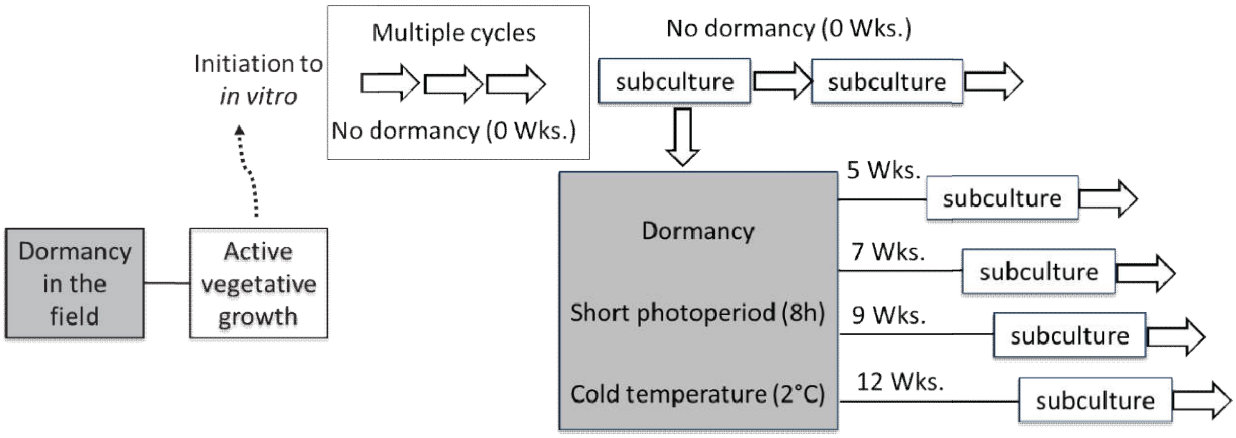


Figure 2-11: Schematic diagram illustrating the prehistory of exposure of explants to dormancy including their growth in the field, initiation in to *in vitro* culture, exposure to continuous vegetative growth for multiple 5-weekly growth cycles, before being used for creating treatments within the current experiment (Experiment 4) by receiving short photoperiod (8h) and cold temperature (2°C) artificially in an incubator (for various durations of 0, 5, 7, 9, or 12 weeks). Arrows represent 5-weekly growth cycles for each subculture.

2.7. Results and discussion

2.7.1. Time to flower *in vivo*

For the dates of de-flask investigated in Experiment 1, spanning July to November, there was a significant reduction in the duration from de-flask to when the first flower bud showed pink colouration ($P \leq 0.05$). The maximum number of days from de-flask to flower was up to 68 days for plants de-flasked in July, but was reduced to 47 days for plants de-flasked in November. Plants de-flasked in November commenced flowering 21 days earlier than those which were de-flasked in July (Figure 2-12). A similar declining trend was observed in the number of plants considered saleable (three flowers open), with the progressive reduction in plant numbers achieving saleable quality with incremental dates of de-flask. Production efficiency for explants which were de-flasked in November (latest date) was reduced by 40% compared to those in July (first date). Since the use of night-break lighting and temperature control is believed to have removed seasonal differences between dates of de-flask, it could be interpreted that non uniformity in time to flower were as a result of changes occurring prior to de-flask. Hence it can be concluded that *in vitro* plantlets with flowers being either initiated or fully developed, should not be considered for de-flask commercially as their subsequent performance as a potted plant is expected to be poor.

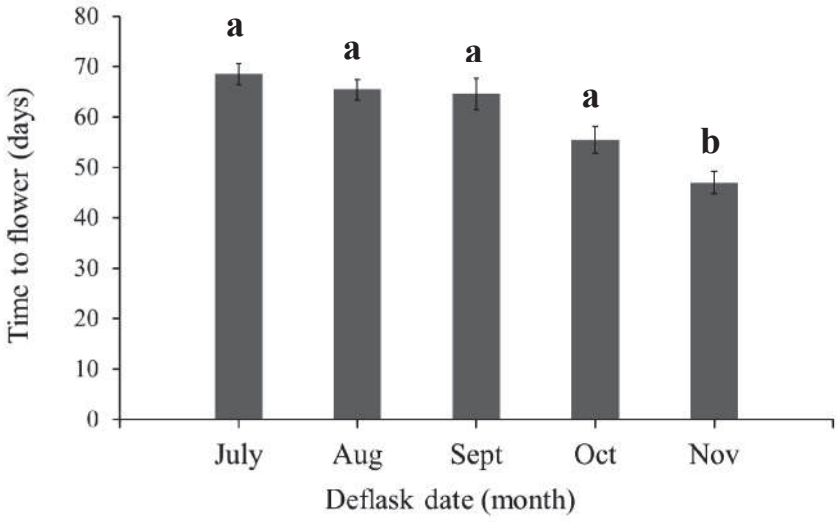


Figure 2-12: The duration from the date of de-flask until when the first apical flower bud on the propagule of gentian 'Little Pinkie' showed colour in the petals, for de-flask dates from July to November. Means with the same letter are not significantly different at $P \leq 0.05$. n = 40 explants.

2.7.2. Position of the explant on flowering

In Experiment 2, the average number of flowering explants *in vitro* was influenced by the position from which explants were derived ($P \leq 0.001$; Figure 2-13). The propensity of explants to flower decreased as the distance of the position of the explant increased from the shoot tip on the donor shoot. From a total number of eight explants per vessel originating from the Tip position, on average ~35% produced flowers (Figure 2-13). In contrast explants derived from the Node 1 and 2 positions achieved about four times less flowering compared to those from the Tip position (Figure 2-13).

The present data confirmed the effect of explant position as an influential factor on the propensity for flowering *in vitro* (Jumin and Ahmad, 1999; Zhang and Leung, 2002), but additionally extended understanding by including the tip of shoots. The difference in flowering propensity between positions has been suggested to be related to the difference in the type of buds, juvenility and capacity of their cells to respond to a gradient of a floral promoter, inhibitor or their combination, existing in each position (Wardell and Skoog, 1969; Fu et al., 1995). As illustrated in Chapter 3 of this thesis, for 'Little Pinkie' shoots arising from Node 1 and Node 2 positions included those derived from pre-existing axillary buds, as well as adventitious buds. As evident with established plants in the field of other cultivars of gentian (Samarakoon et al., 2015), flowering shoots in the current growth cycle *in vivo* typically arise from crown buds formed in the previous growth cycle. As hypothesised by Samarakoon and colleagues, it also was considered likely in the current experiment that pre-

existing buds (i.e. both apical and axillary) may have already progressed to flower induction, and would differ in their propensity to flower from buds which might have developed later on during growth (i.e. adventitious; refer to Chapter 3). Similarly in mature trees, basal (collar) sprouts arising from the buds which might have developed later during the growth season were reported to be more juvenile than shoots arising from pre-existing buds in the canopy and, therefore, the base (collar) of the tree could be physiologically considered as a specialized organ of regeneration and rejuvenation (Fortanier and Jonkers, 1975; Del Tredici, 2001). As compared to pre-existing apical and axillary buds therefore, those adventitious buds produced at the base of the explants cultured *in vitro* might be of a younger physiological age, with less propensity to flower.

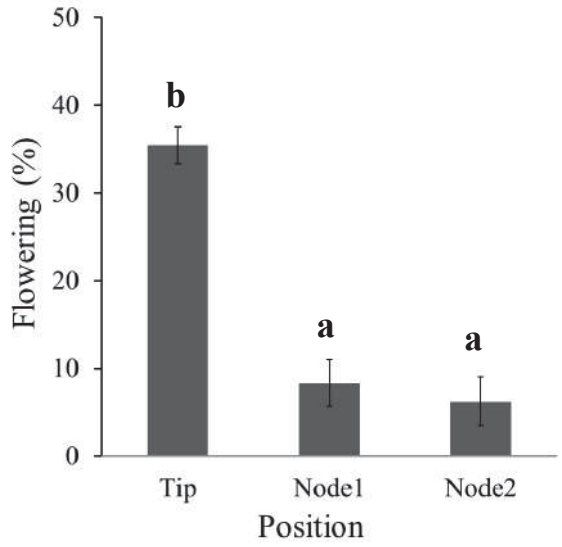


Figure 2-13: Flowering proportion of explants of gentian 'Little Pinkie', per culture vessel after 5 weeks culture *in vitro*; explants were taken from different positions on the donor shoot: Tip, Node 1 (first node below the tip) or Node 2 (second node below the tip). Means with the same letter were not significantly different at $P \leq 0.05$ (Fisher's protected least significant difference). Vertical lines represent ± standard error of means, n=6 vessels each containing 8 explants.

The concept of juvenility was first used by (Goebel, 1900) to explain the differences in growth between propagules taken from various parts of the plant. Meristems forming at the tip are of younger tissue, they may be physiologically 'less juvenile' than meristems forming on the lower part of the stem (Fortanier and Jonkers, 1975). A decrease in juvenility with position along the shoot can be defined as ontogenetical aging, which is genetically programmed in the apex of the initial explant and effectively counts the number of cycles of cell division to make various positions along the shoot. While many factors might be involved, it is likely that the cells at various nodes which differ in their age and juvenility vary in propensity to flower due to the difference in their sensitivity to, and/or concentration

of, floral promoters and/or inhibitors (Firn, 1986). Further research is, however, still required in order to substantiate these possibilities in 'Little Pinkie'.

As reported in earlier experiments, the propensity for 'Little Pinkie' to flower *in vitro* increased up to 71% when the duration of the culture cycle increased from 5 to 8 weeks (refer to Table 2-4,). In the current experiment however, despite keeping time intervals between sub-cultures to no longer than 5 weeks, some explants still continued to flower while *in vitro*. We interpret this to indicate that induction/initiation of flowering in 'Little Pinkie' will occur in explants, and that a duration of culture of more than 5 weeks is not the only factor leading explants toward flowering *in vitro*.

A declining trend in propensity to flower for the explants taken from various positions along the donor shoot, from Tip to Base, resulted in a progressive decline in the number of non-vegetative explants discarded from the sub-culturing cycle and, consequently, a significant increase in multiplication rate ($P \leq 0.001$). The multiplication rate of explants taken from Node 1 and Node 2 positions were, respectively, 2.3 and 3 times more than the multiplication rate of the explants taken from the Tip (Figure 2-15). In a commercial tissue culture laboratory therefore, it would be possible to minimise flowering and increase the multiplication efficiency by excluding the Tip position from the propagation material in sub-cultures. Multiplication rate of the explants taken from various positions in current experiment was different and less than the number of usable explants (equivalent to multiplication rate) in Experiment 2 (refer to Section 2.3.1.4). Explants which had been used as plant material in Experiment 2 were probably of more active vegetative growth than the explant used in current experiment. In Experiment 2, which similar positions as current experiment were used, explants taken from Tip, Node 1 and Node 2 positions all produced similar average of four usable explants (Table 2-6) whereas, in the current experiment, explants taken from Tip, Node 1 and Node 2 positions respectively produced an average of 1, 2 and 4 usable explants.

With a determinant shoot growth habit in gentian, flowering either stops or at least slows down the vegetative growth of the explant, and results in a decline in the number of nodes and internodes on flowering shoots as compared with the vegetative shoots (Figure 2-14). This interpretation is consistent with the concept of an irreversible alteration in the shoot apex, which is a concomitant with the phase change from vegetative to floral (Fortanier and Jonkers, 1975). The multiplication rate in flowering explants would decrease because of the reduced number and length of vegetative shoots arising as shoots per explant. However,

identification of flowering while explants are *in vitro* could save time and resources and, therefore, benefit the overall production efficiency by eliminating flowering explants from the propagation cycle at an early stage, before being grown *in vivo*. If flowers only become visibly evident once de-flasked, but earlier than expected, not only will the multiplication rate be reduced but, as evident by Experiment 1 (Section 2.2.1), when included as plants to be grown *in vivo*, they do not produce shoots and the production efficiency within the greenhouse phase was reduced by up to 40%. From anecdotal observations, although early flowering in pot plants of 'Little Pinkie' does not necessarily permanently stop the subsequent production of multiple shoots, it reduces and/or delays the shoot formation and, therefore, negatively impacts on plant quality and timing (Sachs et al., 1976). Therefore early flowering appears to be detrimental both in terms of effective crop scheduling and production efficiency.

Figure 2-14: Vegetative growth of the explants of gentian 'Little Pinkie' after 5 weeks growth *in vitro* in a flowering explant (right) was less than the vegetative growth in non-flowering explant (left).

As illustrated in Figure 2-16, the percentage of flowering explants per culture vessel almost halved following the application of a treatment that might be inferred to provide a rejuvenation (i.e. using donor shoots arising from adventitious buds located at the node of the explants of the Base position) compared to those without rejuvenation (i.e. taken from positions other than the Base) ($P \leq 0.05$). Similarly, juvenile explants taken from relatively young and vegetative plants of various species, even under conditions which were suitable for flower bud formation, produced only vegetative buds (Lang, 1965; Matsoukas, 2014; Sgamma et al., 2014). In these other reported experiments the reason that flowering did not occur in plantlets developed from the juvenile plant material was hypothesised to be due to

either incapability to produce flowering factor(s) or having non-responsive meristems to flowering factors (Lang, 1965; Hackett, 1985).

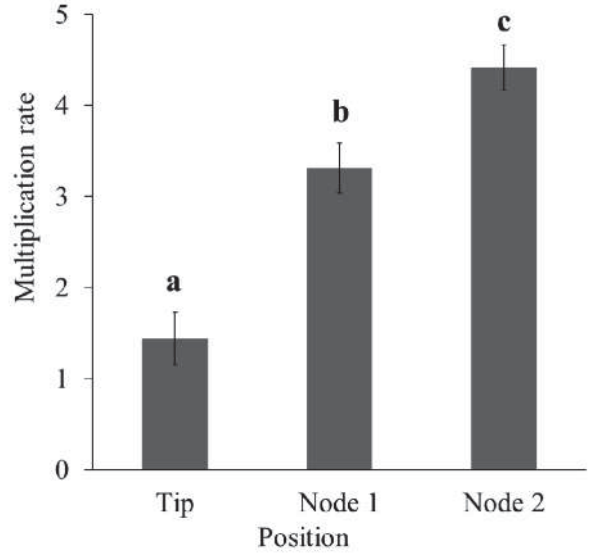


Figure 2-15: Multiplication rate (number of usable explants taken from donor explant) of gentian 'Little Pinkie' explants taken from different positions: Tip (shoot tip), Node 1 (first node below the tip) or Node 2 (second node below the tip) after 5 weeks' growth *in vitro*. Means with the same letter are not significantly different at $P \leq 0.05$ (Tukey). Vertical lines represent ± standard error mean, n=6 vessels each containing 8 explants.

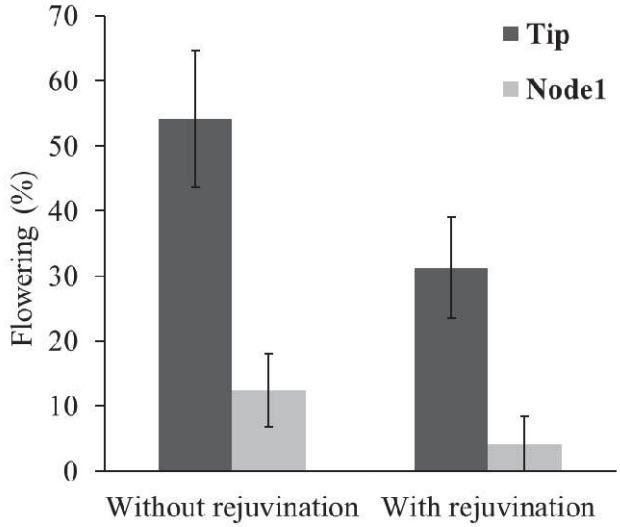


Figure 2-16: Flowering explants of gentian 'Little Pinkie' with rejuvenation (developed from the buds at the node of the Base position) or without rejuvenation (developed from the buds at the node of other positions than the Base position) as a percentage of explants per culture vessel after 5 weeks growth *in vitro*; explants were taken from various positions: Tip or Node 1 (first node below the tip). Means with the same letter are not significantly different at $P \leq 0.05$ (Fisher's protected least significant). Vertical lines represent ± standard error of means, n=6 vessels each containing 8 explants.

With rejuvenation, the percentage of flowering explants per vessel developed from Node 1 (just below the tip) was 28% less than the percentage from the Tip (Figure 2-16). The

difference between explants taken from various positions in their endogenous growth substances could be responsible for the difference between Tip versus Node 1 in their flowering frequency (Scorza and Janick, 1980; McDaniel et al., 1989). Although a positional trend with flowering was similar to the trend observed for explants without rejuvenation, however after rejuvenation flowering decreased even with the application of the positions with the highest propensity to flower (i.e. Tip and Node1). The incidence of flowering was not completely prevented in the explants after rejuvenation, reiterating the likelihood of multiple factors controlling flowering or factors with stronger influence than juvenility on flowering in 'Little Pinkie'.

2.7.3. *In vitro* medium on flowering

Application of the modified culture medium (half MS and PGR-free) was another influential factor which caused a six-fold reduction in flowering frequency of explants in comparison with explants cultured in commonly used medium (with PGRs and full MS) (Experiment 3, Table 2-5; $P \leq 0.05$). With application of modified medium in the current experiment, the position of the explants which previously (refer to Section A, Experiment 1) had been reported to be effective, did not have a significant influence on flowering. However, with application of the common medium, similar to all previous experiments utilising explants derived from various positions along the donor shoot, the number of flowering explants developed from the Tip position was the highest compared to other positions ($P \leq 0.05$; Table 2-5). Therefore, it seems that the effect of medium on flowering was stronger than the effect of the position which explants originated from along the donor shoot. With regard to flowering propensity, there was a significant interaction between the two factors, i.e. medium and position ($P \leq 0.05$). With no PGR in the medium the frequency of flowering in explants from the Tip position was reduced by 16.25% compared to those with a prehistory of PGRs and higher amount of macro salts. Except for the Tip position, none of the other positions produced any flowers in the medium which was free of PGRs (Table 2-5). Explants derived from the Base however, never produced any flowers irrespective of the different media used, which could be due to rejuvenation effect.

Table 2–5: Percentage of flowering explants per vessel containing; culture media (commonly used medium in tissue culture lab with full MS and PGRs or modified medium with MS at half strength and without PGRs). Explants were taken from various positions (Tip (tip), Node 1(first node below the tip), Node 2 (second node below the tip) or Base (base)) along the donor shoots of gentian 'Little Pinkie', cultured for 5 weeks *in vitro*.

Position	Flowering (%)	
	Common medium	Modified medium
Tip	18.75 a[Z]	2.50 b
Node 1	6.25 ab	0.00 b
Node 2	3.75 b	0.00 b
Base	0.00 b	0.00 b

[Z] Groupings for mean separation were based on Fisher's protected least significant test. Means followed by different letters within rows and column were significantly different at $P \leq 0.05$. n = 5 vessels each containing 8 explants.

The decline in flowering of the explants cultured using the modified medium as compared with the commonly used medium, was assumed to be related to their differences in PGRs, confounded with changes in macro salts. *In vitro* flowering occurred in explants cultured using the common medium, which included a combination of cytokinin (BA), auxin (IBA) and gibberellin (GA_3) together with MS salts at full strength. By using the modified medium (without PGRs, and MS salt at half strength) in the current experiment, flowering only occurred in explants taken from the Tip and decreased by 11.5 times less than with the commonly used medium (with PGRs and MS salts at full strength) (Table 2-5). While the MS component might also be influential on early flowering, as discussed below, if the focus was only on the effect of PGRs, cytokinins would be the most likely PGR to induce early flowering (Bernier, 1977; Jumin and Ahmad, 1999; Wang et al., 2002; Vu et al., 2006; Saritha and Naidu, 2007; Ishimori et al., 2009; Jana and Shekhawat, 2011). In contrast, the effect of gibberellins applied *in vitro* have been reported to only be promotive of flowering in some species and inhibitive in others (Chang and Hsing, 1980). Using *Cymbidium niveo-marginatum*, application of gibberellins (GA_3) delayed *in vitro* flowering even when a flower-promoting treatment was included (Kostenyuk et al., 1999). However, in a cultivar of gentian (*Gentiana triflora* Pall. var. *axillariflora* Akita Blue) application of GA_3 did not influence flowering of the explants *in vitro* (Zhang and Leung, 2002). Hence it was considered unlikely that the GA_3 component within the current experiment would induce *in vitro* flowering. In contrast, with other plants cytokinins (BA) have been shown to play a crucial role in the process of flowering through making changes such as initiation of mitosis and regeneration of cell division and organ formation (Taylor et al., 2005). Application of exogenous cytokinin encouraged the apical meristems of *Sinapsis alba* to flower by the effect on the mitotic cycle (Bernier, 1977). BA has also induced *in vitro* flowering in other plant genera (Jumin and Ahmad, 1999; Wang et al., 2002; Vu et al., 2006; Saritha and Naidu, 2007; Ishimori et al., 2009; Jana and Shekhawat, 2011). Therefore, within the current

experiment as far as the effect of PGRs was of concern, it was considered plausible that the difference in flowering frequency between the two medium would be related to their cytokinin content. However, future studies on evaluation of the effect of every individual PGR, especially cytokinins, on growth of the explants would be needed to substantiate such a statement.

The difference in flowering between explants developed on the modified medium compared to the common medium might also have been due to their difference in the strength of MS salts, as well as their possible interaction with PGRs. In a study using *Arabidopsis thaliana*, reduction in nitrogen within the medium following the decrease in the strength of MS salts and, therefore, increasing the carbon/nitrogen ratio, was reported to encourage flowering (Roldan et al., 1999; Corbesier et al., 2002). Other researchers also reported an increase in flowering *in vitro* with other plant species following the decrease in the strength of MS salts (Thakur et al., 1998; Bodhipadma and Leung, 2003), which was in contrast with the results achieved in the current research. The contrast between the results of other researchers compared to the results achieved here, could be possibly related to the interaction with PGRs included in the medium and/or the PGR prehistory of tissue. Research reported by (Rubio et al., 2009) would support this possibility, wherein the biosynthesis of PGRs was reported to be closely related and influenced by the nutrient (MS salts) condition of the culture medium. As an alternative hypothesis, the decrease in flowering observed within the current study following the decrease in the strength of MS salts could be probably related to removal or decrease in the stress induced by an excess quantity of nutrients when applied at full strength. If so, it was possible that full strength MS salts was surplus to the plant's need for the metabolic activities, vegetative growth and, therefore, would accumulate in the explants tissue as a result of several culture cycles, causing toxic effects and stress on the explants. As evident with early flowering of other plant species when under stress (Wingler, 2011; Kumar et al., 2012), this alternate hypothesis could be investigated in any future research.

2.7.4. Temperature and photoperiod on flowering

Application of environmental conditions associated with dormancy induction/breakage such as cold temperature of 2 ºC and short photoperiod of 8h, decreased the frequency of flowering as compared to the explants remaining under normal conditions (Experiment 4, Table 2-5; $P \leq 0.001$). The frequency of flowering in explants following the exposure to cold temperature and short photoperiod for a duration of 7 and 9 weeks, decreased three-fold and completely stopped by a duration of 9 weeks as compared to when explants, had not received

cold temperature and short photoperiod for several sub-culturing cycles *in vitro* (Table 2-5; $P \leq 0.001$). Photoperiod and temperature both play a significant role in the induction and breaking of dormancy in various plant species (Horvath et al., 2004). Although information on inducing and breaking the dormancy in gentian is limited, in a study using the cultivar 'Showtime Spotlight' cultured within a protected environment *in vivo*, with natural short photoperiod but no cold, shoots did not emerge in the subsequent growth cycle from the preformed crown buds (Samarakoon et al., 2015). This was considered to be probably related to their chilling requirement for breaking their dormancy. Dormancy provides an opportunity for cessation of active vegetative growth and development, perhaps resetting the endogenous developmental programs which control biological activities for the next growth season (Horvath et al., 2004).

Table 2–6: Frequency of flowering (%) in explants of gentian 'Little Pinkie' per vessel as influenced by duration of cold temperature (0, 5, 7, 9 or 12 weeks at 2°C) under short photoperiod (8h), on explants taken from various positions on the donor shoot (Tip (tip), Node 1 (first node below the tip), Node 2 (second node below the tip) or Base (base)) *in vitro*.

	Flowering (%)				
	Duration of cold temperature (Weeks)				
Position	**0**	**5**	**7**	**9**	**12**
Tip	45 a[Z]	20 b	20 b	0 b	0 b
Node 1	10 b	0 b	0 b	0 b	0 b
Node 2	5 b	0 b	0 b	0 b	0 b
Base	0 b	0 b	0 b	0 b	0 b

[Z] Groupings for mean separation were based on Fisher's protected least significant test. Means followed by different letters within rows and column were significantly different at $P \leq 0.05$. n = 5 vessels each containing 8 explants.

Apart from a similar positional effect as that reported in Chapter 2; there was also an interaction between position of the explants and the duration of cold temperature and short photoperiod needed to prevent flowering ($P \leq 0.001$). The duration of treatment to prevent flowering decreased as the position of the explant increased from the tip of the donor shoot. Explants which originated from the Tip position needed more than 7 weeks exposure to stop flowering, whereas the explants originated from the first and second node below the tip stopped flowering after receiving the treatment for only 5 weeks. Explants of the Base position never produced any flowering shoots. Such an interaction could be related to the difference between various positions in their response to endogenous (hormones and sugar) and/or environmental signals (Horvath et al., 2004).

An effective duration of winter dormancy in the natural annual growth cycle of plant species which are dependent on photoperiod and/or temperature, would differ among species (Heide, 2001; Rademacher, 2015), but would probably be more consistent within a species. In gentian (*G. scabra*), application of 2-3 weeks of cold temperature at 2 ºC had been reported to be sufficient for breaking the dormancy of seeds (Bicknell, 1984) and in plants of gentian 'Showtime Spotlight' (*G. triflora* x *G. mirandae*) monthly temperature of as low as 8 ºC during winter encouraged shoot emergence in the next growth cycle (Samarakoon, 2012). While there was no attempt to determine cold unit requirements, the current study provided some information on inducing and breaking the dormancy in gentian 'Little Pinkie' which could probably be applicable to other cultivars of gentian if needed. Application of cold temperature and short photoperiod inhibited flowering on explants of gentian cultured *in vitro* been continuously under several vegetative growth cycles before.

The most influential factor on inhibition of early flowering which could completely prevent it was application of cold temperature and short photoperiod on the explants. From all factors investigated in the current section for their possible influence on early flowering in 'Little Pinkie, continuous sub-culturing of the explant seems to be a primary determinant. Explants of the Tip position produced flowers regardless of any strategy applied to prevent it, except for when they were exposed to a period of cold temperature and short photoperiod. The second most influential strategy to prevent early flowering in explants cultured *in vitro* would be modification of the culture medium to include half strength of MS salts instead of full strength and removing all PGRs. Although it did not inhibit early flowering completely, rejuvenation of the explants as detailed in Experiment 2 would also help to decrease it to some extent.

A combination of all the aforementioned strategies was eventually utilized to overcome early flowering in explants used for the later experiments in this thesis (n.b. regrettably some experiments were conducted before this strategy was developed). However, to identify factors which control early flowering in 'Little Pinkie, the physiological and environmental factors regulating bud development, dormancy and flower initiation need further study. As a result of the findings within the present chapter, to reduce the non-uniformity among the explants they can be categorised according to the position of their origin while sub-culturing to produce plant material for the subsequent experiments. Also, in order to avoid early flowering in explants of gentian cultured *in vitro*, and probably subsequent reduction in the

pot plant production *in vivo,* modifications to the protocol for sub-culturing plant material for future experiments included;

- only using explants from the nodes of donor shoots as the source of plant material,
- preferentially use PGR-free medium at half strength MS when sub-culturing,
- limit the duration of the culture cycle for sub-culturing material to no longer than 5 weeks and,
- monitoring explants for their flowering regularly and exposing them to dormancy in case of increase in early flowering in order to regain their vigour.

While early flowering might have disadvantages within the propagation system, and in the current research, future studies might benefit from introducing 'Little Pinkie' as a cultivar susceptible to early flowering *in vitro*. Flowering *in vitro* does potentially provide a controlled system necessary to study molecular and genetic mechanisms of flower induction and morphogenesis, as well as the commercial production of flowers and specific compounds from floral organs (Zhang and Leung, 2002; Pennazio, 2003; Da Silva et al., 2014; Teixeira da Silva et al., 2014). As noted by (Nakatsuka et al., 2009) gentian as a perennial plant typically has a juvenile phase of one to three years from seed, and there is no effective cultivation method to induce early flowering if desired. Therefore, *in vitro* flowering or *in vitro* induction of flowering could be of potential value in commercial applications to reduce the time to flower (Rose et al., 1998; Nakatsuka et al., 2009). Some researchers have tried to decrease the time to flower in gentian by introducing the *Arabidopsis* FLOWERING LOCUS T (FT) gene and develop transgenic gentian plants which shortened flowering to 4 months (Nakatsuka et al., 2012). Also, by inducing apple latent spherical virus (ALSV) vector that express FLOWERING LOCUS T (AtFT) from *Arabidopsis thaliana* or its homolog GtFT1 from *Gentiana triflora* in three cultivars of gentian, their juvenile phase shortened from 1-3 years to 3-5 months (Fekih et al., 2016). 'Little Pinkie' is probably introduced as the first early flowering cultivar released from a conventional breeding method. While still further study is needed to improve the scheduling of flowering of propagules of 'Little Pinkie' *in vivo* when derived from an *in vitro* system, it might be very useful as an early flowering cultivar. In breeding programmes, *in vitro* flowering could be used to speed up the process of multiple generational cycles, enabling pre-selections of progenies based on likely breeding objectives including flower colour (Nakatsuka et al., 2009; Da Silva et al., 2014).

2.8. Conclusions

The maximum number of days from de-flasking 'Little Pinkie' to flower *in vivo*, was up to 68 days but there was a declining trend in the number of days to flower as well as the number of saleable plants, with incremental dates of de-flask. Early flowering was also observed *in vitro*, affected by the position from which each propagule was taken during propagation. Since explants taken from the tip had the highest propensity to flower, using nodes from subtending positions resulted in significant increases in multiplication rates. However, explants of the base position never produced any flowers. Flowering of rejuvenated explants, which had been developed from the base position, decreased in comparison with the explants without rejuvenation. Application of the culture medium with half MS and free of PGRs caused reduction in flowering of explants. While none of the other strategies; rejuvenation or modification of the medium did inhibit *in vitro* early flowering completely, no explant flowered after being exposed to the combination of short photoperiod (8h) and cold temperature (2 ºC) for more than 7 weeks.

As noted previously (refer to Section 2.1), while the series of experiments presented in this chapter were presented last, the need to resolve the problematic situation of non-uniformity in vegetative growth of plants as well as increased flowering frequency and reduced multiplication rate *in vitro*, became apparent well into progress with experiments presented within the entire thesis (i.e. Chapters 3-7). Hence while the results presented in the current chapter might infer these problems were successfully managed, in part, this chapter was presented last as a means to explain some of the variability in results between experiments throughout the entire research.

Chapter 3. Microscopic studies on shoot formation and development *in vitro*

3.1. Introduction

Shoot formation of gentians is associated with the growth and development of buds as secondary (2º) shoots; hence studies on the type, origin, development and emergence of these buds were of importance in this research. Secondary shoots of explants of gentian cultured *in vitro* become macroscopically visible after arising from axillary buds within nodes on the stem (Figure 1-3B). Each nodal explant was a metamer consisting of two nodes and a pair of developed leaves with internode tissue (primary stem; Chapter 1, Figure 1-3B). Therefore, for application in the current thesis occasionally "node/nodal position" was utilized which was equivalent to metamer (refer to Chapter 1, Figure 1-3). A maximum of two 2º shoots are normally expected to develop from the two axillary buds at the node of gentian explants (Ho, 2001; Struwe & Albert, 2002). It was considered likely that axillary buds were initiated at the nodes of the explants taken from the developed shoots of the mother plantlets, but not macroscopically visible (i.e. not developed beyond a bud initial) at this time. In explants of 'Little Pinkie' cultured *in vitro*, multiple vegetative shoots (up to 10) arose from the node/metamer of explants as a result of ethephon application (refer to Appendix 13). Therefore, in 'Little Pinkie' the origin and type of buds from which multiple vegetative shoots were coming following the application of ethephon needed to be studied. Microscopic examination would provide the opportunity to observe the nodal position closely and describe the microscopic features.

To the best of author's knowledge, no previous report was found on microscopic features of shoot formation of gentians *in vitro*. In gentians *in vivo* the over-wintering crown bud, which develop to become floral shoots later, formed within the stem/root transitional zone, i.e. below the lower node of the plants (Samarakoon et al., 2013; 2014). In that research, the initiation of a cluster of crown buds in gentian cultivars like 'Showtime Diva' was reported to present characteristics typical of adventitious buds, but subsequent buds developed within the cluster as axillary buds (Samarakoon et al., 2013). While not associated with microscopic data, nodal explants of 'Showtime Diva' *in vitro* at high sucrose concentration developed crown buds which were considered to be axillary buds (Wang, 2014). Given the far greater number of shoots arising from explants of 'Little Pinkie' when treated with ethephon

(Appendix 13) within the current study therefore, the question to be addressed, was what type of buds initiate and subsequently develop into shoots in gentian 'Little Pinkie' cultured *in vitro* treated with ethephon in culture medium.

Different cultivars of gentian vary in the number of 2º shoots which arise from nodal explants (metamer) while cultured *in vitro*. Cultivars with low number of shoot normally produce one 2º shoot per nodal explant, whereas cultivars with high of shoots may produce more. When treated with ethephon the cultivar with low number of shoot 'Showtime Diva' has been reported to slightly increase in shoot number (Wang, 2014). In contrast, cultivar 'Little Pinkie' was capable of producing up to ten 2º shoots following the application of ethephon in the medium (refer to Appendix 13). *In vitro* explants of different cultivars with high and low response in shoot number were therefore hypothesized to differ in the type and/or origin of their buds and, therefore a microscopic study of cultivars differing in their shoot number was designed to test this hypothesis. Also, there was a difference in the number of shoots arising from the explants taken from various positions of 'Little Pinkie' along the donor shoot (refer to Chapter 2, Section A) which was hypothesised to be related to the difference in the number and/or type of bud primordia at the node (metamer).

Axillary buds are typically of exogenous origin (relatively superficial tissues) at the node (metamer) whereas adventitious buds develop either endogenously or exogenously from any plant organ (Evert, 2006). In studies on other herbaceous perennials, the type of buds has been identified by attempting to trace the vascular connection of the buds to the tissue from which it originated. If there were no evidence of a vascular connection, then it was likely that the bud would be adventitious (Chiatante et al., 2008; Samarakoon et al., 2013). This strategy was similarly followed to identify the type of buds arising from the nodal position of explants in the current study.

Therefore, utilising two cultivars of gentian ('Little Pinkie' and 'Showtime Diva'), within an *in vitro* system, the objectives were to:

- describe the microscopic features of the stem at the node (metamer) of explants,
- identify the origin and type of buds arising from the node (metamer) of explants,
- describe the ontogeny of development of buds to shoots in nodal explants (metamer), and

– microscopically describe the morphological origin for the difference in shoot number in explants of various positions in the presence or absence of ethephon.

3.2. Materials and methods

Plantlets of the gentian cultivars 'Little Pinkie' and 'Showtime Diva', which had been previously grown and sub-cultured on a five-weekly cycle *in vitro,* were used as source material for explants. Donor plantlets had previously been sub-cultured using a medium containing a range of plant growth regulators (PGR) in line with the general protocol for gentians within the tissue culture lab (Morgan et al., 1997). The base medium was prepared as detailed before (refer to Chapter 2, Section 2.2). For the ethephon treatment, ethephon (Ethrel®; 480 $g.L^{-1}$ chlorethephon; Bayer CropScience, New Zealand) at a final concentration of 10 $mg.L^{-1}$, was added to the base medium as the comparative treatment after filter sterilisation (0.2 µm Minisart® filter; Sartorius Stedim Biotech, Germany) at 50°C (Purnhauser et al., 1987) (refer to details within Chapter 2). Culture vessels were similar to those utilised in former experiments (refer to Chapter 2, Section 2.2). Each culture vessel contained 50 mL of medium. Nodal explants were inserted into the medium within half an hour of its preparation, with the nodes being positioned above its surface. Explants were cultured at 25 ± 1 °C, at a photosynthetic photon flux density (PPFD) of 30 ± 5 $\mu mol.m^2.s^{-1}$ and 16 h photoperiod, provided by cool-white fluorescent tubes.

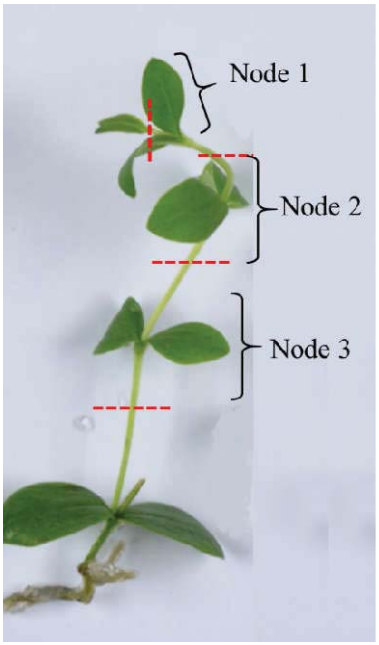


Figure 3-1: Donor plantlet of gentian 'Showtime Diva'illustrating the nodal positions (Node 1, 2 or 3) explants were derived from. Red dashed lines indicate the positions of cutting to take explants from each position.

Shoots selected for production of explants were of a similar physiological age, being of uniform length (40-50 mm) and number of nodes (generally three nodes (metamer) with expanded internodes above and below), which were divided into single node explants, each 10 mm in length (Figure 3-1 & 3-2A). The experiment comprised 12 culture vessels as replicates arranged in a factorial design including two factors; position along the donor shoot (Node 1, 2 or 3) which explants were taken from, and presence or absence of ethephon. Each culture vessel contained eight single-node explants all from the same position in medium in order to eliminate the effect of variation of explants due to nodal position (refer to Chapter 2, Section A), either with or without ethephon. For microscopic examination, samples of explants from each nodal position in presence or absence of ethephon were taken after 8 weeks separately. In addition, explants were taken from only Node 2 at 0 and 4 weeks to be compared with 8 weeks after insertion into the medium. Apart from morphological features no other data were recorded.

External morphological features of fresh samples of explants were recorded under a binocular microscope (Leica DFC550, Switzerland), with a Leica M 205 FA digital camera attached. The nodal area of explants were sampled and dissected by first removing the leaves and leaf sheath, with the latter comprising the petioles of the two leaves enveloping the node (metamer). It was necessary to dissect and remove the leaf sheath so as expose the stem tissue upon which developing bud primordia were positioned.

Scanning electron microscopy (SEM) was used to study the external morphology of the nodal region, beyond that achieved under a binocular microscope, when sampled at 8 weeks after commencement of the experiment. For preparation of imaging, samples were dissected to expose the node, fixed overnight in 3% glutaraldehyde, 2% formaldehyde in 0.1M phosphate buffer under vacuum, buffer washed, dehydrated in a graded ethanol series, and critical point dried using liquid CO_2 as the critical point fluid (Eaton et al., 2010; Becker et al., 2016). Dried samples were mounted on double-sided tape on SEM specimen stubs, sputter coated with gold, and viewed in an FEI Quanta 200 SEM (FEI Electron Optics of Eindhoven, The Netherlands). SEM images were prepared from samples of Node 1, 2 and 3 of both cultivars cultured both in the presence and absence of ethephon.

Internal details of node anatomy, at each treatment position, was investigated utilizing the method described by Eaton et al. (2010) and Samarakoon et al. (2013). Samples were fixed using FAA (formaldehyde: glacial acetic acid: ethanol: distilled water at 2:1:10:7 by volume)

overnight at 4°C, and dehydrated using a graded ethanol series. Tissues were then prepared for wax infiltration through a graded series of Ethanol: Histoclear™ (National Diagnostics, Atlanta, Georgia, USA). The Histoclear was subsequently gradually replaced with Paraplast X-tra (McCormick Scientific, Germany), and embedded in 100% Paraplast X-tra. Depending on the tissue, transverse sections were cut at 8 to 12 μm thickness, using a Leica RM 2145 Rotary Microtome, and mounted onto polysine-coated slides. Transverse sections were taken progressively along the length of node, so as to identify the vascular connections to any buds, and their arrangement around the node (Samarakoon et al., 2013). Slides were stained with 0.05% toluidine blue for 10 minutes, washed in distilled water, and dehydrated in a graded ethanol series diluted with reverse osmosis water. After the final transfer to 100% fresh ethanol, the Paraplast X-tra was removed from the sample using Histoclear. Slides were mounted with cover slips using Entellen (Merck, Darmstadt, Germany). Morphological features of buds were recorded by observing the sections using a bright field microscope (Olympus BH2, Japan) with a Leica DFC 500 digital camera attached.

3.3. Results and discussions

The results from this experiment are presented in two sections, the first describing the origin of shoots in the absence of ethephon within the agar medium, followed by those results from when ethephon was present.

3.3.1. Origin of shoots; adventitious or axillary bud primordia

At the commencement of the experiment (day 0) when nodal explants (Node 2 only; Figure 3-1) were inserted into the medium, apart from the two axillary buds (one in each leaf axil), no other meristematic activity was evident at the node of either cultivar (Figure 3-2B). In the absence of ethephon within the agar medium, after 4-8 weeks of growth *in vitro*, explants of both cultivars produced one or two visible 2º shoots (Figure 3-3A). After 8 weeks in the absence of ethephon, in addition to 2º shoots, a zone of meristematic activity was observed under the binocular microscope at the node (Node 2) of dissected explants of ‘Little Pinkie’ (Figure 3-3B). The meristematic zone, which was arranged as a ring at the node of ‘Little Pinkie’, was clearly formed after the experiment commenced (day 0). However, in ‘Showtime Diva’ while one or two 2º shoots were observed arising from the node (Figure 3-3C), no other meristematic activity was evident (Figure 3-3D).

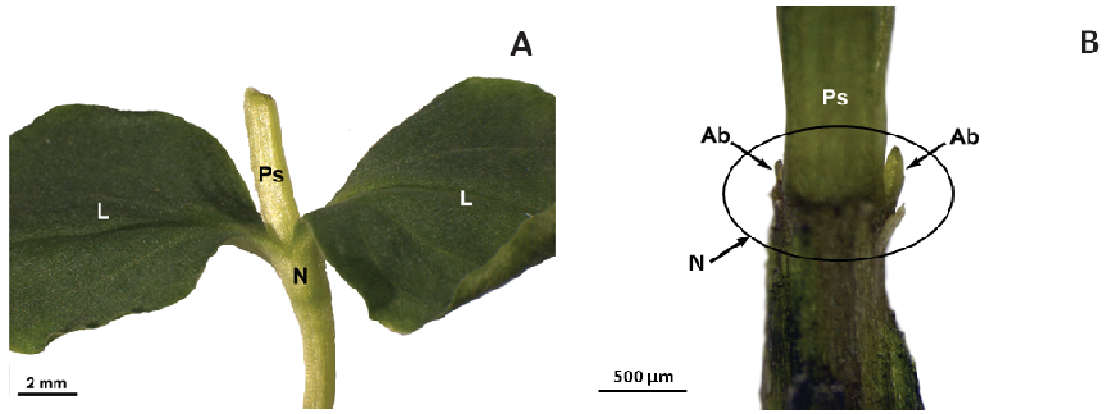


Figure 3-2: Morphological features of Node 2 explants of gentian at day 0 with; **(A)** leaf (L), primary stem (Ps), and node (N); **(B)** axillary buds (Ab) only visible once the leaf sheath was removed from the stem, as viewed under the binocular microscope. Image presented is of 'Little Pinkie'.

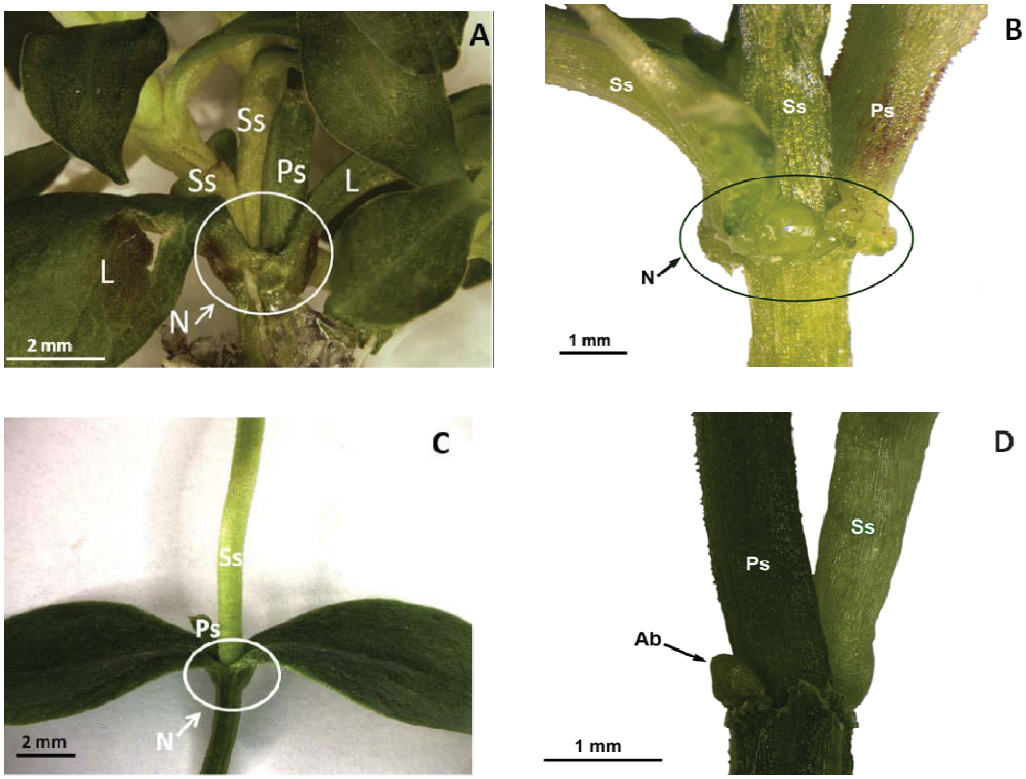


Figure 3-3: Morphological features of the node (N) of Node 2 explants of gentian after 8 weeks growth *in vitro*, **(A, B)** 'Little Pinkie' and **(C, D)** 'Showtime Diva'. **(A)** leaf (L) secondary shoots (Ss), primary stem (Ps) and node (N) inside the circle (A); **(B)** area of meristematic activity at the node under the binocular microscope (leaf sheath removed); **(C)** primary and secondary shoot; **(D)** axillary bud (Ab) within magnified image of the node of 'Showtime Diva' under the binocular microscope.

While external features of the meristematic zone at the node were studied under the binocular microscope, transverse sections assisted with identification of the origin and type of buds. Transverse sections showed the vascular tissue was arranged in a circular vascular cylinder (Vc) with pith (P) at the centre (Figures 3-4A & 3-4C), as it is in other dicotyledonous plants

(Bowes, 1996) including gentians (Samarakoon, 2012). The vascular cylinder was composed of xylem (X), cambium and phloem (Ph). The cambium, phloem, cortex (C) and epidermis were external to the xylem (Figure 3-4B). There were two zones of meristematic activity associated with the nodes within the axil of the leaves (Figure 3-4A). Based on microscopic observations on basipetal sections at the commencement of the experiment, within the meristematic zone, vascular traces of the bud primordia which would connect them to the vascular cambium of the main stem were presumed to not be developed enough and, therefore, not found at this stage. However, bud primordia were located in close association with where the leaf vascular traces left the vascular ring of the stem at the axil of leaves (Figure 3-4A). In addition there were two gaps within the vascular ring of the basipetal sections at the node (Figure 3-4C), which was similar to that previously reported on gentian (Samarakoon et al., 2013). In their study, these gaps were considered to be part of the distortion of the vascular ring at the node (i.e. leaf gaps) in association with the vascular bundles of axillary buds and leaves. Therefore at Node 2 (Figure 3-1), the pre-existing buds at the axil of leaves of the explants at the time of insertion into the culture medium (day 0) were considered to be axillary, albeit primordial. Further confirmation of the buds being axillary was evident after 8 weeks growth, where these buds developed into 2º shoots with vascular connections clearly connected to the main stem (Figure 3-5).

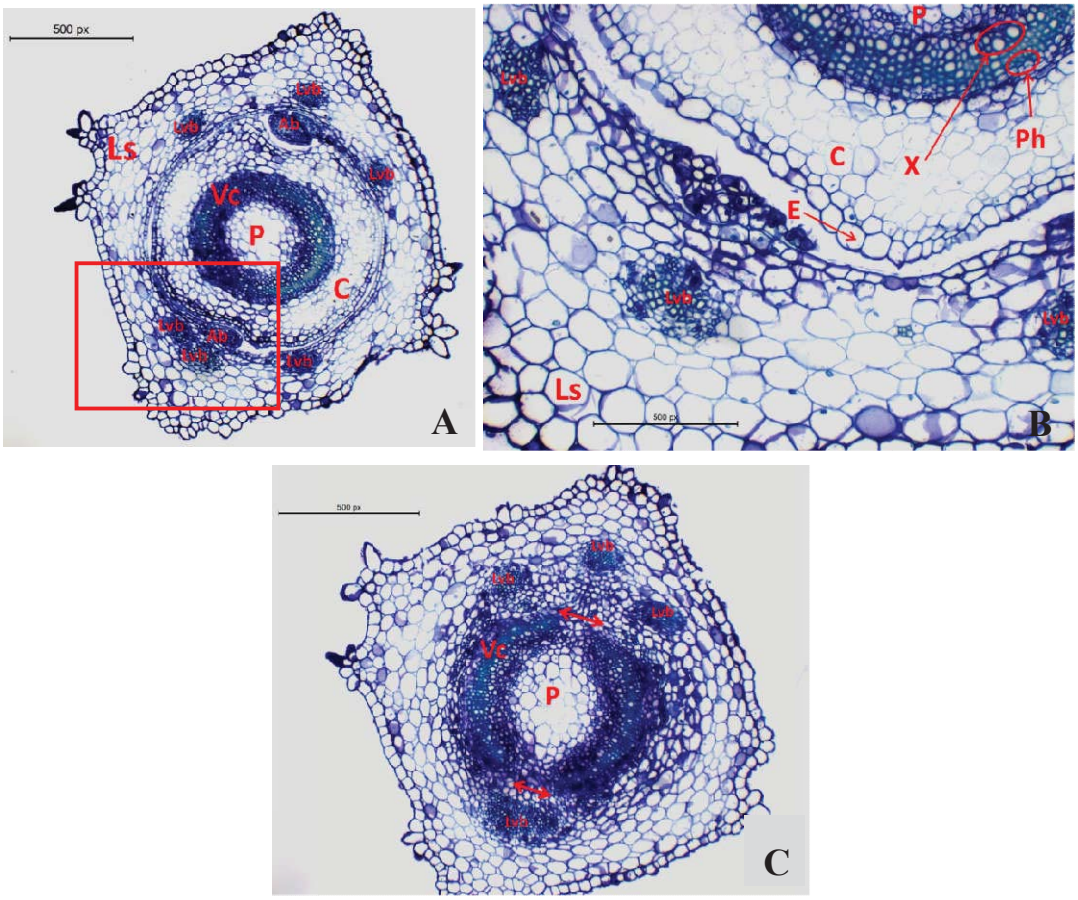


Figure 3-4: Transverse section of the node of gentian 'Little Pinkie' at day 0 *in vitro*, **(A)**; Full section indicating pith (P), vascular cylinder (Vc) and axillary buds (Ab) surrounded by leaf sheath (Ls) with leaf vascular bundles (Lvb), **(B)**; Magnified view of a part of node (red square in Figure 3-4A) indicating the arrangement of xylem (X) cells inside the vascular cylinder, with cambium, phloem (Ph), cortex (C) and endodermis (E) external to the vascular cylinder **(C)** basipetal section with red arrows that show the gaps in vascular cambium of main stem where axillary bud primordia form. Tissue stained using Toluidine blue.

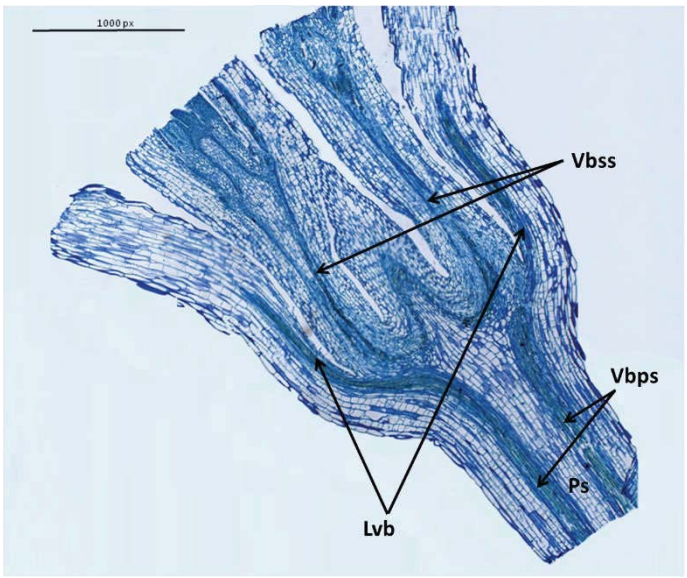


Figure 3-5: Longitudinal section of the node at Node 2 of gentian 'Little Pinkie' after 8 weeks growth *in vitro*, with Primary stem (Ps), vascular bundle of 2º stem (Vbss), vascular bundle of primary stem (Vbps), and leaf vascular bundle (Lvb). Tissue stained using Toluidine blue.

SEM images provided enough contrast to distinguish multiple bud primordia at the node of the explants after 8 weeks, which previously in Figure 3-3B had been showed a ring of meristematic activity under the binocular microscope (Figure 3-6A&B). By rotating the sample under the microscope and taking images from different angles of the node, it was obvious that after 8 weeks growth, there were additional bud primordia (8 to 10 in Figure 3-6A&B) positioned all around the node, and arranged either individually or in clusters. It is common for some species to have multiple bud primordia as accessory/co-lateral buds, in addition to the axillary bud associated with a single leaf (Evert, 2006). Accessory buds are secondary buds formed beside a principal bud (axillary or terminal). The first accessory bud originates from the axillary bud, and the second accessory bud from the first one (Shah and Unnikrishnan, 1971). Collateral buds are accessory buds arranged on either side of an axillary bud. In the current study however, some of additional bud primordia were not associated with the axil of the leaf nor any other buds and, therefore, were considered likely to be adventitious in origin. The presence of adventitious buds as well as axillary and probably co-lateral buds in 'Little Pinkie' observed here, was consistent with previous reports on other cultivars of gentian (Samarakoon et al., 2013).

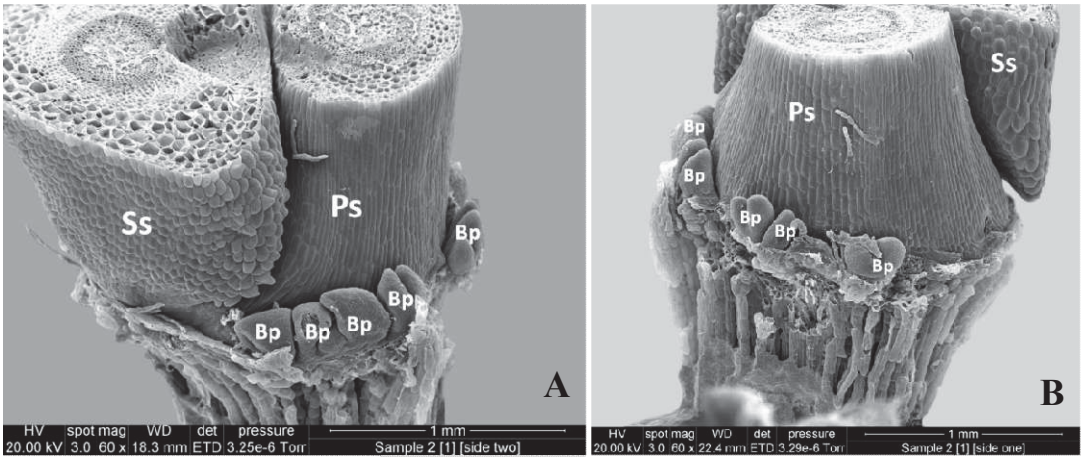


Figure 3-6: Morphological features under SEM of Node 2 of gentian 'Little Pinkie' after 8 weeks growth *in vitro* in the absence of ethephon, after leaf sheath removal, **(A)** with secondary shoots (Ss), primary stem (Ps) and a ring of bud primordia (Bp) around the node; **(B)** 90° rotation of the same sample.

The whole node (Node 2) and buds, regardless of their type (axillary, co-lateral and adventitious), were enveloped under a sheath made of the base of the leaf lamina (petiole) of the two leaves (Figure 3-3) previously described as sub-petiolar (Powell, 2008) and became visible only after leaf was separated. As evident within longitudinal sections of the node of gentian 'Little Pinkie', vascular bundles of each leaf clearly branched from the vascular cylinder of the main stem (Figure 3-5). There were three vascular bundles (leaf vascular

bundles) connecting to each of the leaves (Figure 3-4A & B). However, in order to give an indication of the type and origin of buds at the node, vascular tracing to individual buds around the node were not identifiable using the longitudinal sections. However, images of the acropetal sequence of transverse sections taken through Node 2 after 8 weeks growth, i.e. when 2º shoots were visible macroscopically, illustrated that some of the new bud primordia (Bp) around the node were associated with the axillary bud (Figure 3-7B). When the transverse sections were studied toward the basipetal end; the location at which new multiple bud primordia were observed, was close to the zone with a gap in the vascular cylinder of the main stem (denoted by arrows in Figure 3-7B). More than one bud primordium was found in close association with pre-existing axillary buds at each location where the new buds formed (Figure 3-7C-E). Some of these buds could be considered as co-lateral to axillary buds and others were probably adventitious. However, there were also other individual bud primordia visibly present, which were not associated with axillary bud locations, with the typical characteristics of adventitious buds (Figure 3-7F).

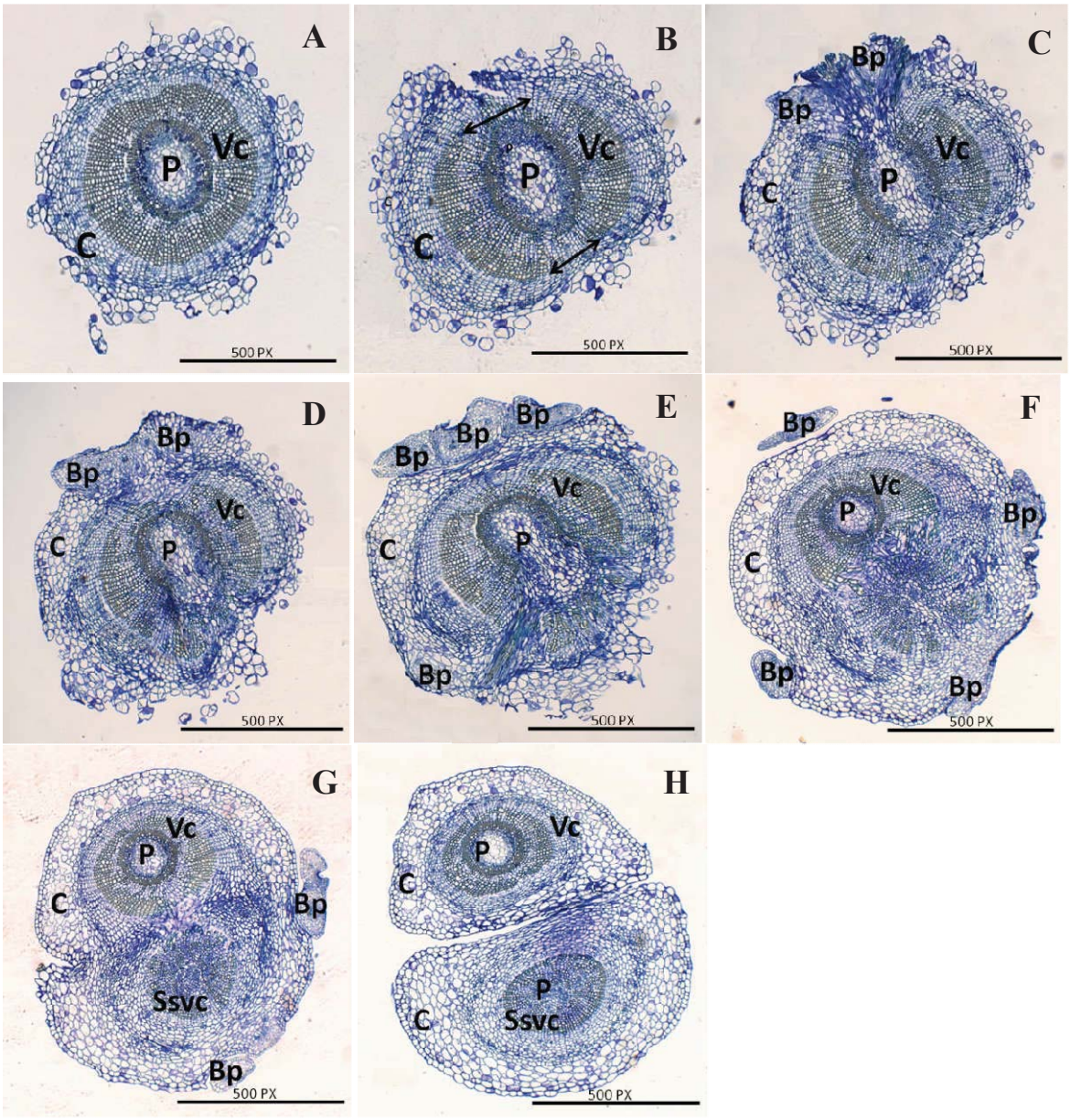


Figure 3-7: Acropetal sequence of transverse sections (A→H) of the node of a single-node explant at Node 2 of gentian 'Little Pinkie' after 8 weeks *in vitro,* with leaf sheath removed. Pith (P), vascular cylinder (Vc), cortex (C), bud primordia (Bp), 2º shoot vascular cylinder (Ssvc). Tissue stained using Toluidine blue.

The gap or distortion in the central vascular ring evident within the sections located at the more acropetal end of the node (Figure 3-7B&C), plus the vascular connections which were visibly connected to the central vascular ring in sections from the basipetal end (Figure 3-7D to H), indicated characteristics of axillary buds as expected in other di-cotyledons (Evert, 2006). In contrast, multiple additional bud primordia which were not associated with the position of axillary buds (i.e. zone with gap in the vascular cylinder; Figure 3-7), originated either within the epidermis or cortex of the stem (Figure 3-8). For these additional buds there also was no gap or distortion in the central vascular ring, suggesting that these new bud primordia were not axillary in origin.

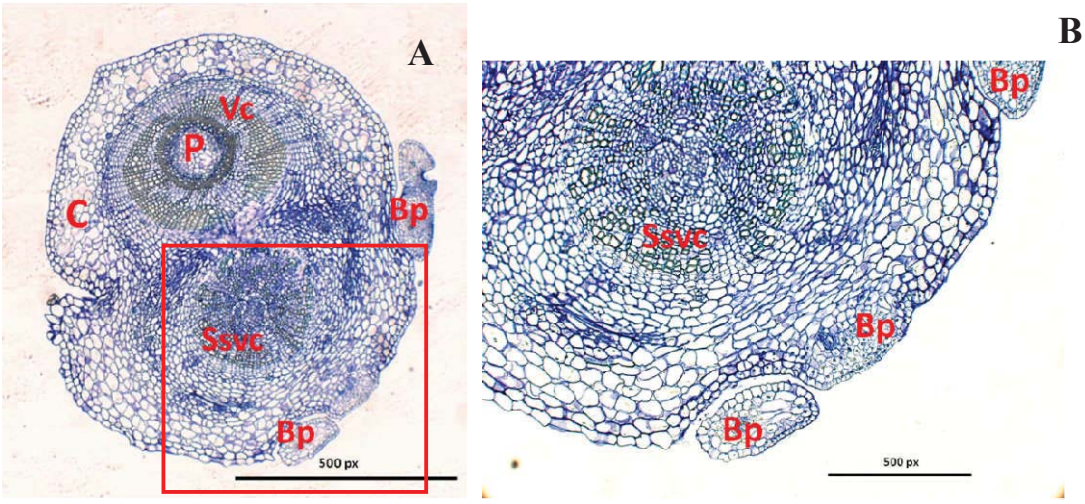


Figure 3-8: Transverse section of the node of 'Little Pinkie' at Node 2 after 8 weeks *in vitro* **(A)**; after leaf sheath removed, illustrating pith (P), vascular cylinder (Vc), cortex (C), bud primordia (Bp), secondary shoot vascular cylinder (Ssvc) and **(B)**; additional individual bud primordia originated either within the epidermis or cortex of the stem, with no association with an axillary bud (i.e. not associated with zone with gap in the vascular cylinder) nor visible vascular connection to the central vascular ring. Tissue stained using Toluidine Blue.

When examined at 8 weeks, within explants at Node 2 the most developed bud clusters seemed to be in association with the axillary bud position, however some individual buds were observed at a distance from the clusters. These might eventually form their own cluster in the future (Samarakoon et al., 2013), but possibly taking a longer period of growth. It was considered that eight weeks might not be enough time to observe development of all buds to form clusters. In cross sections taken after 8 weeks, there was no vascular bundle distinguishable connecting buds which were at a distance from the clusters to the main stem and/or the axillary bud (Figure 3-7 & 3-9). Sampling and further sectioning was repeated after 8 weeks in order to include the leaf sheath around the node, which was missing in previous sections due to it being removed to facilitate first observing bud primordia under the binocular microscope. Images previously taken from samples without the leaf sheath (e.g. Figure 3-7), showed samples had lost some tissue around the node, which might have included some bud primordia. Also, the presence of the sheath with some structures such as leaf vascular bundles were considered important to help identify the position of axillary buds as they appeared to be next to the leaf vascular bundles. With the leaf sheath intact there were three vascular bundles evident for each leaf, and axillary buds appeared to be close to the middle bundle. While providing more supportive/definitive information, the acropetal sequence of transverse sections with the leaf sheath intact (Figure 3-9) did not change the overall conclusion derived from those without the leaf sheath (i.e. Figure 3-7). Hence, as concluded before, in addition to the bud primordia which were in association with axillary buds, there were some other bud primordia which were at a distance from the clusters and,

therefore, originated adventitiously. In longitudinal sections however, the existence of xylem cells, where the bud primordia were located around the node, was clearly evident, but it was not possible to trace these connections to the main stem and/or the axillary bud (Figure 3-10). Therefore this evidence, together with that indicated in the preceding paragraph, collectively supports the notion that these additional bud primordia which were at a distance from the clusters, originated adventitiously.

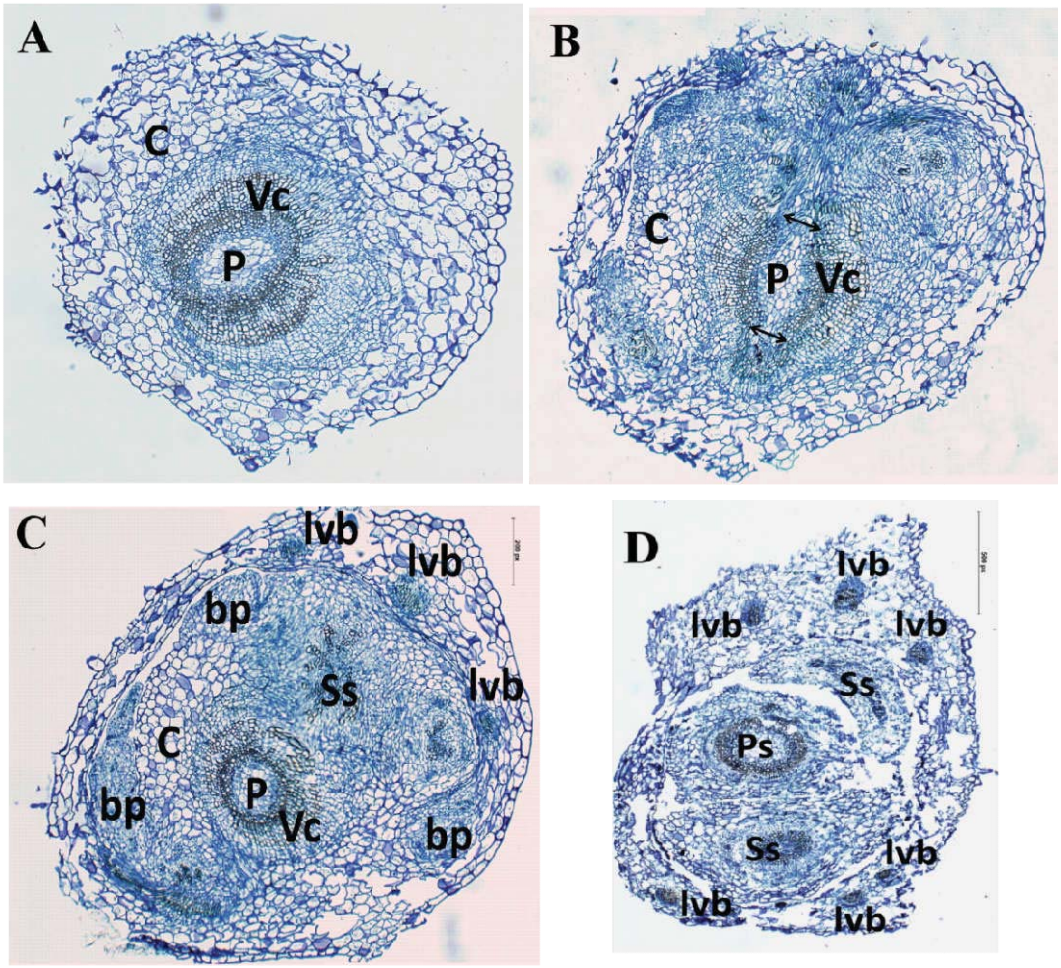


Figure 3-9: Acropetal sequence of transverse sections (A→D) of the node of the explant of gentian 'Little Pinkie' at Node 2 after 8 weeks *in vitro*. Pith (P), vascular cylinder (Vc), cortex (C), bud primordia (Bp), primary shoot (Ps), secondary shoot (Ss), leaf vascular bundle (lvb). Tissue stained using Toluidine blue.

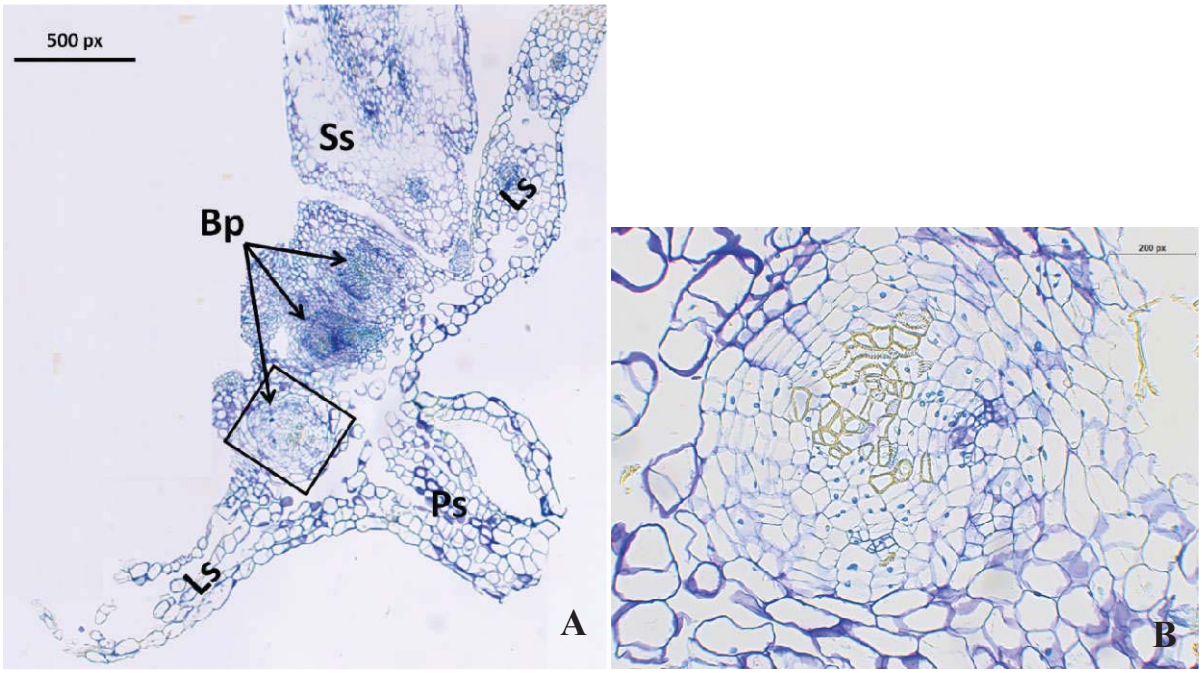


Figure 3-10: Longitudinal sections of the node of **(A)** explants of 'Little Pinkie' at Node 2 after 8 weeks *in vitro*, with primary stem (Ps), secondary stem (Ss), leaf sheath (Ls), bud primordia (Bp); **(B)** magnified image of bud primordium within square of (A) with xylem cells, as evident by yellow-brown colour. Tissue stained using Toluidine Blue.

3.3.2. Ethephon's effect on formation and development of bud primordia

In the presence of ethephon in the medium, after 8 weeks the number of developed 2º shoots from the explants of gentian 'Little Pinkie' increased (Figure 3-11A) over that evident in the absence of ethephon (Figure 3-3A). No such increase was noted with explants of 'Showtime Diva'. For 'Little Pinkie', these results confirmed the earlier observations of an increased number of 2º shoots as a result of ethephon application (refer to Chapter 1, Section 1.1.3). Images taken from transverse sections of 'Little Pinkie' were utilized to demonstrate that multiple bud primordia which emerge around the node after 8 weeks growth in the absence of ethephon *in vitro*, could continue to develop to 2º shoots as a result of the inclusion of ethephon in the medium (Figure 3-7 vs 3-11B). Given the existence of adventitious buds within the nodal region of explants if 'Little Pinkie' was in the absence of ethephon, a new question arising therefore was whether ethephon was only effective in stimulating the development of existing primordia, or also initiated new primordia. As described in the following sections, this question was explored by attempting a range of microscopic examinations.

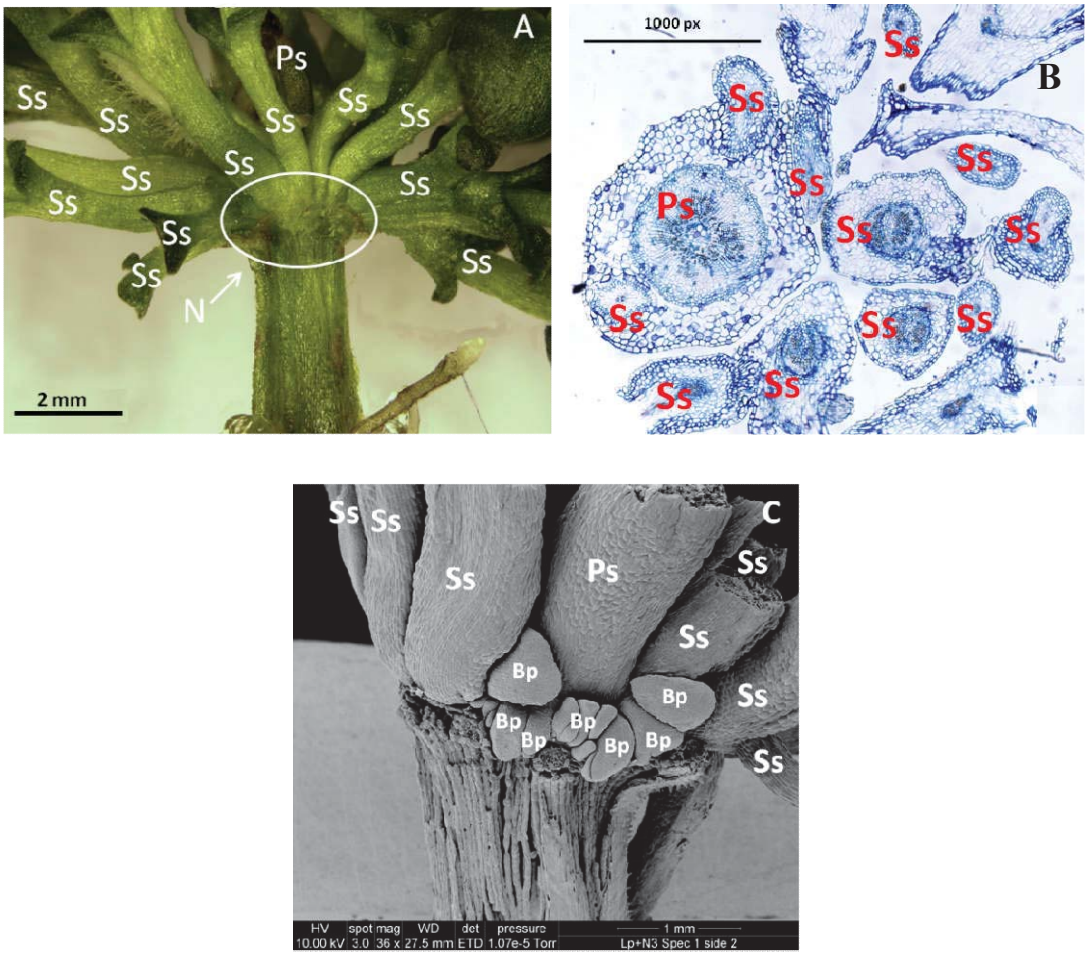


Figure 3-11: Microscopic features of Node 2 of gentian 'Little Pinkie' (leaves removed) after 8 weeks *in vitro* with medium amended with 10 mg.L^{-1} ethephon, **(A)** under the binocular microscope with primary stem (Ps), secondary shoot (Ss) and node (N) inside the circle, **(B)** transverse section of the node illustrating several numbers of developed secondary shoots each included in an individual vascular ring in the middle. Tissue stained using Toluidine Blue; tissue stained green-yellow are xylem vessels and, **(C)** SEM of the node with secondary shoots, primary stem and bud primordia (Bp), after leaf sheath being removed.

It was not possible to identify the presence or absence of some non-developed primordia by using images taken from transverse sections. Increased tissue maturity at this stage (8 weeks of growth *in vitro*) made sectioning difficult. Also, since there was a notable variation in frequency of shoot formation in the explants of 'Little Pinkie', simply counting the numbers of bud primordia and shoots was not considered helpful to answer this new question with accuracy. SEM was subsequently employed to investigate the influence of ethephon on initiation of new primordia. Multiple non-developed bud primordia were observed additionally to several developed 2º shoots of 'Little Pinkie' under the SEM (Figure 3-11). As noted within Section 3.3.1, in the absence of ethephon 'Little Pinkie' tended to produce multiple bud primordia at the node after growing *in vitro* for 8 weeks (Figure 3-6 & 3-7). Those bud primordia were logically assumed to have been the source of the multiple number of developed 2º shoots observed (e.g. Figure 3-11). New bud primordia observed in Figure 3-

11 were obviously additional to those that could have been developed in 'Little Pinkie' without inclusion of ethephon in the medium. Therefore, it can be concluded that ethephon is effective both in stimulating the development of existing primordia and, initiating new primordia.

In the absence of ethephon, bud primordia were first observed after 8 weeks growth of 'Little Pinkie' (Figure 3-7), whereas they were observed at the same stage of development earlier, at week 4, in the presence of ethephon (Figure 3-12). Based on comparing the morphological features within the node it can be concluded then: in 'Little Pinkie' ethephon clearly accelerated the development of existing primordia to form shoots (Figures 3-11 & Figures 3-12 compared with Figures 3-7). As noted within Section 3.3.1, because the additional bud primordia of 'Little Pinkie' were not in association with an axillary bud, they were considered to have originated adventitiously. Similar logic was also applied to the origin of additional bud primordia and 2º shoots in the presence of ethephon, i.e. these were adventitious in origin.

In the absence of ethephon, with 'Little Pinkie' the number of bud primordia increased as the distance of the position that the explant was taken from (Figure 3-1), increased from the tip. There were clearly more bud primordia (at least 7) in the explants taken from Node 3 as compared with explants taken from Node 1 (only one) (Figure 3-13 A, B and C). Also, explants taken from Node 2 produced bud primordia which appeared to be more developed and of larger size that as noted by Xu et al. (2015) affects shoot architecture, as well as having a higher number of 2º shoots (three) as compared with the explants of Node 1 (only one). Microscopic observations in the current study were also found to be in line with those presented in Chapter 2, supporting the conclusion of a gradient in shoot number with nodal position. Therefore, additional bud primordia, which increased in frequency as the distance of the node position increased from the tip to lower nodes, was identified as the primary source of the non-uniformity in Chapter 2. As a result of ethephon application, both the number of 2º shoots and bud primordia increased as the distance of the position increased from the tip. For 'Little Pinkie', after 8 weeks explants of Node 2 produced up to 15 2º shoots as compared with the explants of Node 1 which produced only 2 (Figure 3-13). Distinction between shoots was not possible for their origin. However, since in a metamer of gentian, maximum of two shoots were expected to develop, as evidence by the microscopy work presented in current chapter, additional shoots presumably were of a mixture of axillary, co-lateral and/or

adventitious origin. Ethephon therefore seem to stimulate buds from all types to develop in to shoots.

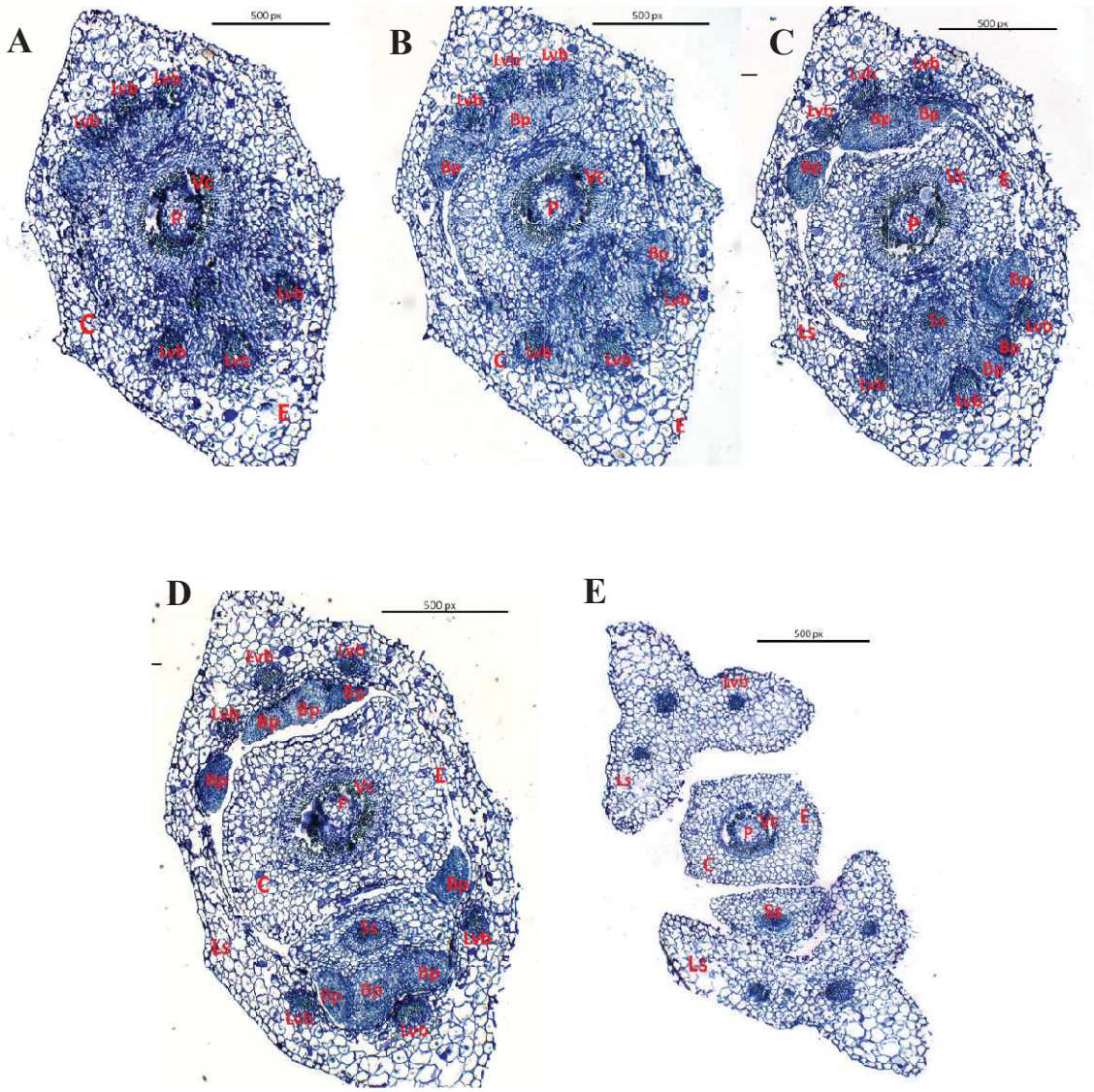


Figure 3-12: Acropetal sequence of transverse sections (A→E) of the node (included the leaf base sheath) of a single-node explant of Node 2 of gentian 'Little Pinkie' after 4 weeks growth in an *in vitro* medium amended with 10 mg.L^{-1}ethephon. Epidermis (E), cortex (C), vascular cylinder (Vc), pith (P), leaf vascular bundle (Lvb), bud primordia (Bp), secondary shoot (Ss), leaf sheath (Ls). Tissue stained using Toluidine Blue.

The difference between various positions could be either related to the endogenous hormonal content or sensitivity of their cells to hormones (Trewavas, 1983; Emery et al., 1998). In a series of articles by Trewavas in the 1980's (Trewavas, 1981a; b; 1982; 1983), it was suggested that the capacity of cells to respond to a growth substance may not be fixed or cells may differ in their sensitivity to the growth substance. Additional interactions between the growth substance and its receptor also may contribute to differences in responsiveness

between cells (Firn, 1986). Firn demonstrated factors which could contribute to sensitivity changes, which included: the growth substance, the receptor for the growth substance and, the affinity of the receptor for the growth substance. These factors can affect the magnitude of the evoked growth response in different positions as well as between different cultivars, which is related to the number of receptors occupied at any time. Each of the potential hypotheses raised potentially warrant further investigation, but has not been the focus of this thesis.

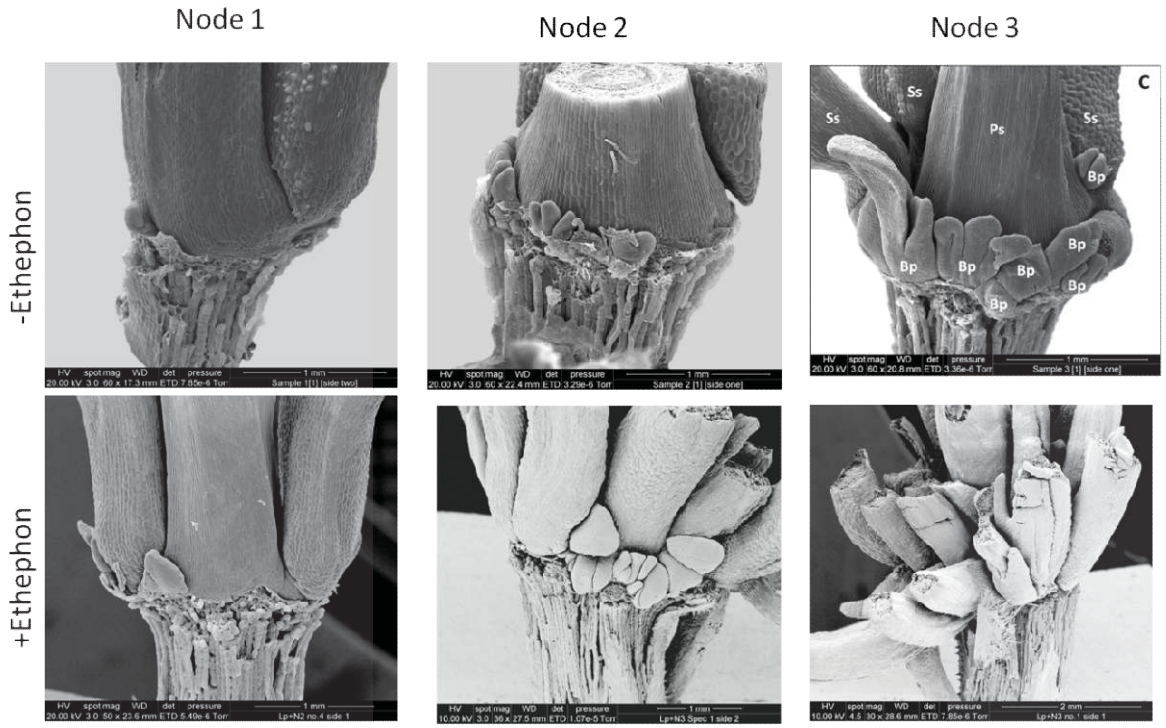


Figure 3-13: SEM features of the node within nodal explants of 'Little Pinkie' (Node 1, Node 2 and Node 3) after 8 weeks growth *in vitro* in the presence (+) or absence (-) of 10 mg.L^{-1} ethephon. Within Node 3, in the presence of ethephon the more developed shoots had been cut back during the sampling and preparation process for SEM observation.

3.3.3. Microscopic differences between cultivars in response to ethephon

In contrast to the results for 'Little Pinkie', multiple bud primordia were not evident around the node of explants of 'Showtime Diva', after 8 weeks growth *in vitro*, irrespective of the presence or absence of ethephon in the medium (Figure 3-3 & 3-14). Only one or two 2º shoots were observed arising from the node/metamer of 'Showtime Diva', whereas from the explants of 'Little Pinkie' there were up to 10 to 15 in the presence of ethephon (refer to Appendix 13). Also for 'Showtime Diva', regardless of the application of ethephon, the number of 2º shoots and/or bud primordia did not increase as the distance of the position that explants were taken from increased from the tip. Explants taken from Node 3 produced up to the maximum of 2 secondary shoots, both with and without application of ethephon, as

compared with the explants taken from Node 1 which produced only one 2º shoot, with the bud primordium within the other phytomer remaining dormant (Figure 3-15). The small numbers of shoots found in 'Showtime Diva' in either the presence or absence of ethephon in this experiment was not expected, as it was previously reported to produce up to 4 shoots (not necessarily limited to secondary shoots however) following the application of ethephon *in vitro* (Wang, 2014). This different result might also be related to differences in durations of time used by Wang (2014) as compared with in the current experiment (i.e. 12 weeks compared to 8 weeks, respectively). Also, it is likely that distinguishing between secondary and tertiary shoots was not carried out in that study. Hence in the current experiment, if any other bud primordia were to arise, they presumably would develop after 8 weeks of the experiment. Furthermore, it is recognised that different cultivars at different developmental stages within the explant, may respond to ethephon differently as a result of differences in their sensitivity, affinity and/or endogenous hormonal content (Trewavas, 1982; 1983; Firn, 1986).

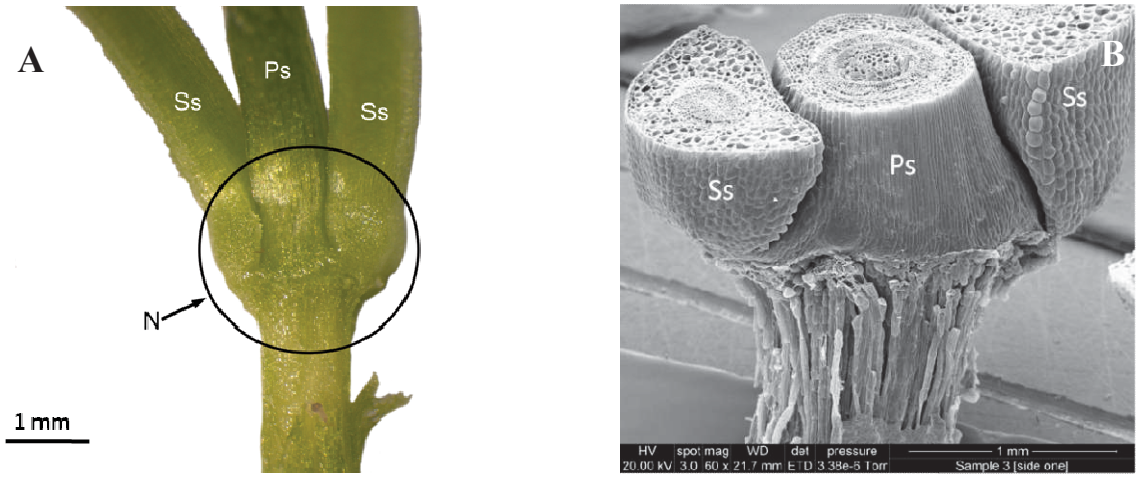


Figure 3-14: Nodal explant of gentian 'Showtime Diva' after 8 weeks growth in an *in vitro* medium amended with 10 mg.L^{-1} ethephon, illustrating the primary stem (Ps) and secondary shoot (Ss) under; **(A)** binocular microscope and, **(B)** SEM, after leaf sheath removed. N.B. developed shoots were cut back during the sampling and preparation process for SEM observation presented in (B).

As a cultivar with a low shoot formation frequency, 'Showtime Diva' naturally does not develop more than 1-2 axillary buds from a node/metamer. If 'Showtime Diva' were able to produce more than 2 shoots following ethephon treatment, future microscopic studies after week 8 could assess whether there is a stimulating effect of ethephon at various concentrations on initiation of new bud primordia. Similarly, microscopic studies could be done on other genotypes such as '26 N 4', which has also been reported as a non-responsive genotype to ethephon (Funnell, pers. comm.).

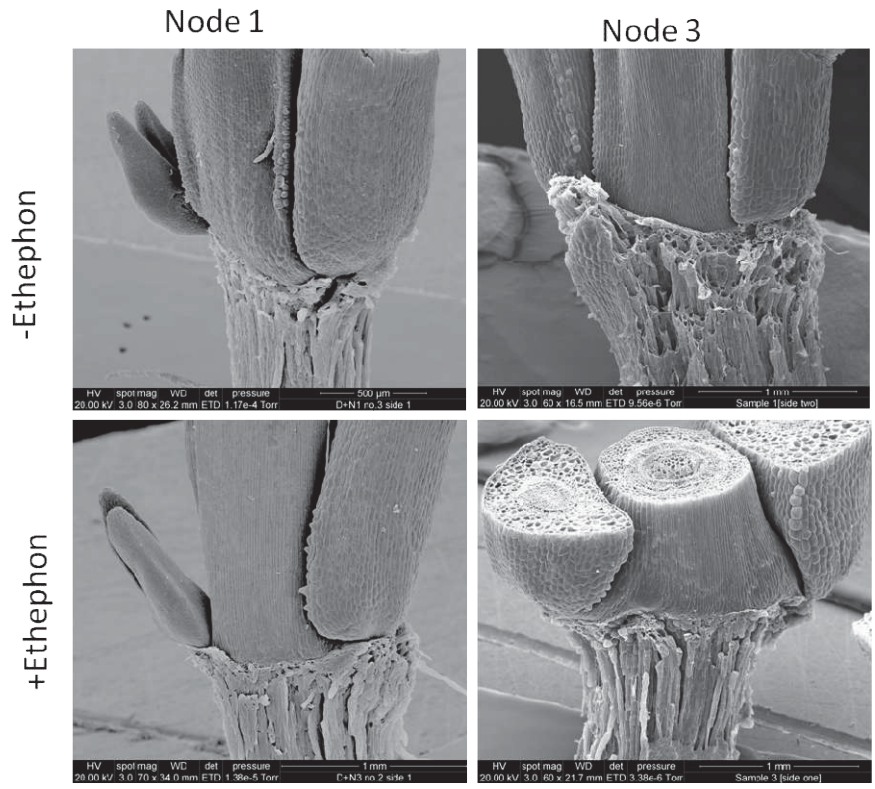


Figure 3-15: SEM features of the node of 'Showtime Diva' (Node 1 and Node 3) after 8 weeks *in vitro* in the presence (+) or absence (-) of 10 mg.L^{-1} ethephon. Within some samples more developed shoots had been cut back during the sampling and preparation process for SEM observation.

Differences between cultivars, stages of culture and/or shoot organogenesis, in their *in vitro* shoot regeneration response to ethephon/ethylene, as the most probable component of decomposition of ethephon in the medium, have previously been reported in other plant species (Huxter et al., 1981; Lakshmanan et al., 1997; Pua & Tiong-Chew Koh., 1999). For other plant species stimulatory effects of ethylene were evident by promoting lateral bud growth and shoot proliferation in rose (Kevers et al., 1992), lavandin (Panizza et al., 1993), eastern white cedar (Nour and Thorpe, 1994), petunia (Dimasi-Theriou et al., 1993), and peach (Dimasi-Theriou and Economou, 1995). In contrast, inhibitory effects of ethylene on shoot development in passion fruit were reported (Trevisan and Mendes, 2005), with the addition of the ethylene inhibitor silver thiosulfate (STS) to the culture medium significantly increasing the differentiation and development of adventitious shoots (Faria and Segura, 1997b). However, the effect of ethylene was not always a one-sided stimulatory or inhibitory effect. In an experiment with tobacco callus, with ethephon and ACC added to the medium, ethylene inhibited shoot initiation early in culture but speeded up primordium formation later during the culture period (Huxter et al., 1981). In summary therefore, several factors such as plant species, concentration, the growth condition and, developmental stage of the explants,

have been suggested to have an effect on the diversity of effects of ethylene on growth in various species (McManus, 2012). In acknowledgement of these potential factors, any experiments within this thesis will need to be done considering these factors.

While ethylene accumulation has inhibited shoot development in many studies (Lai et al., 1998; Arigita et al., 2003; Reis et al., 2003), application of culture vessels with vented lids has been reported to favour shoot elongation and plantlet development (Trevisan and Mendes, 2005). Having confirmed microscopically what effect ethephon has on explants of 'Little Pinkie', for the plant system used in this thesis therefore, the new question which immediately arises is what concentration of ethylene explants were exposed to when the medium was amended with ethephon. The next chapter therefore covers a series of experiments that address this question.

3.4. Conclusion

Those bud primordia which formed at the node in explants of gentian 'Little Pinkie' derived from *in vitro* culture had a combination of axillary, co-lateral and adventitious origins. Number of bud primordia, increased in frequency as the distance of the node position increased from the tip to lower nodes and was identified as the primary source of the non-uniformity. In nodal explants buds formed either individually (adventitious), or in cluster/s associated with pre-existing axillary buds, either in the form of co-lateral and/or adventitious buds. Combining the results of macroscopic and microscopic studies on 'Little Pinkie', as a cultivar with high number of shoots, it was evident that ethephon had its effect on increasing the shoot formation both by accelerating development of pre-existing buds into shoots, and stimulating new adventitious buds to form. The proposed mode of ethephon's action through the modification of auxin to cytokinin ratio was investigated in subsequent chapters of this thesis. In contrast to the results for 'Little Pinkie', explants of 'Showtime Diva' did not produce multiple shoots beyond those derived from pre-existing axillary buds, irrespective of the presence or absence of ethephon. Regardless of their origin, ethephon seem stimulate buds from all types to develop in to shoots.

Chapter 4. Ethylene production from the decomposition of ethephon *in vitro*

4.1. General Introduction

Following the observation of increased shoot formation in gentian 'Little Pinkie' as a result of ethephon application *in vitro* (refer to Chapter 3 and Appendix 13), it is hypothesised that the increased number of 2º shoots can be attributed to both accelerated growth of existing buds and additional adventitious shoots being induced by ethephon. Ethephon is stable below pH 4; as the pH is raised above this, the compound decomposes to ethylene, phosphate and chloride ions (Yang, 1969; Goudey et al., 1987a; Biddington, 1992; Marin et al., 2006). The reaction is catalysed by hydroxyl ions. The half-life of ethephon at pH 6 is about 96 hours; at pH 7, under the same conditions, about 80% conversion to ethylene occurs over the same duration of time (Domir and Foy, 1978). The *in vitro* growing medium which was utilised in this thesis, and resulted in increased shoots in explants of 'Little Pinkie', was buffered to be at pH 5.7 as a part of general lab protocol (refer to Chapter 2, Section 2.2). Although for any experiment pH adjustment in the medium was carried out after autoclaving and before the inclusion of ethephon, adding ethephon at such a small volume of concentrated ethephon was not expected to make a significant change in the final pH of the medium. It is hypothesised therefore that, within the current *in vitro* system, ethephon exerts its effect on increasing shoot formation through its decomposition to ethylene.

Previous studies on the application of ethylene *in vitro* have been mostly focused on the negative/positive effects of ethylene/ethephon on growth variables of the plants (Harbage and Stimart, 1996; Kerbauy and Colli, 1997). No published paper was found detailing an ethephon-ethylene relationship *in vitro*, nor the concentration(s) in an amended agar medium. As presented in this chapter, so as to develop greater understanding of the efficacy of ethylene/ethephon in increasing the number of shoots in gentian 'Little Pinkie', a series of experiments were conducted. The overall aim of these experiments was to develop an effective methodology to measure the concentration of ethylene released following the decomposition of ethephon within the atmosphere around the explants (i.e. headspace of the culture vessels) (Figure 4-1).

Figure 4-1: Non-sealed culture vesselused as part of the experimental system within this thesis, illustrating the agar medium, free headspace, and the lid which afforded gas exchange.

4.2. Ethylene concentration in vessels and pattern of release from ethephon

4.2.1. Introduction

The first experiment in this chapter focused on developing techniques for quantifying the concentration of ethylene in the headspace, when released from the decomposition of ethephon contained within the amended *in vitro* medium. It was acknowledged that the concentration of atmospheric ethylene would directly be affected by the type and volume of the culture vessel, physical characteristics of the agar-based medium, and the duration of ethephon decomposition. As part of the general lab protocol, the culture vessels used were disposable vessels with snap-on lids (Alto Packaging, Hamilton, New Zealand). The design of the lids of the culture vessels were considered to provide adequate gas exchange needed for plant growth, while simultaneously preventing contaminating organisms getting into the vessels. This design included 240 very small, gas permeable, channels all around the sections of the lid which were in contact with the base, providing spaces between the edge of the base and the lid (Figure 4-2). Gas exchangeable vessels as compared with sealed vessels were hypothesised to result in different concentrations of ethylene in the headspace when containing medium amended with ethephon. In some preliminary trials this hypothesis was tested by comparing the concentration of ethylene in the sealed glass vessels and gas exchangeable vessels, i.e. those which were used for culturing explants *in vitro* within this thesis.

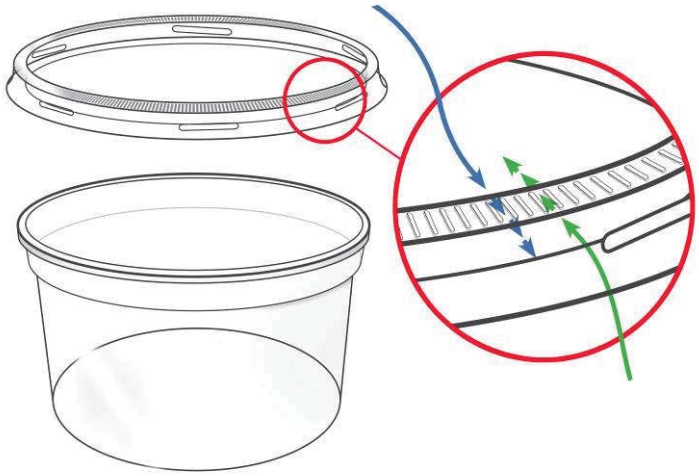


Figure 4-2: An illustration of the culture vessels used, highlighting the ability for gas exchange provided by the moulded channels within the section of lid which rests on the top edge of the container. Graphic image by T. Corbett (Plant & Food Research).

Different equipment is available for measuring the concentration of ethylene in the headspace of vessels. Gas chromatography (GC), is a common type of chromatography used for analysing the different components of a gaseous mixture and determining the relative amounts of such components (James and Martin, 1952; Kevers et al., 1992; Feng et al., 2003; Huang and Chen, 2005). As a technique it should be able to accurately detect ethylene at a wide range of concentrations down to ca. 50-100 ppb (part per billion). As described in this chapter, the concentration of ethylene in sealed and non-sealed vessels was attempted to be measured using GC, in a preliminary experiment. When the concentration was confirmed to be lower than detectable by GC, another piece of equipment, a commercial laser-based ethylene detector (type ETD-300 (ETD), Sensor Sense B.V., Nijmegen, the Netherlands), provided an opportunity to monitor ethylene based on laser photoacoustic spectroscopy in real time without incubation periods, and measure it at concentrations down to 300 pptv (1 pptv = 1 part per trillion volume = $1:10^{12}$) and time resolution of 5 s (Woltering et al., 1988; Cristescu et al., 2008; Clarke et al., 2009; van den Dungen et al., 2011; Forni et al., 2012). For application in the current study therefore, as a progressive process both pieces of equipment were used for ethylene measurement, in order to develop a protocol which could be used to measure the concentration of ethylene in the headspace of the vessels accurately.

The ETD has been designed to perform two measurement methods: ‘continuous flow’ and ‘Stop and flow’. Additionally however, there is another method which uses the ‘Stop and flow’ method with some modifications, which is called ‘sampling’. As described in the

following sections, all three measurement methods were evaluated for their application to this research.

'Continuous flow' method

The 'continuous flow' methodology provided a consistent flow of ethylene-free air to a chamber of interest (i.e. culture vessels evaluated in this research). As stated in the manual developed by the manufacturer, this method has been designed to be used with completely sealed vessels, so as to provide a consistent flow rate through the system continuously. When using the 'continuous flow' method, even a very tiny hole in the system made establishing the required consistent flow rate difficult to achieve. Only gas-tight vessels therefore were utilised when this measurement method was used.

The equipment provides an open system with the option of sending an adjustable flow rate of air to carry the gas sample through the cuvettes of the detector, before eventually exhausting this out of the system (Millenaar et al., 2009)(Figure 4-3). A stream of ethylene-free air which has been scrubbed by the catalyser flushes through cuvettes continuously. The valve controller distributes ethylene-free air into each cuvette and related culture vessel through an individual outlet. Gas samples then are taken from the culture vessel connected to each cuvette into the valve controller through an individual inlet. Air that has left the cuvette passes through scrubbers to remove all CO_2 and H_2O and then entered a laser-driven photo acoustic ethylene detector (Sensor Sense B.V.). Measurement of the ethylene concentration in the gas sample takes place in the detector. The distinguishing characteristic of conducting 'continuous flow' measurements is that through all selected cuvettes the air keeps flowing throughout the full experiment, even when the cuvette is not being measured (i.e. one of cuvettes is measured at any one time). As a result therefore, there is no accumulation of ethylene either in the vessels which are, or are not, measured at any point in time. This method could be used to measure real-time ethylene release and could be considered to present a reflection of the rate of ethylene production at the time of measurement.

Before conducting the measurement, it was considered likely that the humidity inside the culture vessels without air flowing into them would be higher than when they are exposed to continuous air flow. Hence, it was considered likely that in following the 'continuous flow' method' a dry skin would develop on the surface of the agar-based medium over time. If so, the dry skin might trap the ethylene released from the decomposition of ethephon, leading to

decreased production of ethylene within the headspace. Therefore, there might be a difference in concentration of ethylene released from the surface of the medium that has been exposed to a continuous flow of dry air over time, as compared with when exposed to a humidified air flow.

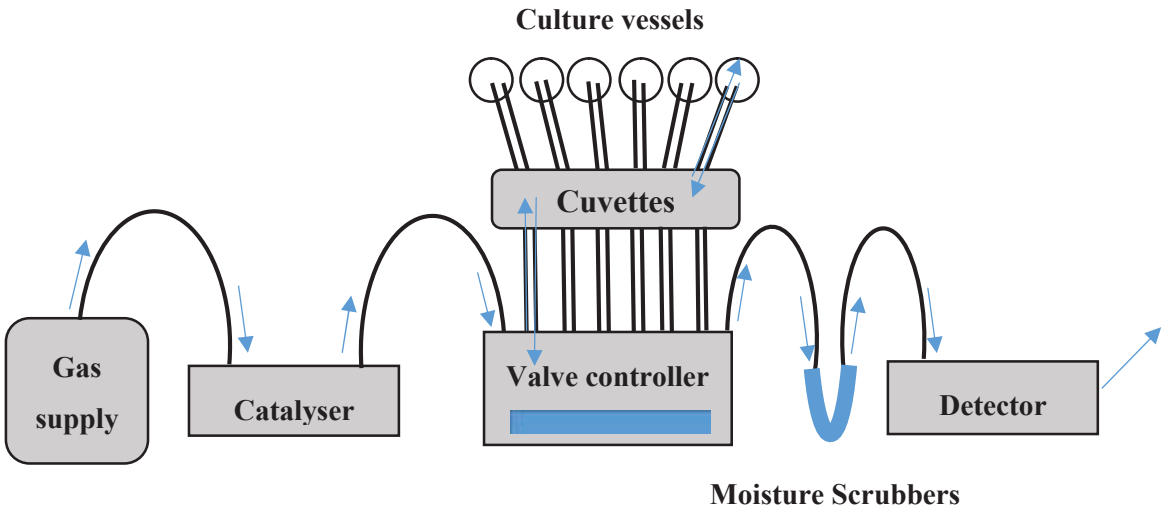


Figure 4-3: Illustration of a typical experimental set up for ethylene measurement using ETD-300 (Sensor sense B.V.) A stream of air passes through a catalyser to remove any possible hydrocarbon and then at a controlled flow rate passes through 6 cuvettes to carry samples. Each cuvette is sampled independently. A valve controller distributes the ethylene-free air into each cuvette and related culture vessel through an individual outlet. A gas sample then is taken from the culture vessel connected to each cuvette into the valve controller through an individual inlet. The gas sample then passes through KOH and $CaCl_2$, which are moisture and CO_2 scrubbers respectively, to prevent CO_2, water or water vapour entering the detector. Measurement of the ethylene concentration in the gas sample takes place in the detector. Only one of six cuvettes is measured at any one time. Blue coloured arrows indicate stream of gas.

'Stop and flow' method

In this method, ethylene accumulates in the headspace for an adjustable amount of time in an enclosed environment, before it is transported to the ethylene detector with an adjustable flow rate. Up to 6 cuvettes are sampled in series in this way. While one cuvette is being flushed out and measured, the other cuvettes are accumulating ethylene for a known period of time (Sensor sense B.V.; Forni et al., 2012).

'Sampling' method

While similar to the 'Stop and flow' method the 'sampling' method utilises non-sealed vessels which are only briefly connected to the ETD. Using a pump the whole internal atmosphere was gradually pulled out of each container and sent to the ETD for ethylene measurement. In contrast to the 'continuous flow method' therefore, between each measurement time vessels were not under a forced air flow, albeit gases were able to diffuse as normal through the small gaps underneath the clip-on lids (Figure 4-2). Hence this method was considered to present a reflection of the concentration of ethylene accumulated in the gas exchangeable vessel during the time interval between sampling times. Since only one vessel could be measured each time using the 'sampling' method, to be able to capture the peak in a reasonable amount of time needed for each vessel, a higher flow rate than when using the 'continuous flow' method was used. At the end of each measurement, a fresh, ethylene-free atmosphere replaced the previous ethylene-containing atmosphere in the vessel. So as to evaluate the impact of this sampling method on the concentration of ethylene inside the culture vessels therefore, two types of measurements were compared, i.e.:

- repeated measurements, and
- non-repeated measurements

Since the 'Sampling method' was applied on non-sealed vessels, it was likely that there might be non-uniformity between culture vessels in gas exchange due to the special structure of the lids (Figure 4-2), and variation in how tightly they were fitted. If this was the case therefore, non-uniformity in the concentration of ethylene in the headspace of culture vessels may occur. Capturing the possible non-uniformity between replications of vessels in their gas exchange would be valuable in designing future experiments, and making any conclusion on the effect of ethephon on explants cultured in these vessels. Hence, the concentration of ethylene which accumulated in the headspace of different replications of the non-sealed culture vessels was measured and compared.

Finally, to develop a complete understanding of how the different sampling methods compared in their methodology of ethylene measurement, the concentration of ethylene in vessels was measured using the 'sampling' method and compared with the result of ethylene concentration measured using the 'continuous flow' method. This knowledge was then used to determine the most effective methodology for ethylene measurement.

As described in the following sections therefore, a series of trials were undertaken in order to evaluate and compare the effect of different containers, equipment and methods of measurement. Therefore for the first experiment of this chapter the objectives were:

- to compare different methods ('continuous flow' versus repeated and non-repeated 'sampling' method) for ethylene measurement inside the headspace of culture vessels containing ethephon in order to identify a practical, effective protocol for quantifying the:
    - concentration of ethylene over time and,
    - rate of ethylene release over time.
- to determine the uniformity of the culture vessels in terms of gas exchange.
- to quantify the effect of RH in the air flow on the concentration of ethylene measured in the headspace.

4.2.2. Materials and methods

4.2.2.1. General materials

The base medium for the experiment, which was a PGR-free medium, was prepared as detailed before (refer to Chapter 2, Section 2.2). The pH of the medium was adjusted to 5.7 with either 0.1 N NaOH or 0.1 N HCl, prior to autoclaving. The medium was cooled after autoclaving to 50°C and then ethephon (Ethrel®; 480 $g.L^{-1}$ chlorethephon; Bayer Crop Science, NZ), was added after being filter sterilized (0.2 µm Minisart®filter; Sartorius Stedim Biotech, Germany) to achieve a final concentration of 10 $mg.L^{-1}$. The same final volume (50 mL) of medium, measured using sterilised measurement cylinders, was inserted in each culture vessel. Culture vessels used for the experiment were disposable 290 mL (80 mm base diameter × 60 mm deep) tubs with snap-on lids manufactured by blow moulding of a general purpose styrene and K-resin plastic (Alto Packaging, Hamilton, New Zealand; Figure 4-1).

4.2.2.2. Method development and experiment

In the following experiments the vessels did not contain plant material. Some preliminary experiments as listed below were necessary in order to develop the methodology of measuring ethylene concentration in vessels. Ethylene was measured using two pieces of equipment and various 'sampling' methods:

– ***Preliminary experiment 1***

Using GC a preliminary experiment was carried out in order to measure the concentration of ethylene released from the medium into the atmosphere of sealed vessels compared to vessels with unsealed lids. For this purpose, two types of vessels of the same size were tested:

- sealed glass jars (sealed using a rubber-lined metal lid with a screw-top ring),
- plastic culture vessels (Figure 4-2) with lids sealed with masking tape and,
- plastic culture vessels without sealed lids (i.e. gas exchange permitted by manufactured lids; Figure 4-2).

Hence in total, this experiment comprised three types of containers as treatments, and was conducted as a Completely Randomised Design (CRD) with five replicate vessels for each treatment.

Lids were perforated and sealed with a rubber septum before inserting the medium into the culture vessels, so as to facilitate sampling. Putting some clear glue, using a hot glue gun, around the rubber septa was effective for making a better seal. Gas samples of the headspace of each container were taken with a 1 mL sampling syringe from two rubber septa installed on the lids of culture vessel and immediately injected into the GC. Each gas sample was injected into a Varian 3400 GC fitted with a flame ionization detector and equipped with an alumina column (Porapak Q 80/100 156.4 mm × 45.72 mm × 2.159 mm, AllTech Associates, New Zealand). The column was set at 70 °C, with N_2 as the carrier gas, at a flow pressure of 200 kPa. Flame ionization detector temperature was set at 150 °C with H_2 and air pressure of 55 and 60 kPa respectively. A β-standard gas (10.3 ± 0.2 $\mu L.L^{-1}$ ethylene in air) was used for calibration (BOC, New Zealand). Output signals were analysed and recorded with an HP 3394A (Hewlett Packard, USA) integrator.

So as to provide environmental conditions similar to that used when culturing plants *in vitro*, containers were held at 25 ± 1 °C, at a photosynthetic photon flux density (PPFD) of 30 ± 5 $\mu mol\ m^2.s^{-1}$ and 16 h photoperiod, provided by cool-white fluorescent tubes in a chamber beside the GC. These conditions being the same as that used for culture of explants throughout this thesis.

On the first day, measurements were done at 2 h and 6 h after adding ethephon to the medium, followed by daily measurements at 24 h intervals. Instead of measuring ethylene

over 4 days, which had been reported to be the half-life for ethephon in a buffer at pH 6 (Domir and Foy, 1978), the duration of the experiment was extended to 5 days. Extending the measurement time from 4 to 5 days was in order to consider the possibility of a longer half-life for ethephon at a lower pH (5.7) as compared with pH 6.

– ***Preliminary experiment 2***

For more sensitive ethylene measurements, the concentration of ethylene in the vessels was measured using an ETD detector, either using the 'continuous flow' or 'sampling' method (refer to Section 4.1). Using 'continuous flow', the effect of RH in the air flow on the concentration of ethylene was also investigated.

To test whether relative humidity (RH) within culture vessels exposed to continuous air flow differed from the RH in vessels without any air flow: three culture vessels of each type containing 50 mL of medium were used. RH loggers (iButton, Maxim integrated Company, USA) were sterilised (dipped in ethanol and dried within a laminar flow hood) and glued onto the interior wall of vessels before the experiment started. Those vessels exposed to continuous air flow were sealed all around the edges using a hot glue gun and connected to the ETD, 2 hours after preparing the medium. The ETD provided a very slow 'continuous flow' of carrier gas inside the vessel's atmosphere (i.e. 1 $L.h^{-1}$, being the smallest possible rate of air flow which runs through the machine). The air through all three cuvettes kept flowing throughout the whole experiment. Another three culture vessels which were not sealed, were used as the control treatment. The RH in both types of vessels was recorded for 7 days after the experiment commenced.

During application of the 'sampling' method, repeated measurement of culture vessels was compared with non-repeated measurement.

– ***'Continuous flow' and humidity***

To test the hypothesis that RH influences the concentration of ethylene in the vessel, the concentration of ethylene was measured in vessels exposed to air without humidification (RH~ 50 to 70% depending on the environmental humidity in the lab) as compared with vessels exposed to humidified air (RH ca. 95%). To achieve 95% humidity in the air, a humidifier was used before sending the air flow into each container. Air was first passed through a flask (placed in the room) containing 300 mL of 21% glycerol in water before

continuing to each container (Forney and Brandl, 1992). Each inflow was also passed through a 0.2 µM membrane filter (MINISART, Global Scientific Company, Germany) to prevent microbial contamination.

The 6 culture vessels were individually connected to one of the 6 cuvettes of the ETD, which was the maximum number of inlets available. Three replications of culture vessels were exposed to non-humidified airflow, and another three were connected to the humidified air flow. All culture vessels were sealed around the edge of their lids using a hot glue gun before being connected to ETD (because preliminary trials determined masking tape to be ineffective). There were two sets of tubes for the inflow and outflow plugs to connect the culture vessels to cuvettes, and to the equipment (Figure 4-3). In consideration of the rapid process of ethephon decomposition (Yang, 1969; Domir and Foy, 1978; Goudey et al., 1987b; Biddington, 1992; Marin et al., 2006), the time duration needed for making the agar medium and setting up the ETD machine was kept as short as practicable; vessels were first connected to the ETD 4 hours after adding ethephon to the medium.

– ***'Sampling' method, repeated versus non-repeated***

At each time of applying the 'sampling' method, non-sealed plastic vessels (i.e. gas exchange via the manufactured lids) with rubber septa on their lids were sequentially connected to the ETD for 20 minutes. A sampling needle and tube were inserted into the rubber septum previously installed on the lid of each vessel. A pump was used to gradually pull out the internal atmosphere of each vessel, at a flow rate of 5 $L.h^{-1}$, and send this to the ETD for ethylene measurement. The first measurements were taken 4 hours after adding ethephon to the medium and closing the lid. Subsequent measurements were recorded at 48 h intervals, for a period of two weeks. Between each measurement time containers had no forced airflow, but gases were able to diffuse as normal through the small gaps underneath the clip-on lids (refer to Figure 4-2). During each 20 minute period of measurement, the ETD determined the concentration of ethylene every 6 s in each vessel. The integrated area under the peak was calculated by the software (Valve controller 1.4.1, Sensor Sense, Nijmegen, The Netherlands). Data derived from application of the 'Sampling method' (non-repeated) were used to calculate the rate of ethylene production and compared with the rate of ethylene production measured by the 'continuous flow' method.

During repeated measurements, three replicates of the same culture vessels were used for every measurement over the two-week period of observation. In contrast, for the non-repeated measurement, three fresh replicates of culture vessels were used at each measurement time.

Using the 'sampling' method, when samples were analysed, the graph which represents ethylene concentration in the detector normally shows a peak (Figure 4-4). Within this figure the red coloured lines connect multiple data points which represent the concentration of ethylene taken every 6 s. Data handling software was used to calculate the area under the graph, which represented the entire quantity of ethylene in the vessel at the time of measurement. The black coloured line is a projection of the base line for the ethylene-free airflow which was flushed through the vessels before each measurement.

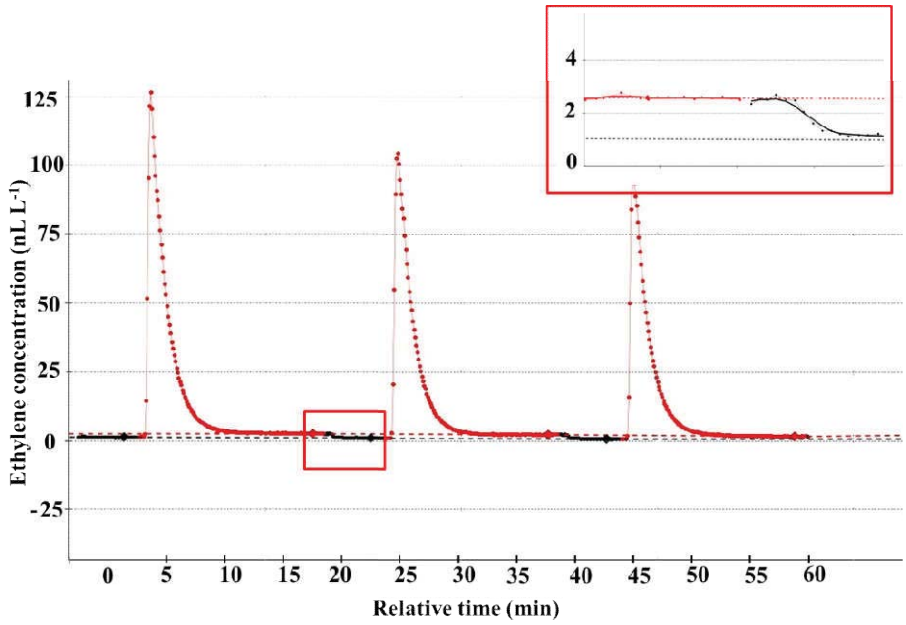


Figure 4-4: Measurement of ethylene concentration in three replicates of non-sealed culture vessels containing 10 $mg.L^{-1}$ ethephon, recorded by ETD at a flow rate of 5 $L.h^{-1}$. Each red coloured peak represented the concentration of ethylene in each replication relative to the time from when the first vessel was connected to the ETD for measurement. Two kinds of measurement can be made from the data: (1) integrating the red peaks allows calculation of the total quantity of ethylene present in the chamber and flushed out by the ETD; and (2) The difference between the red asymptote and the dotted black line represents the instantaneous rate of ethylene release from the agar medium into the airflow at the time of measurement. In the magnified area (red coloured square): When the container was disconnected, the concentration (black line) returned quickly to the original (dotted black) base line representing 0 $nL.L^{-1}$ ethylene.

A continuous measurement of the rate of ethylene release from the medium could be achieved by using the 'continuous flow' method. Data handling software provided hourly

average values for the amount of ethylene which had been quantified by the ETD every 6 s. When following the 'sampling' method, the rate of ethylene production was also calculated from the difference between the stabilized levels of the peak of ethylene concentration (end of the red coloured line in Figure 4-4) with the stabilized level for the base line. For calculation, the average value derived from 15 data points of the base line after each measurement time, was subtracted from the average value of 15 data points of the end of each measurement time after reaching a stabilized level.

Both the experiment comparing sampling methods and that comparing the influence of RH were based on a completely randomised design. Data were analysed using General Linear Model in Minitab (version 16.1.1, Minitab Inc., State college, Pennsylvania, USA) and Genstat (Genstat, version 17, 2014, VSNi Ltd, Hemel Hempstead, UK). Replications of vessels were considered as a random factor, and both method and time of measurement as fixed factors. Mean separation was done using Fisher's protected least significant test at $P \leq 0.05$. For comparison between non-repeated versus the repeated method another analysis; the REML (Restricted Maximum Likelihood) or mixed effect analysis in Genstat, was also carried out.

4.2.3. Results and discussion

– *Preliminary experiment 1*

Results indicated that while the amount of ethylene was detectable using the GC, it did not exceed 5 $mg.L^{-1}$ during a five-day measurement period in the sealed glass vessels (data not presented). In the plastic containers however, no ethylene was detected by GC even when the vessel's lids were 'sealed' with tape. It was not clear whether the plastic container walls themselves were porous to ethylene; but it was considered more likely that the tape did not provide an adequate seal to retain this volatile material. No ethylene was detected by GC in gas samples taken from the headspace of non-sealed culture vessels at different time intervals (2, 6 and 24 hours, and 5 days) after ethephon was added to the medium. It was concluded that the concentration of ethylene released from the medium was very low and likely below the detectable level of the equipment (GC). Hence, the ETD with more sensitivity to ethylene (down to 300 ppt) was subsequently used for ethylene measurement in the headspace of culture vessels.

– ***Preliminary experiment 2***

There was a significant decrease in RH (on average 2.7%) between vessels exposed to air flow compared to the vessels without air flow ($P \leq 0.05$)(Figure 4-5). Continuous air flow apparently decreased RH inside vessels by taking moisture out of the vessel and replacing it with fresh dry air continuously. As a result, a stable value of RH (ca. 92.5 to 93%) was established within 2 days, and continued over a 7 day period. The lowest RH was measured on day 6 and 7 at the end of the measurement period. In contrast, average RH in vessels without any air flow increased slightly (from 95 to 95.5%) which was probably due to the accumulation of humidity inside the vessel over time.

In spite of statistically significant changes in the averages of daily RH for both types of vessels over time (Table 4-1) however, the fluctuation was only up to 0.5% RH and, therefore, was practically negligible. Hence, at a room temperature of 25 ± 1 °C the average RH within non-sealed culture vessels containing agar medium without air flowing through averaged about 95% and in flowing air about 92.5%. Whether this subtle decline in RH in vessels with air flow compared to vessels without air flow influences the concentration of ethylene or not, was addressed in the next experiment of this chapter.

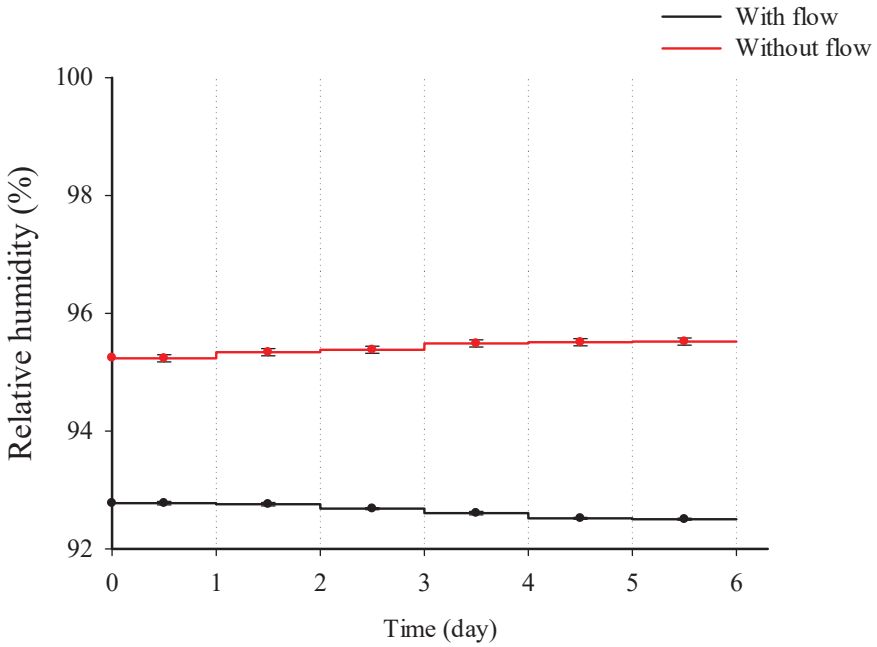


Figure 4-5: Daily averages of relative humidity inside culture vessels containing 50 mL agar medium measured continuously over time. There was a 1 $L.h^{-1}$ continuous air flow inside the sample vessel (black line). There was no air flow rate inside the control vessel (red line). Vertical bars represent ±standard error of means, n=3.

Table 4–1: Relative humidity inside culture vesselscontaining 50 mL agar medium with and without being exposed to 1 $L.h^{-1}$ continuous air flow, n=3.

Day	No flow	Flow
1	95.244 f[Z]	92.776 a
2	95.234 g	92.776 a
3	95.338 e	92.757 ab
4	95.380 d	92.682 bc
5	95.487 c	92.606 c
6	95.508 b	92.518 d
7	95.519 a	92.503 d

[Z] Groupings for mean separation are based on Fisher's protected least significant test. Means followed by different letters within a column were significantly different at $P \leq 0.05$.

- ***Continuous air flow and humidity***

There was clear evidence of a rise in rate of ethylene production for the first 48 hours (day 2) in both humidified and non-humidified air streams (Figure 4-6). The rate of ethylene production subsequently stabilised over the subsequent day (i.e. day 3). It was hypothesised that such stabilization might be as a result of achieving an equilibrium between the rate of ethylene production from the medium and the amount of ethylene taken out by the outflow, with a constant rate of 1 $L.h^{-1}$ to the detector. Such equilibrium (~19.5 ± 0.08 $nL.h^{-1}$ for non-humidified and ~10 ± 0.07 $nL.h^{-1}$ for humidified air flow) therefore, was considered to be the maximum rate of release possible in the volume of the culture vessel flushed with 1 $L.h^{-1}$ of air. The rate of ethylene production after day 3 started to decline over time, and reached a minimum at the end of the measurement duration. Daily average of the ethylene production at day 6 was about ~ 82-83% less than the ethylene production in day 3 using both humidified and non-humidified air flow. Given the declining rate of production beyond day 3, it is possible that media in both treatments may not be able to sustain a consistent rate of production/release of ethylene over the 6 days investigated, as well as over a duration longer than 6 days. Since the initial ethylene release was faster, the headspace probably saturated at a higher flow rate than at subsequent days, and then as production from the medium declined the headspace concentration would probably drop and the time which it would take to saturate the vessel would be longer than at the start. Given that the half-life of ethephon is likely to be of the order of 96 h (Klein et al., 1979; Beaudry and Kays, 1987), the early peak in rate of ethylene production is easily understandable.

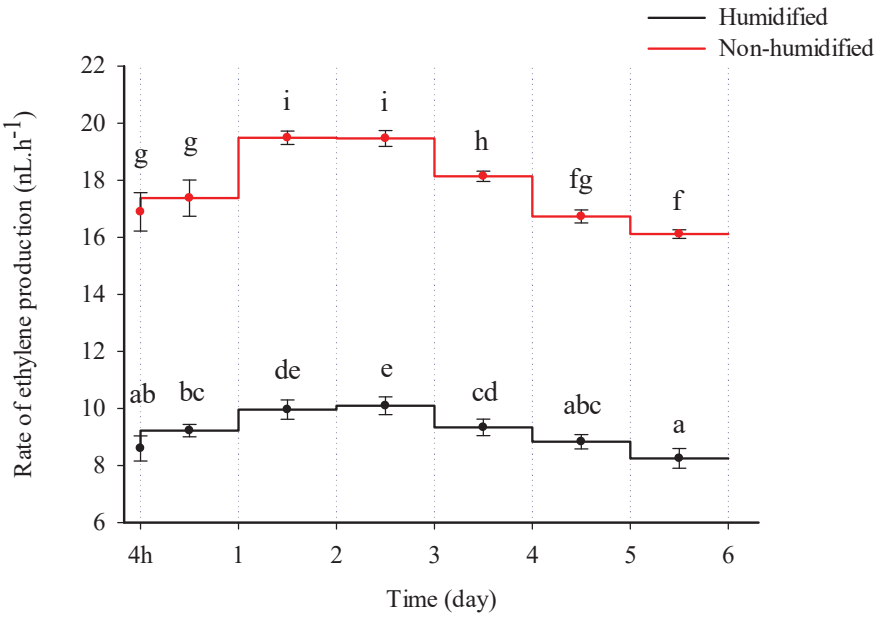


Figure 4-6: Daily averages of rate of ethylene production (nL.h^{-1}) from medium containing 10 mg.L^{-1} ethephon in sealed culture vessels (i.e. glued lid) over time as recorded by ETD operating at a flow rate of 1 L.h^{-1}. Duration on x-axis is the time (hours) after adding ethephon into the medium and closing the lids. Culture vessels were connected to the ETD 4 hours after adding ethephon to the medium, and ethylene production was recorded continuously over time from then. Daily averages of the rate of ethylene production were calculated from the data recorded continuously using either a 'continuous flow' of non-humidified air (red line) or humidified air (black line). n = 3 for both treatments. Vertical bars represents ± standard error of mean. Fisher's protected least significant test was used for mean comparison. Means accompanied by different letters were significantly different at $P \leq 0.05$.

Although it was not expected, but increasing RH significantly decreased the overall rate of ethylene production quantified in vessels by 1.9 times (Figure 4-6). The higher rate of ethylene production using non-humidified compared to a humidified air flow could result from micro-cracks forming on the surface of the desiccated medium, which would increase the surface area for gas exchange. Alternatively, it could be due to the smaller space available for ethylene molecules within humidified air within the headspace, as a consequence of some spaces being occupied by the water vapour molecules within the total volume of the headspace of the culture vessels; i.e. the humidified air 'saturated' more quickly with evolved ethylene, changing the diffusional gradient. In support of this hypothesis it has been reported that vapour pressure has a considerable effect on the rate of ethephon decomposition at a certain temperature and pH (Klein et al., 1979; Beaudry and Kays, 1987). Breakdown of ethephon increased up to a certain level, at a constant temperature, as a result of an increase in the vapor pressure. Ethephon breakdown then decreased above an optimum vapor pressure. At pH 6.3 and temperature of 20 °C, half-life of ethephon at vapour pressure of 1.70 g.mL^{-1} was about 60 hours. Half-life of ethephon dropped to about 30 hours by decreasing the

vapour pressure from 1.70 to 1.40 $g.mL^{-1}$. However, by decreasing the vapour pressure to 1.25 or 1.10 $g.mL^{-1}$, half-life of ethephon increased to 87.5 or 94 hours, respectively. In the current study, irrespective of the underlying causes for the difference in the rate of ethylene production measured in non-humidified and humidified air flow, ethylene release continued throughout the entire measurement time of 144 hours. It was likely that the half-life of ethephon in this study was more than the study done by Klein, which could result from the lower pH (5.7) and presence of other ingredients together with ethephon in the culture medium. Due to a lower rate of ethylene production in samples exposed to humidified air flow compared to non-humidified air flow, a higher amount of non-released ethylene should remain in the medium. While it was not investigated further in the current study, the amount of ethephon remaining in the medium could be measured in future experiments.

The 'continuous flow' method could be used to determine both the rate of ethylene production, and also to calculate the concentration of ethylene inside the vessels at any single time of measurement during *in vitro* culture. 'Continuous flow' method had been applied previously by other researchers to quantify ethylene in various biological systems (Millenaar et al., 2009; Salman et al., 2009; Hermans et al., 2010; Lloyd et al., 2011; van den Dungen et al., 2011; McDaniel and Binder, 2012; Mur et al., 2012; Nitsch et al., 2012; Pranamornkith et al., 2012). For example; ethylene release was measured continuously in real-time on control and heat-stressed or low light intensity-treated plants of *Arabidopsis thaliana* under standard growth chamber conditions (Clarke et al., 2009; Millenaar et al., 2009). Also, in another study production of ethylene was measured continuously by ETD in kiwifruit during up to 50 hours of being either wounded or inoculated with Botrytis (van den Dungen et al., 2011). Using the 'continuous flow' system it was possible to quantify the entire amount of ethylene released from the medium because culture vessels were sealed with a glue gun and ETD was designed in a way to stop measurement if any leakage from the vessels was diagnosed. Therefore, apart from the presumed negligible quantity of ethylene diffusion through the plastic vessels, there was not any ethylene lost from the system. However, there were some limitations with the 'continuous' method, even after repeating the experiment for several times, which had to be considered:

- There were only 6 inlets for the ETD (Salman et al., 2009; van den Dungen et al., 2011), therefore from a statistical point of view it was not possible to consider multiple treatments with replications.

- Furthermore, despite endeavouring to keep vessels sterilised, and filter sterilising the incoming air flow, some replications of each treatment (i.e. vessels) had to be removed from the experiment due to contamination. For example, out of three vessels used for each treatment, only one remained non-contaminated for the entire period of observation (refer to Appendices 1 & 2).
- A further limitation could be related to the fact that making the vessels sealed and flushing with a continuous airflow would possibly change the actual atmosphere of the headspace and the concentration of ethylene which explants would experience in a non-sealed culture vessel without the air flowing into it. The rate of ethylene production determined using the 'continuous flow' method, even with humidification, would be different from the actual condition and the rate of ethylene production inside a closed, non-sealed culture vessel. Therefore, in addition to the risk of contamination, the 'continuous flow' method may not be suitable for measuring the actual concentration of ethylene which explants in vessels are exposed to, especially for long durations eg. more than 6 days.

Hence, while the 'continuous flow' method could be used for comparative measurements, as used to compare humidified against non-humidified air, a method of measurement which would be representative of the atmospheric conditions experienced by explants when in the vessels needed to be developed. The 'Stop and flow' method was another mode which had been used for the ETD by other researchers before (Roeder et al., 2009; Yordanova et al., 2010; Hermans et al., 2011; Forni et al., 2012). This method was to measure very small amounts of ethylene concentration after allowing it to accumulate in sealed vessels. However, for application within the current study, considering a potentially high rate of ethylene production in a small volume of the culture vessels, there was no need to apply long accumulation times. Furthermore, opposite to all previous research utilizing ETD to measure ethylene in sealed vessels, culture vessels in the current study were gas exchangeable and, therefore, not able to accumulate all of the ethylene that has been produced. Therefore, there was a need to develop a method that could measure the ethylene production in the headspace of non-sealed vessels at specific points of time. Hence a third method of measurement, as described in the following section, was considered for sampling in subsequent experiments. To the best knowledge of the author, as described in the following section the application of the 'sampling method' for quantification of ethylene in non-sealed *in vitro* culture vessels has not been previously reported.

– ***'Sampling' method, repeated versus non-repeated***

The concentration of ethylene detected in the vessel using both 'sampling' methods utilised here, described a similar pattern of ethylene release over time. There was a peak of ethylene concentration accumulated between 4 to 48 hours after adding ethephon to the medium (Figure 4-7). When measured repeatedly the peak concentration, between 4 to 48 hours (day 2), was greater than the concentration from day 10 until the end of the experiment ($P \leq 0.05$). However, with non-repeated measurements the difference in the concentration of ethylene detected in culture vessels at different times of measurements was not statistically significant. The concentration of ethylene measured using the two methods fluctuated between 40 and 80 $nL.L^{-1}$ (Figure 4-7). These concentrations can be viewed as being a result of the equilibrium between releasing ethylene from the medium and leakage via the non-sealed status of the culture vessels. In a non-sealed vessel containing ethephon in the medium, the concentration of ethylene in the headspace released from the decomposition of ethephon, and accumulated in the vessel at the time of measurement, would be the net result of any ethylene lost from the headspace via the edges of the lids (i.e. leakage), and the amount of ethylene released from the agar medium, as summarized in the following equation:

$$C_{Tm} = (R_{MR} - R_L) \times t$$

Where:

C_{Tm}= Concentration of ethylene at the time of measurement ($nL.L^{-1}$)

R_{MR} = rate of release from medium ($nL.h^{-1}$)

R_L = rate of leakage from vessel ($nL.h^{-1}$)

t = duration of accumulation in vessel (h)

Following the addition of ethephon to the medium, within a closed, but non-sealed culture vessel, it is possible that the rate of leakage is not constant; it may begin as a slow diffusion, but accelerate into mass flow as ethylene (and hence pressure) builds up within the culture vessel, to reach an equilibrium which may be dependent on the initial rate of release and how tightly the lid fits on the vessel. Other variables such as the position of the vessels in the culture room/incubator might also affect the rate of leakage.

With 'sampling' method, the atmosphere inside a non-sealed culture vessel flushes out over a few minutes; the integrated area under the peak is all the ethylene that was present in the chamber at the start. Air from the surrounding environment gradually flowed into the vessel through the moulded channels on the edges of the lids (Figure 4-4 & Figure 4-2), resulting from the negative pressure caused by flushing the interior atmosphere out to the equipment. As a result therefore, a continuous dilution happened over time. When the culture vessel was fully flushed the ethylene concentration ultimately reached a stabilised level. As evident by the red coloured lines within the Figure 4-4, the stabilized level was slightly higher than the initial ethylene-free air level shown by the black line. This was because the agar medium was continuously producing ethylene. The little residual 'tail' of ethylene production represents the steady-state rate of production at the particular flow rate applied. Using this 'sampling' method, it was possible to detect even the smallest amount of ethylene production at the time of measurement.

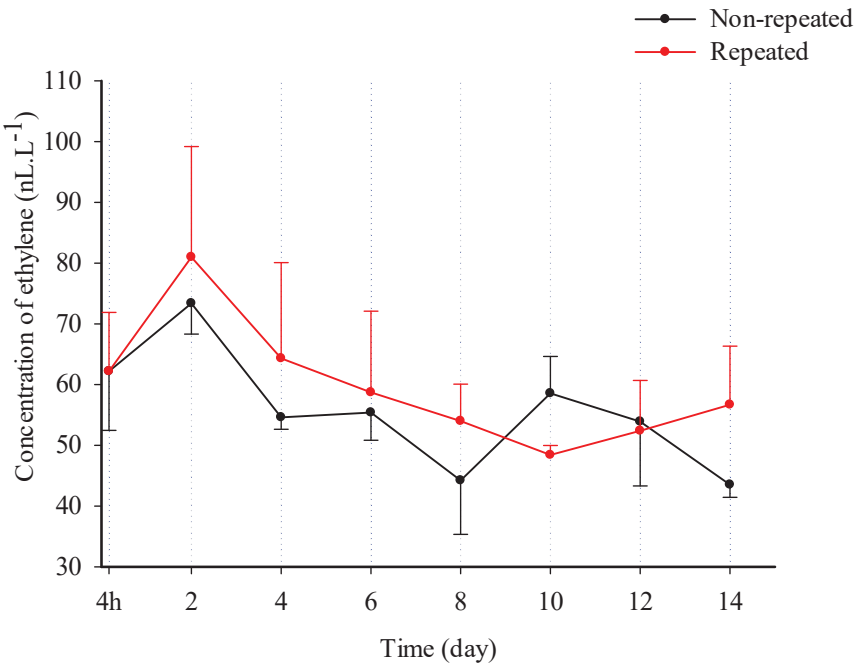


Figure 4-7: Averages of the concentration of ethylene in the headspace of non-sealed culture vessels,measured by non-repeated (black line) which in every vessel was sampled only once at one point of time or repeated (red line) 'sampling' method which in every culture vessel was sampled at every point of time, over the duration from adding ethephon to the medium, as recorded by ETD at a flow rate of 5 $L.h^{-1}$. First measurement was done 4 hours after adding ethephon. Average values at each measurement time were connected by lines over the time only to show the trend, n = 3. For clarity vertical lines only represent + or - standard error bars on the mean values. Fisher's protected least significant test was used for mean comparison at $P \leq 0.05$.

Despite very subtle fluctuations which were not statistically significant, concentration of ethylene measured by both repeated and non-repeated methods seemed to remain almost constant over the 14 days of the experiment albeit with a very gradual decline over time. Concentration of ethylene reached a peak at day 2 for both repeated and non-repeated methods (not statistically significant), followed by a drop which was considered negligible due to the non-significant slope for the regression line (non-repeated: slope = 0.00 and P = 0.982, repeated: slope = -0.16, P = 0.233). For the vessels measured repeatedly, at each measurement time the headspace of the vessel was fully evacuated and replaced by ethylene free air and, therefore, concentration of ethylene was not expected to remain at a level which was almost constant over the entire experiment. It could be concluded therefore, that the rate of ethylene decomposition in vessels measured repeatedly was fast enough to return the concentration of ethylene in the headspace of the vessel to the equilibrium concentration again after each full evacuation (Figure 4-7). With non-repeated measurements, also similar to the repeated 'sampling', the headspace of the culture vessels was occupied by the concentration of ethylene in air, which was not significantly different between each measurement interval. It is acknowledged however, that there might be a significant difference if shorter measurement intervals than that were used here (24 h), or when the decomposition rate of ethephon in the medium was slower or had stopped.

There was a notable difference between individual culture vessels in the concentration of ethylene detected in them using both repeated and non-repeated 'sampling' methods (Figure 4-8). For example in non-repeated 'sampling' at day 2, the highest amount of ethylene during the entire experiment was detected in one of the vessels (80 $nL.L^{-1}$), while the other two replications of vessels measured at the same time contained ~37 % less. Also, the magnitude of variation was different at different measurement times. For example, variation between the concentrations of ethylene measured repeatedly in three replications of vessels at day 12 was 4-fold the variation between vessels at day 4 (Figure 4-8). Variable environmental conditions dependent to the position of the vessels in the culture room/incubator (light and temperature), human error, and variable construction of the vessels are examples of factors that could have influenced the concentration recorded. As an example of this, since these vessels were not sealed, any pressure on the lids, such as the pressure caused by inserting the sampling needle and tube to each vessel, might result in sending some of accumulated ethylene molecules out of the headspace through the bevelled edges of the lids.

Vessels were considered as a random factor in the analysis, and both time and method of measurement as fixed factors, within the General Linear Model, making it a repeated measures analysis. There was no significant difference between the two methods of measurement ($P = 0.68$). In contrast there was a significant difference in concentration of ethylene between vessels ($P \leq 0.01$), which comprised a combination of variability between vessels used for each method, as well as within each method. Another analysis; the REML (Restricted Maximum Likelihood) or mixed effect analysis in Genstat, which deals with multiple sources of variability in data, was subsequently used in order to make a more robust conclusion. The analysis allowed for the extra variability (from vessel to vessel differences) in the non-repeated data, as compared to the repeated. The result of the REML analysis confirmed no significant difference between the two methods of measurements. The variability between vessels when using the non-repeated sampling method was 1.5 times the variability when using repeated measurements on the same vessel (mean square of 255.0 (= 170.4+84.6) compared to 170.4).

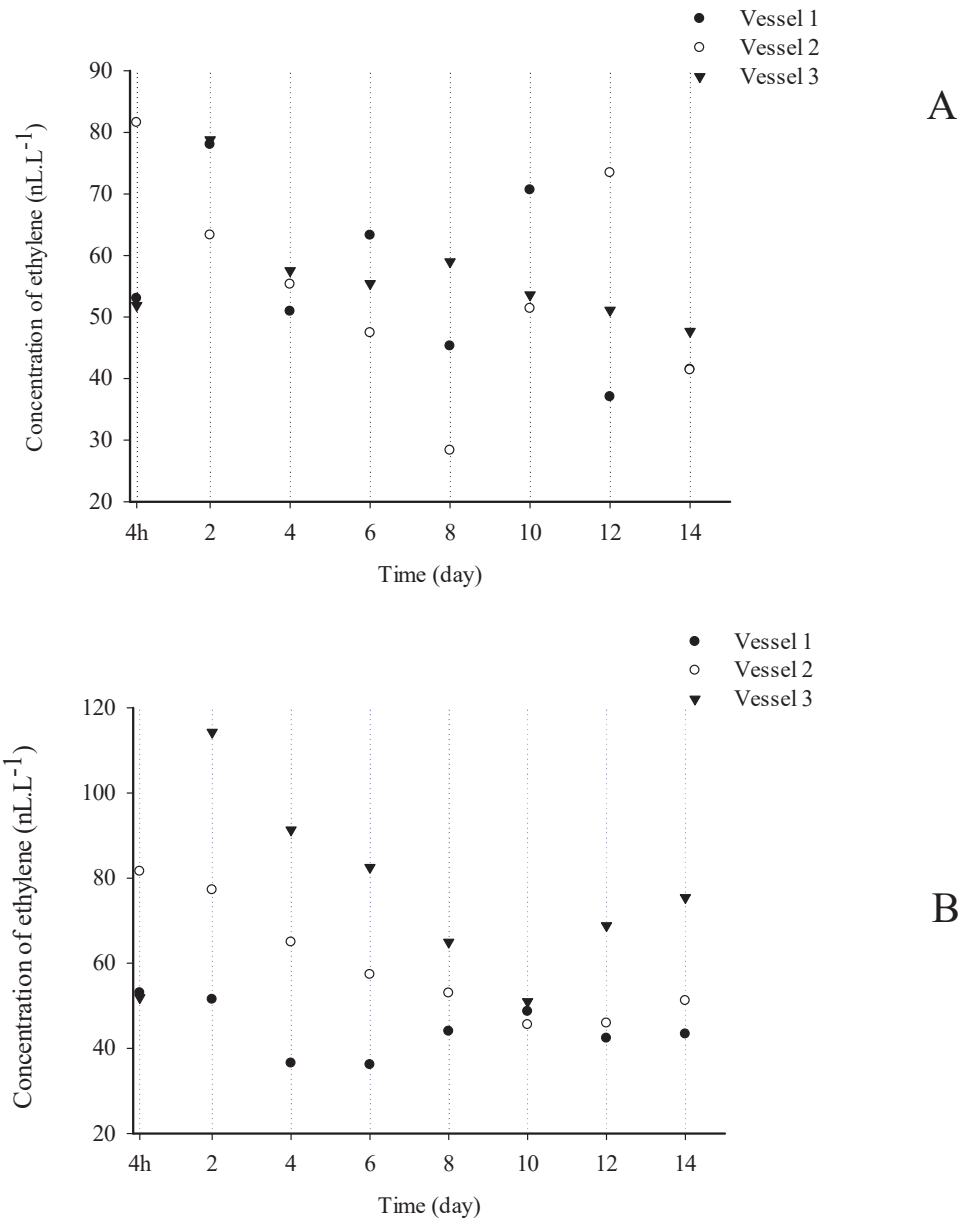


Figure 4-8: Scatter plots representing the variation in concentration of ethylene in the headspace of 3 replications of non-sealed culture vessels containing 10 mg.L^{-1} ethephon, measured either (A) non-repeatedly: every culture vessel was sampled at every point of time or, (B) repeatedly, where every vessel was sampled only once at one point in time, and then was removed from the experiment. Both (A) and (B) present data recorded during a two week duration, by ETD, at a flow rate of 5 L.h^{-1}. Each data point represents an individual culture vessel, n = 3.

As explored further below, the non-repeated 'sampling' method was considered likely to be more representative of the actual condition in vessels compared to the repeated 'sampling' method. Because of the risk of microbial contamination, the repeated 'sampling' method was not practically ideal to be considered as a measurement method every 48 hours for a duration of measurement longer than 10 days. With the repeated 'sampling' method multiple replication of vessels (more than 5) would be needed because there was a high risk of losing

some vessels during the experiment as a result of contamination. Contamination presumably resulted from using a frequently unintentional intake of non-sterilized air through the moulded, bevelled feature of the lids into the culture vessels (Figure 4-2). Therefore, despite variability between concentrations of ethylene measured in vessels using both methods, the non-repeated 'sampling' method was preferred for future use.

Despite all the practical disadvantages mentioned for application of the repeated sampling method over non-repeated, the two methods were not statistically different from each other. The repeated sampling method can still be applicable in circumstances in which resources or plant material would be limited, so as to provide a statistically robust number of replications (vessels) to be measured over a long period of time. However if the repeated sampling method is used, due to the risk of microbiological contamination, the number of vessels (replicates) needs to be large.

The concentration of ethylene in the headspace measured by 'sampling' methods, irrespective of which sampling method, remained unchanged over the 48 hour intervals and the entire measurement period (Figure 4-7). In the future therefore, investigations using time intervals shorter than 48 h is suggested in order to capture any possible difference in ethylene production over time. As an example presented in Appendices 1 & 2, at a rate of ethylene production of 5.65 $nL.h^{-1}$ at the first measurement time (4 hours after adding ethephon to the medium), it would take 2.64 h to produce 62.17 $nL.L^{-1}$ of ethylene in the headspace. While the duration actually required might be slightly increased after taking into consideration the effect of leakage, the duration required would still be far less than the 48 h time interval used in the experiments presented in this chapter. If the rate of ethylene production in the subsequent measurements were different from the rate of ethylene release recorded at the first measurement, the expected time duration for the interior atmosphere of the culture vessel to reach an equilibrium concentration of 60-80 $nL.L^{-1}$ of ethylene, would possibly differ, being dependent on the rate of ethylene production for each measurement time. The higher the rate, the less time it would take to reach to the equilibrium after evacuation and vice versa. From the data achieved previously (refer to Section 4.2.2.2 and Appendices 1 & 2) there was a slight increase in the rate of ethylene production measured continuously during the first 2 days and then a slight decrease over 6 days of measurement (Figure 4-6). However, despite subtle changes in the rate of ethylene production, the concentration of ethylene in culture vessels averaged ~ 60-80 $nL.L^{-1}$ albeit with a gradual decline over time.

The concentration of ethylene builds up quickly in the headspace (around first 2-3 hours) and, despite the half-life of ethephon (96 hours), ethylene release from the medium remained high thereafter throughout the entire two weeks of measurement (Figure 4-7 and Appendix 1). Therefore, explants would not be exposed to a sudden 'flush' of ethylene production within the first 48 hours. However, despite not being statistically significant, there was an overall gradual decline in the concentration of ethylene over the two weeks of measurement, acknowledging the decline could be more significant if extended to the duration explants are normally in the culture vessel for (i.e. 4-5 weeks). When ethylene production from ethephon at different concentrations was measured over a 4-week duration, the bulk of the ethylene was released within the first 2 weeks of culture (Tisserat and Murashige, 1977). Similarly, in another project, ethylene production from 70 µM ethephon in the agar medium, which equates to the 10 $mg.L^{-1}$ used in the current research, slightly declined but was not statistically significant during the first 15 days (Kong and Yeung, 1994). In that study however, the concentration of ethylene then decreased by 90 times after day 29 as compared with day 8 which could be suggested to be due to presence of somatic embryos of white spruce in culture vessels in their study. In contrast the ethylene concentration measured in culture vessels of the first experiment of this chapter did not involve plant material. It is likely that the presence of the explants would affect the concentration of ethylene quantified in the system (Biddington, 1992) and, therefore, such biological effects of the explants on the concentration of ethylene in the system need to be considered. Furthermore, investigations would be needed over the duration of exposure that explants of gentian are cultured for to show the biological response of increasing the number of secondary shoots.

– ***'Continuous flow' and 'sampling' method comparison (rate of production)***

The overall rate of ethylene production measured by the continuous method was 1.3 times more than when it was measured using the 'sampling' method (Figure 4-9) ($P \leq 0.05$). However, both methods presented very similar patterns of change in rates of ethylene production over time. The rate of ethylene production using both methods were not different from each other for the first 4 days (Figure 4-9), then significantly dropped after day 4, but only when the 'sampling' method was applied. In contrast, using the 'continuous flow' method, the rates of ethylene production did not significantly change over the entire measurement period. The higher rates of ethylene production recorded by the 'continuous flow' method compared to the 'sampling method' may be due to the possibility of air flow

across the medium to release more ethylene, by taking the ethylene away from the surface, and thereby allowing more to diffuse into the headspace of the vessel.

Flushing air through the culture vessel resulted in continuous dilution of the atmosphere within the headspace, and thereby changed the relationship between the rate of ethylene production from the medium and concentration of ethylene in the headspace. With the 'continuous flow' 'sampling' method the outflow was always constant, and the rate of ethylene release ended up at an equilibrium which was affected and limited by the air flow. In contrast however, using the 'sampling' method, the rate of leakage could change depending on the gas pressure inside the vessel, as affected by any change in the rate of ethylene release from the medium. Hence, the rate of ethylene release measured by the 'sampling' method wasn't limited by any factor, and was probably closer to the real conditions which explants would be exposed to. With both methods the concentration ended up at equilibrium, because some of the released molecules of ethylene in the headspace could always be returned to the medium if they cannot leave the vessel. Flushing the air through the vessel would alter the gas pressure inside the vessel, giving rise to a different pressure from that normally found in the culture vessel when not flushed with air. The relationship between rates of ethylene production and concentration of ethylene in the headspace will change following any changes in gas pressure. Hence, the 'sampling' method was suggested for use in future research using culture vessels designed to have gas exchange, as well as the future experiments in this thesis.

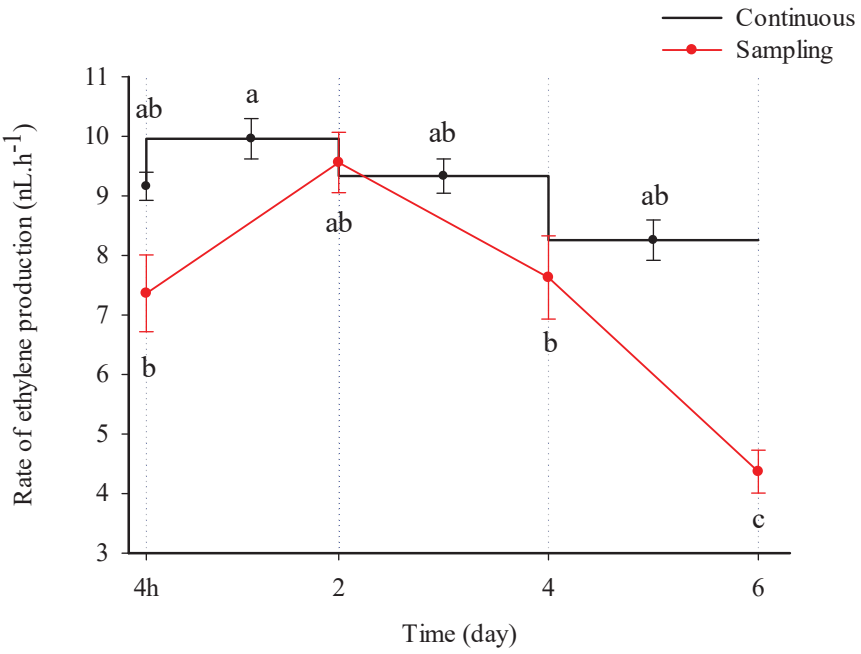


Figure 4-9: Rate of ethylene production within culture vessels containing 10 mg.L^{-1} ethephon, as measured by ETD either using the 'sampling' (red line) or the 'continuous' (black line) method, with humidified air flow. n = 3. The rate of ethylene production when measured continuously was averaged over each 2 days and compared with the rate of ethylene production measured by use of the sampling method at only one point of time every 2 days. Average values at each measurement time for the sampling method were connected by lines over time only to show the trend, Vertical lines represent ± standard error of the mean. Fisher's protected least significant test was used for mean comparison. Means followed by different letters were significantly different at $P \leq 0.05$.

4.2.4. Conclusion

Increasing RH in the air flow during continuous measurement, decreased the concentration of ethylene measured in the vessels. Due to limitations of the 'continuous flow' method such as risk of microbial contamination, limitation in the number of treatments and replications, the 'sampling' method was identified as clearly superior for measuring the ethylene concentration in the headspace of *in vitro* culture vessels. Also, the non-repeated 'sampling' method was preferred to the repeated 'sampling' method, as it more closely simulates the actual condition which explants would be exposed to. However, the non-uniformity of the vessels needs to be taken into consideration and, depending on the resources (i.e. explants and other general material), a larger number of replications is desirable. Despite very subtle fluctuations which were not statistically significant, concentration of ethylene measured by both repeated and non-repeated methods seemed to remain almost constant over the 14 days of the experiment and concentration of ethylene in culture vessels averaged ~ 60-80 nL.L^{-1} but with a gradual decline over the time.

4.3. Ethephon-ethylene concentration in a non-sealed vessel containing agar medium

4.3.1. Introduction

The concentration of ethylene in the headspace was fairly constant over a duration of 14 days of measurement in spite of changes in the rate of ethylene production (refer to Section 4.2). An average concentration of 60 $nL.L^{-1}$ ethylene was measured in the headspace of vessels containing 10 $mg.L^{-1}$ ethephon in the medium. It now needs to be established what concentration of ethylene develops in the headspace at different concentrations of ethephon in the medium. This was tested by applying various concentrations of ethephon in the medium of culture vessels (1 $mg.L^{-1}$ versus 10 $mg.L^{-1}$) and measuring the concentration of ethylene in the headspace.

Having developed a reliable methodology for determining the concentration of ethylene in the headspace of vessels (refer to Section 4.2), this methodology was utilised to address the concepts introduced within the General Introduction (refer to Section 4.1) via the following objectives:

- to quantify the relationship between concentration of ethephon within the agar medium, the rate of production of ethylene, and the concentration of ethylene in the headspace and,
- to compare the rate of production and concentration of ethylene released from different concentrations of ethephon within the agar medium over time.

4.3.2. Materials and methods

The basic agar medium (PGR-free) was prepared as described earlier in Section 4.2.2. However in the current experiment the media was amended with either 1 or 10 $mg.L^{-1}$ ethephon, as treatments. The concentration and rate of ethylene production was measured using the non-repeated 'sampling' method, as detailed earlier (refer Section 4.2.2.2). Measurement was done every 48 hours over 10 days. Three replications of vessels were measured non-repeatedly at each measurement time and the experiment was based on a completely randomised design. Concentration and the rate of ethylene production in the headspace of vessels containing ethephon were calculated following the methodology explained in Section 4.2.2.2. Concentration of ethylene was calculated from the area under

the peaks for each replication (Figure 4-4). The rate of ethylene production was also calculated from the difference between the stabilized levels of the peak of ethylene concentration (end of the red line in Figure 4-4) with the stabilized level for the base line. Statistical analysis using the General Linear Model was done in Minitab (version 16.1.1, Minitab Inc., State college, Pennsylvania, USA) and Genstat (Genstat, version 17, 2014, VSNi Ltd, Hemel Hempstead, UK), using Fisher's protected least significant test at $P \leq 0.05$ to do mean comparisons. So as to address the variability in the data, log transformation was done on the data related to the concentration of ethylene.

4.3.3. Results and discussion

The concentration of ethylene in the headspace of vessels including 1 $mg.L^{-1}$ ethephon in the medium peaked during the first 48 hours (2 days), then slightly decreased over the rest of the measurement period (8 days) (Figure 4-10A) ($P \leq 0.05$). Although the decrease in concentration of ethylene did not seem to be statistically significant from day 4 to the end of measurement, the negative slope for the regression line was significant ($P \leq 0.05$, slope = -0.053). For the concentration of ethylene released from 10 $mg.L^{-1}$ ethephon, a similar increasing trend was observed during the first 2 days (peak was not statistically significant), followed by a slight decrease from day 2 onward. For the 10 $mg.L^{-1}$ treatment however, the changes in actual concentrations over time were not statistically significant and, the slope of the regression line was, just above the normal level of $P \leq 0.05$ ($P = 0.054$, slope = -0.16) but probably significant at $P \leq 0.10$. Therefore, the decrease in concentration of ethylene released from 10 $mg.L^{-1}$ ethephon over the time was not as clear as the decrease in the concentration of ethylene with 1 $mg.L^{-1}$, and for practicable purposes was considered to be negligible. Such a fairly stable amount of ethylene released from 10 $mg.L^{-1}$ ethephon measured in the current study could be due to the equilibrium between releasing ethylene from the medium and leakage via the non-sealed status of the vessels, which was detailed earlier in Section 4.2.3. These observations were similar to a previous report (Kong and Yeung, 1994) in which regardless of the concentration of ethephon applied (1 or 10 $mg.L^{-1}$) in the agar medium within Petri dishes, the concentration of ethylene decreased over the 29 days of measurement, but the decrease was not statistically significant. Hence, they interpreted the data as with 10 $mg.L^{-1}$ ethephon, the concentration of ethylene remained statistically unchanged in vessels.

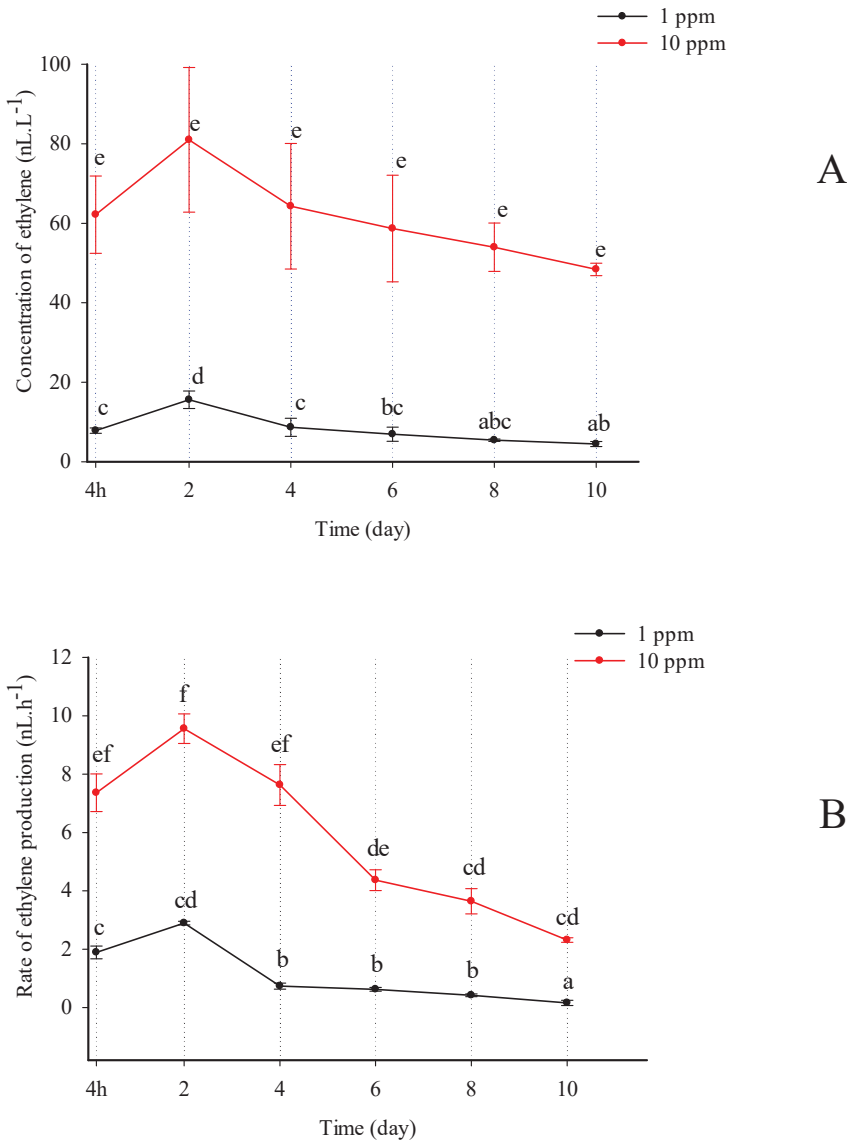


Figure 4-10: **(A)** Concentration and **(B)** rate of ethylene production in the headspace of culture vessels containing either 10 (red line) or 1 mg.L^{-1} ethephon (black line) in the media, measured by 'sampling' method (non-repeated), at only one point of time every 2 days recorded by ETD at 5 L.h^{-1} flow rate. Average values at each measurement time were connected by lines over the time only to show the trend. Vertical lines represent ± standard error bars on actual mean values. The letter classifications are based on log-transformed data and the least significant difference at $P \leq 0.05$.

Across the 10 day period, the average ethylene concentration in vessels containing 1 mg.L^{-1} ethephon was 7.54 nL.L^{-1}, as compared to 60.13 nL.L^{-1} in vessels with 10 mg.L^{-1} ethephon (Figure 4-10A). Thus, decreasing the concentration of ethephon in the medium by 10-fold resulted in approximately an 8-fold decrease in the concentration of ethylene in the headspace. As examined further in the following paragraphs, it was hypothesised that these differences in ethylene concentration between treatments were due to different rates of ethylene production (Table 4-1). The cumulative release of ethylene was also reported to be proportional to the ethephon concentration by Tisserat and Murashige (1977). Kong and Yeung (1994) also found the concentration of ethylene released a medium containing 10

$mg.L^{-1}$ ethephon was more than the concentration of ethylene in Petri dishes containing ethephon at a concentration of 1 $mg.L^{-1}$ in the agar medium. However, the proportion between the concentration of ethephon and the ethylene released was not similar to that achieved in the current study. In their study, which included plant material in culture vessels, 1 $mg.L^{-1}$ ethephon gave rise to a headspace concentration of ~ 8 nL (g fresh weight (FW) tissue)$^{-1}$. h^{-1} compared to the control, whereas 10 $mg.L^{-1}$ ethephon produced ~ 32 nL (g FW tissue)$^{-1}$. h^{-1} ethylene, i.e. 4 times more. They deduced the plant tissue applied in their system was probably modifying the perceived headspace concentration of ethylene. This therefore explain the difference between the proportions of ethephon concentration to ethylene released in their study, as compared with in the current study.

The rate of ethylene production with 10 $mg.L^{-1}$ ethephon in the medium was about 6 times higher than when 1 $mg.L^{-1}$ was applied ($P \leq 0.05$). Apart from the initial increase to a peak at 48 hours which was similar for both treatments (1 and 10 $mg.L^{-1}$ ethephon), the rate of ethylene production followed a decreasing trend but slightly of different pattern for each treatment during the rest of the measurement time (Figure 4-10B). For the 10 $mg.L^{-1}$ ethephon treatment, the slight increasing trend in ethylene production during first 48 hours was similar to the trend of concentration of ethylene illustrated in Figure 4-8. Due to having a higher rate of ethylene production within the 10 $mg.L^{-1}$ treatment, it is estimated that it took less than 4 hours from adding the ethephon to the medium to reach the peak rate of ethylene production. Therefore, the true peak for the 10 $mg.L^{-1}$ treatment could have possibly happened earlier than the 48 hour measurement time. After 48 hours, the rate of ethylene production was not statistically significant between each two subsequent mean values, but the negative slope for the regression line was significant ($P \leq 0.01$, slope = -0.01). The rate of ethylene production from the medium containing 1 $mg.L^{-1}$ ethephon after 48 hours significantly decreased to a value which statistically remained unchanged till day 8, and then was followed by another significant decrease at the end of the period. However, when regression analysis was applied between day 4 to the end of measurement time, the negative slope for the regression line was again significant ($P \leq 0.01$, slope = -0.04). Therefore, it was concluded that the rate of ethylene production after 48 hours (2 days) decreased over time for both concentrations of ethephon in the medium.

Comparing the results for the rate of ethylene production with the concentration of ethylene in the headspace, it can be concluded that the concentration of ethylene measured in vessels

followed a similar trend as the rate of ethylene production, for the 1 $mg.L^{-1}$ treatment. The mean separation values of both rate and concentration of ethylene production indicated a progressive decline over the time after the peak at day 2 for the 1 $mg.L^{-1}$ treatment (Figure 4-10). For the 10 $mg.L^{-1}$ treatment, in spite of the decrease in rate of ethylene production from day 2 onward, the decrease in concentration of ethylene released over time was not as clear as the decrease in the concentration of ethylene with 1 $mg.L^{-1}$ ethephon and, therefore, was considered to be negligible. The reason for such a difference could probably be related to either the variation in the data as well as the higher rate of ethylene production within the 10 $mg.L^{-1}$ treatment being enough to keep the concentration of ethylene in the vessels almost unchanged during the measurement time. However, it was likely that in experiments lasting longer than 10 days, such as those within the current thesis using plant material, the concentration of ethylene when 10 $mg.L^{-1}$ ethephon was applied would also decline, and potentially affect the plant's response. It would be interesting to investigate in the future, how important is the initial exposure to ethylene, as compared to long term exposure, in terms of the physiological response to ethylene.

The concentration of ethylene maintained in the headspace, is an equilibrium resulting from an interaction between ethylene released, volume of the vessel, gas pressure inside the vessel and leakage. In addition, ethephon does have a half-life (about 96 h in aqueous solution as reported by (Domir and Foy, 1978)), which would affect the concentration of ethylene released over the time. Therefore, the concentration of ethylene released from ethephon would be expected to drop in the vessel over the time. As evident for the concentration of ethylene in the vessel containing 1 $mg.L^{-1}$ ethephon (Figure 4-10), the declining trend would also eventually occur for the vessel containing 10 $mg.L^{-1}$. It is possible that the difference in the concentration of ethylene in the vessels containing 10 $mg.L^{-1}$ ethephon was not captured because of the high rate of ethylene production which occupied the volume of the vessel with an almost constant concentration of ethylene. In contrast, the lower rate of ethylene production within the 1 $mg.L^{-1}$ treatment, was probably not enough to occupy the entire volume of the vessel and maintain the constant concentration in it. Therefore, the possible relationship between the surface of the medium and the ethephon content, in addition to relationship between gas pressures inside the vessels with the rate of ethephon decomposition and leakage, could be some of the reasons for the differences between rate of ethylene production and the concentration of ethylene in vessels containing different concentration of ethephon. Now, having the knowledge that application of 1 $mg.L^{-1}$ ethephon in the medium

would result in less concentration of ethylene in the atmosphere of the vessels as compared with 10 $mg.L^{-1}$, it might be interesting to find out in the future whether both concentrations cause the same physiological alteration in shoot formation of gentian 'Little Pinkie.

4.3.4. Conclusion

It can be concluded that the rate of ethylene release as well as the concentration of ethylene, which was a result of an equilibrium between releasing ethylene from the medium and the leakage from the vessel, was highly dependent on the concentration of ethephon added to the medium. Decreasing the concentration of ethephon in the medium by 10-fold (10 $mg.L^{-1}$ compared to 1 $mg.L^{-1}$) resulted in approximately 6 and 8-fold decrease, respectively, in the rate of ethylene production and concentration of ethylene in the headspace. Also, for 1 $mg.L^{-1}$ ethephon, regardless of the concentration of ethephon applied, the rate of generation and concentration of ethylene decreased after day 2 and over the rest of the measurement time. Application of 10 $mg.L^{-1}$ compared to 1 $mg.L^{-1}$ ethephon in the medium produced a higher rate of ethylene production which resulted in a concentration of ethylene in the headspace of vessels which, after day 2, only slightly decreased over the rest of the measurement time and, therefore, was considered negligible and almost constant.

Chapter 5. Ethephon/ethylene uptake by explants *in vitro*

5.1. General Introduction

There was a positive effect of ethephon application on increasing shoot formation in gentian 'Little Pinkie' (refer to Chapter 3), which was hypothesised to be due to the increased number of 2º shoots developed from the axillary, co-lateral and/or adventitious buds in explants been exposed to ethephon in their culture medium. Ethylene released from the decomposition of ethephon has been hypothesised in Chapter 1 to be responsible for the increased shoot formation response and therefore quantified in culture vessels (refer to Chapter 4). This hypothesis is supported by evidence from other plant species: for example, ethylene has been shown to be involved in adventitious bud/shoot formation from bulb-scale explants of *Lilium* × *hybrida* (lily) (van Aartrijk et al., 1985a). Despite evidence supporting the positive involvement of ethylene in formation and development of adventitious buds, the literature is not uniformly consistent in this regard. The application of ethephon modified the architecture of seedlings of *Petunia* x *hybrida* (petunia) to form a short-bushy plant, as a result of the outgrowth of lateral buds (Haver et al., 2002). However, another study on *Brassica oleracea in vitro* indicated an increase in callus proliferation and concentration of ethylene as a consequence of applying ethylene precursors S-adenosylmethionine (SAM) and 1-aminocyclopropane-1-carboxylic acid (ACC), while the application of inhibitors of ethylene biosynthesis aminoethoxyvinylglycine (AVG) and cobalt chloride ($CoCl_2$), and ethylene antagonist silver nitrate ($AgNO_3$), increased shoot differentiation and reduced ethylene concentration (Sethi et al., 1990). In some studies ethylene or ethylene precursors have been reported to enhance shoot formation, whereas in some other studies ethylene inhibitors or absorbents promoted shoot growth. Therefore it seems that the effects of ethylene on growth are species dependent, and also depend on the concentration of ethylene, growth condition and developmental stage of the plant (McManus, 2012).

As mentioned earlier, ethephon decomposes to ethylene (which is volatile), and phosphate and chloride ions which are non-volatile (Yang, 1969; Goudey et al., 1987b; Biddington, 1992). As hypothesised in Chapter 1, ethephon exerts its effect on increasing shoot formation through decomposition to ethylene as a volatile compound, and a methodology was developed to quantify the concentration of ethylene released from the decomposition of ethephon in the *in vitro* medium (refer to Chapter 4). However, there is a possibility that the

non-volatile components of ethephon induce the biological effects on explants of gentian. As introduced in the following sections, experiments in this chapter were designed to investigate this possibility, as well as determining the duration that explants of gentian would need to be in contact with ethephon/ethylene *in vitro* to induce the physiological response of increasing shoot formation.

5.1.1. Experiment 1- Ethylene production by explants as affected by ethephon *in vitro*

5.1.1.1. Introduction

In order to investigate the overall hypothesis on the possible role of ethylene released from ethephon on shoot formation in explants of gentian, ethylene measurements were made with explants in the system. However, it was possible that ethylene consumption or production by explants may contribute to the shoot formation response (Huxter et al., 1981; Sethi et al., 1990; Trujillo-Moya & Carmina, 2012). Hence, the experiments in this section were designed to study the effect of including plant material when determining the effect of ethephon on the concentration of ethylene in the headspace of culture vessels.

As noted earlier, cultivars of gentian respond to ethephon as a shoot formation stimulant differently (refer to Chapter 3, Figures 3-13 & 3-15). In the presence of ethephon, explants of 'Little Pinkie' produced 5 times more 2º shoots compared to those of 'Showtime Diva'. As a potential cause of this difference, it was considered possible that explants of cultivars may differ in consumption and/or production of ethylene. If so, then the concentration of ethylene measured in the headspace of vessels containing different cultivars was hypothesised to differ (Zhang et al., 1998). Any difference in the concentration of ethylene within vessels containing different cultivars of gentian, might help to gain more understanding of the mechanism of ethephon's effect on shoot formation.

Due to the logistical limitations of handling several vessels connected to the ETD for non-repeated sampling (refer to Chapter 4, Section 4.2.3), in the current experiments, the repeated sampling method was used to measure the ethylene concentration in the headspace of non-sealed vessels.

Therefore, the current experiments were planned with the objectives of quantifying and comparing the concentration of ethylene in the head space of culture vessels:

- with and without explants cultured in the medium in the presence or absence of ethephon,
- containing gentian cultivars differing in their shoot formation response to ethephon in the medium.

5.1.1.2. Materials and methods

Basal agar medium (PGR-free), was prepared as detailed in Chapter 2. For the current experiments, however, two types of media were required; base medium with and without 10 $mg.L^{-1}$ ethephon. To examine the potential effect of the biological activities of explants of gentian on the ethylene production inside culture vessels, culture vessels containing 50 mL of media and explants of gentian were connected to the ETD. 'Sampling' had been selected as the preferred method for measuring the ethylene concentration by the time the current experiment was conducted. However, as discussed earlier (refer to Chapter 4) the 'sampling' method required multiple replications of each treatment despite there being a limitation in the amount of plant material available. Therefore, a preliminary experiment using only six vessels (maximum number of inlets for ETD) was undertaken to provide an initial indication of ethylene production in culture vessels in the presence of explants before propagating a larger number of explants for a bigger experiment (second experiment).

For the preliminary experiment six culture vessels were prepared and connected to the ETD using continuous flow for a duration of six days. There was only one replicate vessel for each treatment. Although the number of replications for each treatment was not enough to undertake statistical analysis and draw a conclusion, the result of the experiment presented in Appendix 2 illustrated some differences between treatments. Therefore, a second, larger experiment was subsequently carried out using the 'sampling' method and multiple replicates.

Within the second experiment treatments comprised a 2 × 2 factorial arrangement of ethephon concentration (0 $mg.L^{-1}$ or 10 $mg.L^{-1}$) and cultivar ('Little Pinkie' or 'Showtime Diva'), plus controls (no explants); i.e.:

- 10 $mg.L^{-1}$ ethephon and explants of 'Little Pinkie',
- 10 $mg.L^{-1}$ ethephon and explants of 'Showtime Diva',
- 10 $mg.L^{-1}$ ethephon and no explants,
- 0 $mg.L^{-1}$ ethephon and explants of 'Little Pinkie',

- 0 mg.L^{-1} ethephon and explants of 'Showtime Diva', or
- 0 mg.L^{-1} ethephon and no explants.

Each of three culture vessels, each containing 8 explants only from the second node below the tip as detailed in Chapter 2, were used as replicates for each treatment. In anticipation of contamination forcibly removing replicate culture vessels from the experiment, more than three replicates were used. Concentration of ethylene in culture vessels was quantified using the methodology described in Chapter 4 (refer to Section 4.2.3). Due to contamination (fungal and/or bacterial growth in the culture medium), data analysis was done on only three vessels of each treatment which remained non-contaminated from the second measurement time onwards. The experiment was analysed as a split plot factorial design, utilising a General Linear Model in Genstat (Genstat, version 17, 2014, VSNi Ltd, Hemel Hempstead, UK) on log transformed data. Mean comparison was done using the alternative Fisher's protected LSD test, where the Studentized Range statistic is used instead of Student's t test at $P \leq 0.05$, being equivalent to Tukey' s test (Williams and Abdi, 2010).

5.1.1.3. Results and discussion

In keeping with the preceding experiments (refer to Chapter 4), in the presence of ethephon in the medium but without explants, the atmosphere inside the vessel contained an average ~59 nl.L^{-1} ethylene, which was significantly higher than in vessels without either ethephon or explants (i.e. 0.4 nl.L^{-1}; $P \leq 0.05$) (Figure 5-1 & Appendix 3). Such a result was expected and consistent with the literature indicating ethylene is released from ethephon at a pH higher than 4.0 (Warner and Leopold, 1969; Yang, 1969; Biddle et al., 1976). Although not statistically significant, the concentration of ethylene peaked at about 48 hours in the presence of ethephon, which was similar to the pattern reported by others (Warner and Leopold, 1969) as well as to previous experiments (refer to Chapter 4, Figure 4-1 A). In the absence of ethephon, regardless of the presence or absence of explants in vessels (Figure 4-1B), the ethylene concentration remained approximately constant and extremely low.

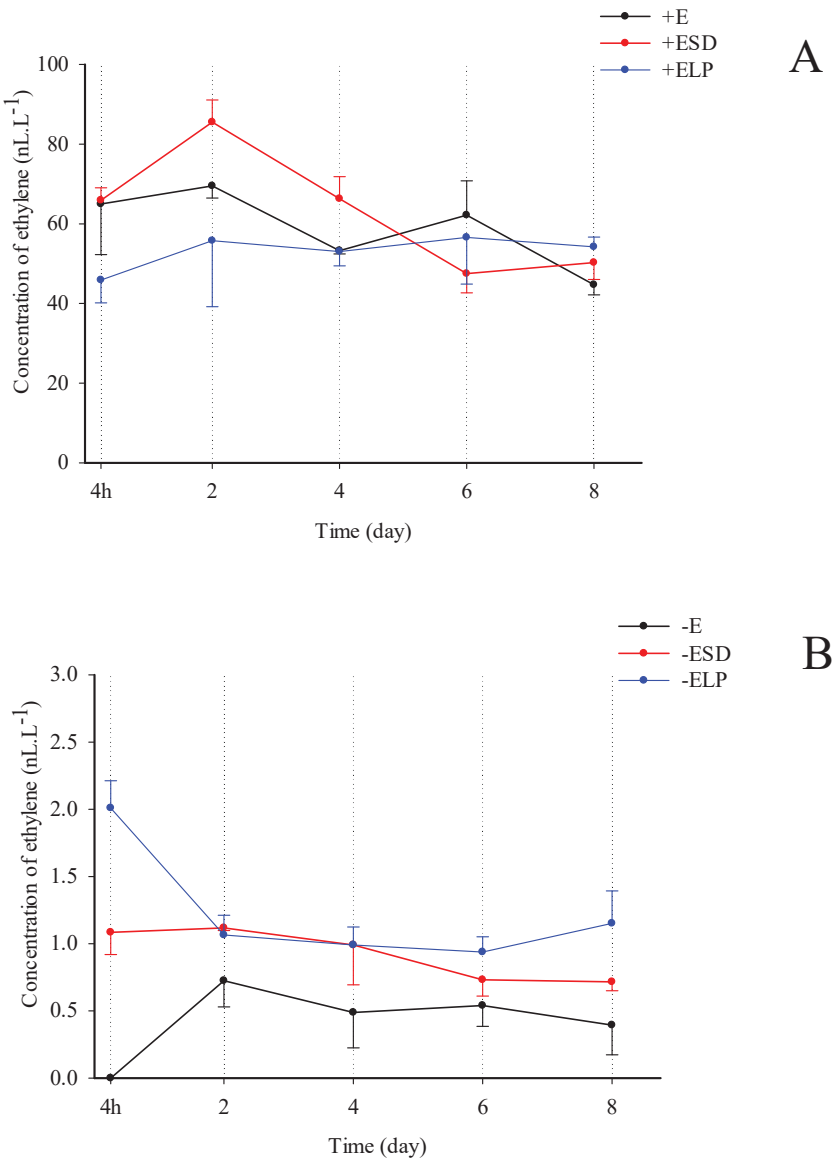


Figure 5-1: Concentration of ethylene over time in the headspace of vessels containing either, **(A)** agar medium amended with 10 mg.L^{-1} ethephon with either no explant (+E) or with explants of 'Showtime Diva' (+E SD) or 'Little Pinkie' (+E LP) mg.L^{-1} or, **(B)** agar medium with no ethephon containing either no explant (-E) or with explants of 'Showtime Diva' (-E SD) or 'Little Pinkie' (-E LP) . Averages were calculated using data from the repeated sampling method in non-sealed culture vessels measured at only one point of time every 2 days, recorded by ETD at 5 L h^{-1} flow rate. n=3 vessels each containing 8 explants. Average values at each measurement time are presented as being connected by lines so as to show the trend. General Linear model was on log transformed data. Mean comparison was done using the alternative LSD test where the Studentized Range statistic was used instead of Student's t test at $P \leq 0.05$. Actual mean values presented here. Vertical lines represent + or – standard errors.

Inclusion of explants in the absence of ethephon resulted in an enhancement in the concentration of ethylene in vessels as compared with vessels without any explants, which was statistically significant only at the first measurement time (4 hours from the commencement of the experiment; $P \leq 0.05$). The concentration of ethylene in vessels with and without explants, in the absence of ethephon, was not statistically significant for the rest of the measurement times. In vessels without explants, there was no ethylene detected at 4

hours whereas it was up to 2 $nL.L^{-1}$ in vessels containing explants, depending on the cultivar ('Little Pinkie' having two times more than 'Showtime Diva'; $P \leq 0.05$) which was almost certainly not enough to cause a biological response.

The ethylene concentration after the first 4 hours remained unchanged for all treatments at a level which did not statistically differ from each other throughout the rest of the experiment ($P \leq 0.05$) (Figure 5-1B). Such ethylene production at the start of the experiment implies biosynthesis of ethylene by the explants possibly due to wounding stress (Hoffman and Yang, 1982; Riov and Yang, 1982; Yang and Hoffman, 1984; Kende, 1993; De Paepe and Van Der Straeten, 2005).

Although in presence of ethephon, inclusion of plant material in culture vessels did not significantly affect ethylene production in vessels ($P \leq 0.05$) (Figure 5-1 A), once compared with the absence of ethephon, the size of the difference between concentrations of ethylene produced with explants seemed to be larger compared to vessels without explants. Also, in the presence of ethephon explants of 'Showtime Diva' produced more ethylene than the explants of 'Little Pinkie'. Although this may imply that 'Showtime Diva' was autocatalytically increasing its own ethylene production under the influence of ethephon, it was only 'Little Pinkie' that responded to ethephon as a shoot formation stimulant (refer to Chapter 3). Ethylene production can result from autocatalysis, most frequently reported in fruit tissues (Burg and Burg, 1965; Rhodes, 1980), however also in vegetative tissues (Aharoni and Lieberman, 1979; Katz et al., 2005). Nevertheless, the difference in concentration of ethylene produced by different treatments in the current experiment was not statistically significant; primarily attributed to the number of sampling vessels. Only three replications of vessels remained non-contaminated in this experiment for sampling; so further repetition of the experiment with more replications per treatment would be needed to strongly support this statement. Therefore it is likely that the difference between concentrations of ethylene produced by different treatments occurred due to variability in data rather than in the biosynthesis by the explants of different cultivars following the exposure to ethephon. Variability in data might have resulted from the variability between vessels (refer to Chapter 4, Section 4.1.3). Together with the results from previous chapters, ethephon is therefore unlikely to cause autocatalytic ethylene production in explants, but does trigger shoot formation in 'Little Pinkie' (refer to Chapter 3).

5.1.1.4. Conclusion

In the presence of ethephon in the medium but without explants, the atmosphere inside the vessel contained an average of ~59 $nl.L^{-1}$ ethylene, which was significantly higher than in vessels without either ethephon or explants. In the absence of ethephon, regardless of the presence or absence of explants in vessels, the ethylene concentration remained approximately constant and extremely low. Inclusion of explants in the absence of ethephon resulted in an enhancement in the concentration of ethylene in vessels as compared with vessels without any explants, but was only statistically significant at the first measurement time possibly due to wounding stress. Ethephon is therefore unlikely to cause autocatalytic ethylene production in explants, but does trigger shoot formation in 'Little Pinkie'.

5.1.2. Experiment 2 - Aerial exposure of the explants to ethylene gas

5.1.2.1. Introduction

Within this section the aim of the experiment was to evaluate the potential involvement of ethylene on increasing shoot formation in 'Little Pinkie'. The logic simply was, if ethephon's decomposition to ethylene was the likely source of the active compound, then application of gaseous ethylene at the same concentration should generate a similar shoot formation response to ethephon. In the current experiment therefore, it was hypothesised that exposing the explants of gentian 'Little Pinkie' to ethylene in the vessel's headspace would increase shoot formation compared to explants exposed to ethylene-free air. The concentration of ethylene measured in vessels containing ethephon in previous sections reached a steady level of about 60 $nl.L^{-1}$, from less than 4 hours after adding ethephon to the medium (refer to Chapter 4). Therefore, explants were planned to be exposed to this concentration of ethylene, utilising an ethephon-free agar medium.

While additional 2º shoots had been recorded after a continuous exposure to the medium treated with ethephon for a duration of 8 weeks (refer to Chapter 3), the data available describing the concentration of ethylene from the decomposition of ethephon recorded in vessels was for only the first two weeks of culture (refer to Chapter 4). In the current study therefore, it was acknowledged that the effect of ethephon on shoot formation in gentian 'Little Pinkie' potentially could have resulted from the tissue response anytime between 0 and 4 weeks of exposure before shoots were macroscopically visible. It was also possible that a duration of exposure longer than two weeks might be needed to influence growth, and that

the effect of aerial exposure of the explants to ethylene could depend on the duration of application. For example, the number of shoots, presumably adventitious, produced by the leaf explants of petunia that had been exposed to 1 $mg.L^{-1}$ ethylene in the atmosphere was significantly higher in the second week of culture as compared to the first or third week (Dimasi-Theriou et al., 1993). Also the number of differentiated adventitious buds per cultured explant of *Lilium speciosum* bulb-scales had been reported to be increased by ethylene provided during the first 3 to 7 days of the culture period (van Aartrijk et al., 1985a). In the current experiment therefore, different durations of exposure needed to be applied, in order to find the critical exposure duration required to produce the biological effect on explants of 'Little Pinkie'.

In order to test the hypothesis that gaseous ethylene is involved in changing the growth variables (number of shoots (1º and/or 2º and 3º), length of shoots, root development and, total fresh weight of the plantlet) in gentian 'Little Pinkie', the specific objectives were to:

- evaluate the effect of ethylene gas exposure on shoot formation and other growth variables in explants and,
- identify the critical exposure duration needed to generate the effect on shoot formation and other growth variables.

5.1.2.2. Materials and methods

Explants of gentian 'Little Pinkie', from a combination of positions (Tip, Node 1, Node 2 and Base; refer to Chapter 2) along the donor shoot were selected to be used as plant material. Eight explants from all positions were distributed evenly in each culture vessel containing 50 mL of standard agar medium (PGR-free), as described previously (refer to Chapter 2, Section 2.2).

Culture vessels were exposed to a flow rate of 1 $L.h^{-1}$ of externally supplied gas flushing to the culture vessels continuously. External gas flow, either containing air with 0 (certified as ethylene-free) or 60 $nl.L^{-1}$ ethylene (BOC Ltd, Christchurch, New Zealand), was sent into manifolds and distributed to culture vessels. As illustrated within Figure 5-2, the experimental set-up utilised two manifolds. The first manifold was to switch between two types of gas flow (i.e. to change the flow from ethylene to ethylene–free, and vice versa). Each gas cylinder was connected to an individual inlet of the manifold, which was equipped with an adjustable flow rate meter (Figure 5-2; Manifold 1), before passing into the second

manifold (Figure 5-2; Manifold 2), which was to distribute the gas into individual vessels. The pressure was set at 5.0 $L.h^{-1}$ for both flow rate meters in Manifold 1. From Manifold 1 there were 9 outlets for 9 treatments of time exposure to ethylene (0, 4, 24, 48 hours, 1, 2, 4, 6 and 8 weeks) (Figure 5-3A). Each outlet in Manifold 2 had an individual switch for each type of gas flow, which could be either turned off to stop the gas flow from getting into the outlet, or turned on to keep the gas flowing. Following the desired time period for exposure to ethylene, the switch for ethylene free gas flow was turned on, and ethylene free gas flowed into the system for the rest of the experiment. The second manifold which was used to distribute the gas to individual vessels had 9 inlets and 27 outlets in total. Each outlet was connected to an individual vessel. Gas flow was humidified at 95-98%, by using humidifiers (sealed containers containing 300 mL of 21% glycerol in water (Forney and Brandl, 1992)) and filtered to reduce microorganism numbers (0.2 µm Minisart®filter; Sartorius Stedim Biotech, Germany) before entering each culture vessel (Figure 5-3B).

Culture vessels including explants, and connected to the system, were held in a culture room which was ventilated continuously in order to prevent accumulation of ethylene within the room itself and gas contamination between treatment vessels. Standard culture room environmental conditions of 25 ± 1 °C, a photosynthetic photon flux density (PPFD) of 30 ± 5 $\mu mol.m^{2}.s^{-1}$ and a 16 h photoperiod was applied during the experiment.

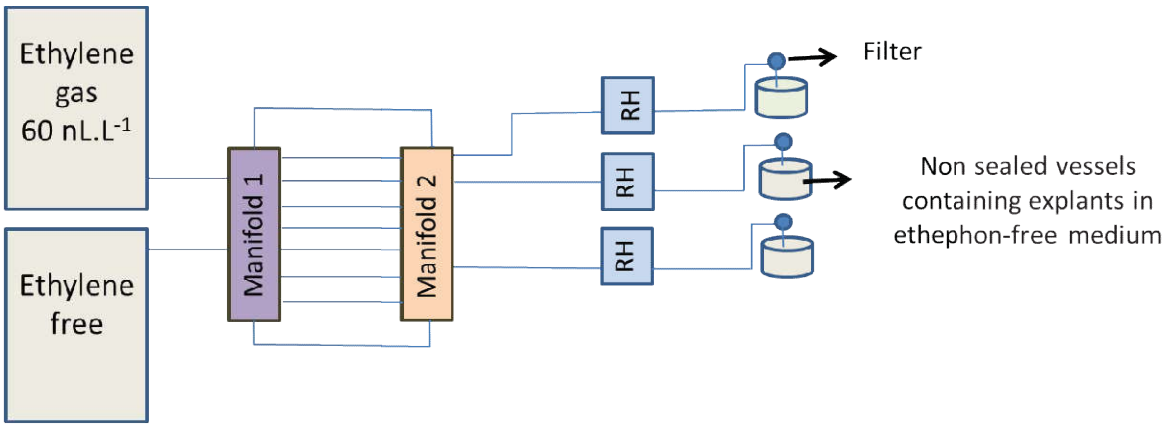


Figure 5-2: Schematic illustration for delivering external gas flow (either containing 0 (ethylene-free air) or 60 $nl.L^{-1}$ ethylene) to an individual inlet of the manifold 1. Manifold 1 enabled a switch between two types of gas flow (change the flow from ethylene to ethylene–free, and vice versa). From Manifold 1 there were 9 outlets for the 9 durations of exposure (0, 4, 24, 48 hours, 1, 2, 4, 6 and 8 weeks). Each outlet in Manifold 2 had an individual switch for each treatment gas, which could be either turned off to stop the gas flow from getting into the outlet, or turned on to keep the gas flowing. Manifold 2 was used to arrange the connections for 3 individual replications of vessels for each treatment (27 outlet in total; only 3 illustrated here) which were not connected in series. External gas was passed through humidifiers (RH) and was also filtered to remove microorganisms before entering the culture vessels.

Treatments comprised a factorial combination of two types of external gases (either 60 nl.L^{-1} ethylene or ethylene-free air) and duration of exposure (0, 4, 24 or 48 hours, 1, 2, 4, 6 or 8 weeks). In order to create the treatments, for example for an initial two weeks of exposure, culture vessels containing explants were exposed to ethylene for two weeks, then the type of gas was changed to ethylene-free air for the rest of the experiments duration (i.e. 8 weeks). Three replications of vessels for each treatment combination were utilized.

Figure 5-3: **(A)** Manifold for distributing the gas to vessels; **(B)** External gas was filtered to remove microorganisms before getting into the culture vessels.

The logic behind choosing growth variables which were recorded in the current experiment was presented earlier in this thesis (refer to Chapter 2, Section 2.1). Growth variables recorded after 8 weeks in culture were; the number of shoots arising from explants, shoot length and root development were recorded. In doing so, the number of short (< 5mm), medium (5 to 20 mm) and long (> 20 mm) shoots (1º and/or 2º) was recorded, as well as the score for root development (0 for no root growth, 1 for 1-2 roots of up to 1 mm length, 2 for 3-4 roots of 1-10 mm length, 3 for 5-6 roots of 10-15 mm length, and 4 for 5-6 roots of more than 15 mm length), and total fresh weight of the plantlet. Data were analysed as a split plot factorial design, including factors such types of external gases (either 60 nl.L^{-1} ethylene or ethylene-free air) and duration of exposure (0, 4, 24 or 48 hours, 1, 2, 4, 6 or 8 weeks).

General Linear Model in Genstat was used to analyse the data. Mean comparison was done as detailed in Section 5.1.1.2.

5.1.2.3. Results and discussion

Atmospheric exposure of *in vitro* explants to ethylene gas into culture vessels, for any of the time periods tested did not significantly affect growth variables such as number of shoots (1º and/or 2º), total weight and root development score of the explants compared to the control ($P \geq 0.05$) (Table 5-1). While it was reported in other experiments in this thesis, as a result of ethephon application in the medium the number of shoots increased and rooting decreased (refer to Chapter 3), application of gaseous ethylene did not result in any change in any of these growth variables. Hence, the hypothesis for current experiment was rejected.

Table 5–1: Analysis of variance for growth variables of *in vitro* cultured explants of gentian 'Little Pinkie' as influenced by duration of exposure (time) to ethylene gas and position along the shoot which explants originated from. Data were analysed using general linear model at $P \leq 0.05$, n=3 vessels each containing 8 explants.

	Number of shoots	Total weight (mg)	No. short shoots	No. medium shoots	No. long shoots	Root score (1-4)
***P* value**						
Time	0.176	0.148	0.788	0.606	0.271	0.528
Position	<0.001	<0.001	0.012	0.924	0.051	0.328
Time × Position	0.226	0.125	0.853	0.382	0.180	0.462

One argument could be that the concentration of ethylene gas applied and absorbed by the explant from the atmosphere was not enough to generate the response. It is possible that a concentration of ethylene higher than 60 $nL.L^{-1}$ might be needed to be effective. On the other hand, there is plenty of evidence in the literature that ethylene accumulation in the vessel would negatively affect growth variables for most plants cultured *in vitro* (Lai et al., 1998; Arigita et al., 2003; Reis et al., 2003; Da Silva, 2013). Therefore, it seems more likely that ethylene uptake from the headspace is not the mechanism that regulates the change in explant physiology. Direct uptake of ethephon from the medium by the explants (Nir and Lavee, 1981), and then decomposing to ethylene in the tissue, potentially could generate a higher effective endogenous concentration of ethylene in the explants which, alone or together with the aerial ethylene, could be responsible for generating response of increased shoot formation. However, it was also possible that non-volatile ethephon metabolites in the tissue such as chloride or phosphate ions would cause the response (Biddington, 1992; McCullough

and Sidhu, 2014). To investigate these possibilities further, further experiments in this chapter were designed and implemented (refer to Section 5.1.3).

From the current experiment, a critical concentration or duration of ethylene exposure for inducing shoot formation was not identified. However, when the results were compared with the use of ethephon-amended media it was considered likely that inserting the explants into the agar medium amended with ethephon exposed the explant's tissue to a pulse treatment of ethylene. It was also likely that the concentration of ethylene released from ethephon during the first few hours after application to produce the pulse treatment would differ, and might even be higher than the concentration of ethylene applied through the atmosphere in the current study. As reported by Nir and Lavee (1981) radiolabelled ^{14}C ethephon broke down rapidly during the first hours after application within the tissue of grapevines. Therefore, such rapid decomposition of ethephon could produce a pulse of high concentration of ethylene in the tissue, which could be responsible for the increase in shoot formation as compared with no response to the aerial application of ethylene. Hence, future experiments within this thesis also aimed to investigate the critical duration of ethephon/ethylene exposure on increasing shoot formation.

As evidence for the difference between a pulse and continuous application of other PGRs on growth variables of explants *in vitro*, explants of *Actinidia deliciosa* exposed to a pulse treatment of BA (first 24 hours of a 45 day culture period) produced approximately a three times higher percentage of rooted explants of greater shoot length as compared with longer-term application (e.g. 20 days of 45 days culture; (Arigita et al., 2005). Such a difference between short-term and long-term application of a PGR could be related to the sensitivity of the tissue at the time of the application (Firn, 1986). Tissue of mung bean were reported to show the most sensitivity for rooting to a 24 hour ethylene 'pulse' 2–3 days after taking cuttings (Robbins et al., 1985). Such differences could possibly explain why aerial application of ethylene to explants did not affect shoot formation, while application of ethephon in the medium did. It was therefore not possible to identify the critical duration of exposure to gaseous ethylene which was needed to make the shoot formation effect on explants. However, in an experiment which will be presented in following chapter, the critical duration of exposure to ethephon in the medium was investigated (refer to Chapter 6, Section 6.3).

Given the lack of any ethylene-related response, and also due to the similarity to the result of experiments presented elsewhere throughout this thesis (refer to Section 5.1.3), the effect of position on growth variables in current experiment was not presented here (refer to Appendix 4).

5.1.2.4. Conclusion

Exposing *in vitro* explants of gentian 'Little Pinkie' for eight weeks to atmospheric ethylene gas at 60 $nL.L^{-1}$ (the concentration previously found in vessels containing 10 $mg.L^{-1}$ ethephon) did not change the number of shoots produced. This meant that it was not possible to determine if there is a critical time duration for exposure to ethylene to affect the growth variables of explants.

5.1.3. Experiment 3 - Ethylene/ethephon uptake by explants *in vitro*

5.1.3.1. Introduction

Aerial exposure to exogenously supplied ethylene, at the concentration which was previously quantified in vessels including ethephon in the medium (60 $nL.L^{-1}$), was not effective in producing a biological response on explants of gentian 'Little Pinkie' (refer to Section 5.1.2). Therefore, in order to make the increased response in shoot formation noted to date (e.g. refer to Chapter 3 and Appendix 13) direct uptake of ethephon from the medium by the explants is probably needed. Ethephon then probably decomposes to ethylene or other constituents within the tissue that could be responsible for making the response. It was also possible that any subtle fluctuations in the concentration of ethylene released from ethephon over time, which may not have been captured during measurement, could also make a difference. From the results of the experiments presented so far therefore, it was not clear that atmospheric ethylene alone or together with ethylene from the decomposition of ethephon inside the tissue would be needed to make the response. While most of the previous studies on the application of ethephon on various crops have been focused on only atmospheric ethylene (Domir and Foy, 1978; Kong and Yeung, 1994; Haver et al., 2002), it is likely that the effect of ethephon would be related to the metabolites of ethephon in plant tissue which eventually would break down to ethylene, and in doing so probably provides a more long-term ethylene emanation (Foster et al., 1992). The current experiment therefore, was designed to address the question of; 'in what form and by what process do explants respond to ethephon', i.e. in the form of ethephon through the medium, ethylene through the atmosphere around the plant, or both?

Explants were exposed to ethylene released from ethephon only through their aerial parts without being in direct contact with ethephon-containing medium, and compared to explants exposed to direct contact with ethephon in the medium (as well as through their aerial parts). If decomposition to ethylene within the headspace is the mode of ethephon's action, then the surface area of the medium as a border between the medium and the atmosphere inside the vessel could potentially play an important role in the concentration and rate of ethylene release. Similarly, the volume of medium within the culture vessel is another factor which could affect the ethylene concentration achieved inside the vessel, since it will control the total amount of ethephon in the container. Hence in designing the current experiment, keeping the total surface area and volume of the medium similar to previous experiments was considered to be important.

Therefore, in order to investigate those questions and to eventually gain more understanding of the mechanism of ethephon's effect on the biological response of shoot formation in gentian, the objective for the current experiment was:

- to quantify shoot formation and other growth variables of gentian 'Little Pinkie' exposed to atmospheric ethylene as compared to explants exposed to ethephon in their *in vitro* medium.

5.1.3.2. Materials and methods

In order to expose only aerial parts of the explants to ethylene released from ethephon without being in direct contact with ethephon-containing medium, a different culture system from that used in previous experiments was utilised. In this culture system the culture vessels used throughout this thesis (refer to Chapter 4, Figures 4-1 and 4-2) were used and contained 50 mL PGR-free medium with either 0 or 10 $mg.L^{-1}$ ethephon as previously detailed (Section 4.1.2 & Chapter 3). Additionally however sterilised plastic Petri dishes (55.6 mm diameter) containing explants inserted into the base medium (PGR-free), were positioned within the main culture vessels (Figure 5-4). Doing so, provided the opportunity of exposing only aerial parts of the explants to ethylene released from ethephon without actually being in direct contact with ethephon-containing medium, unless included within the treatment combinations (Figure 5-5).

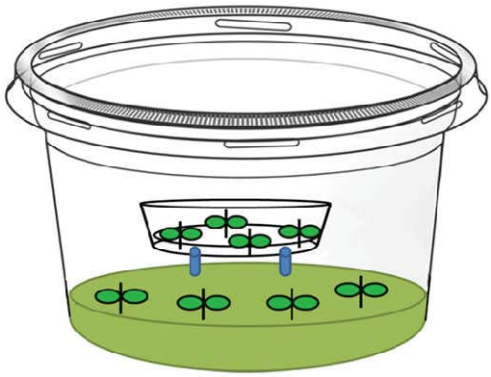


Figure 5-4: An illustration of the physical arrangement of 4 explants in a Petri dish inserted in a culture vessel also containing 4 explants in the basal medium. The Petri dish and main culture vessel provided the opportunity to expose explants to combinations of media with and without ethephon (refer to Figure 5-5).

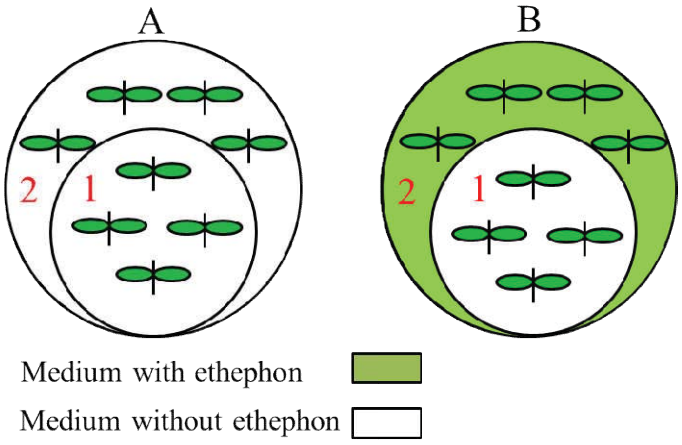


Figure 5-5: Schematic representation illustrating the arrangement of treatments applied within the experiment during cultivation of gentian *in vitro*; **(A) 1**: explants without any exposure to ethylene either from the medium or the atmosphere in the headspace, by being cultured in a Petri dish containing 25 mL of ethephon-free medium and located in the main vessel containing 50 mL of ethephon-free medium, **2**: explants without any exposure to ethylene either from the medium or the atmosphere in the headspace, by being cultured in the vessel containing 50 mL of ethephon-free medium **(B) 1**: with only aerial exposure to ethylene in the headspace of the culture vessel containing 50 mL of medium amended with 10 $mg.L^{-1}$ ethephon, by being cultured in a Petri dish containing 25 mL of ethephon-free medium and located into the main vessel, **2**: explants with aerial exposure to ethylene in the headspace as well as absorption from the medium by being cultured in the main culture vessel containing 50 mL of medium amended with 10 $mg.L^{-1}$ ethephon.

Irrespective of treatment, a similar total volume of medium to that used in former experiments (i.e. 50 mL) was applied in all vessels. In order to prevent covering a part of the surface of the medium with the Petri dish, Petri dishes were held above the main medium surface on short lengths of narrow (7 mm diameter) support structures (i.e. pipette tips; Figure 5-4). There were five of those columns needed to bear the weight of the Petri dish containing 25 mL of medium (providing a similar depth of medium to the main culture vessels for explants; Figure 5-4). So as to allow interpretation of the shoot formation response to the form of ethephon uptake, there were 4 treatments, arranged into two experimental combinations (Figure 5-5) as:

Combination A, no ethephon:

1. Explants without any exposure to ethylene either from the medium or the atmosphere in the headspace, cultured in a Petri dish containing 25 mL of ethephon-free medium suspended within the main culture vessel containing 50 mL of ethephon-free medium.
2. Explants without any exposure to ethylene either from the medium or the atmosphere in the headspace, cultured directly in the culture vessel containing 50 mL of ethephon-free medium only.

Combination B, aerial ethylene versus ethephon-medium:

1. Explants with only aerial exposure to ethylene in the headspace of the culture vessel, cultured in a Petri dish containing 25 mL of ethephon-free medium suspended within the main culture vessel containing 50 mL of 10 $mg.L^{-1}$ ethephon in the basal medium.
2. Explants with both aerial exposure to ethylene in the headspace as well as potential absorption from the medium, cultured directly in the culture vessel containing 50 mL of 10 $mg.L^{-1}$ ethephon in the basal medium.

Explants of gentian 'Little Pinkie' used for this experiment were taken from the most uniform population of donor plantlets grown *in vitro* in a PGR-free medium under standard growth conditions, as described in Chapter 2. Explants comprised tip (Tip), first node below the tip (Node 1), second node below the tip (Node 2) and the rooted base (Base) (refer to Chapter 2, Figure 2-2), with resulting nodal positions distributed evenly amongst the treatments. There were four explants in each Petri dish, and similarly another four explants in the main culture vessel (Figure 5-5).

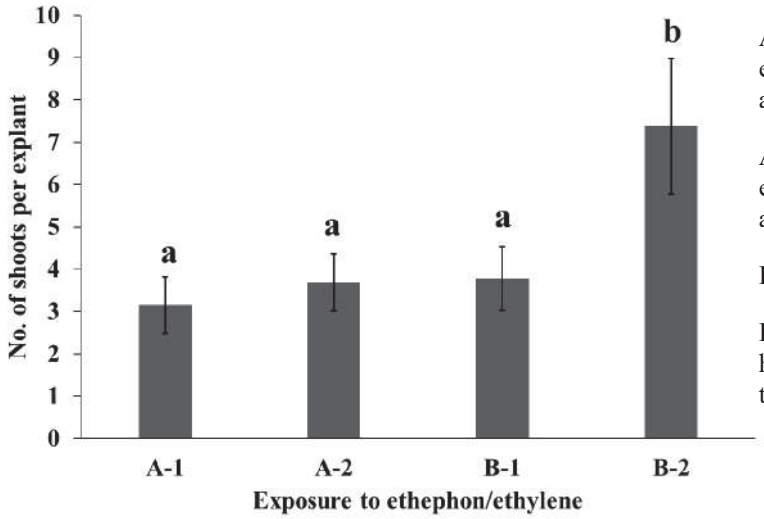
The experiment was a factorial design, comprising two factors: type of exposure to ethylene/ethephon (i.e. from medium, from atmosphere, or no exposure), and position along the original shoot which explants were taken from (Tip, Node1, Node2 and Base). Each treatment comprised eight culture vessels or petri dishes as individual replicates and each contained four explants from various nodal positions evenly distributed. In anticipation of an increased risk of contamination due to construction of the new culture system, the number of replications was increased from that used in previous experiments. After 8 weeks of growth data were recorded for; the number of 2° shoots per explant, explant fresh mass and root development score (refer to Chapter 2, Section 2.2). Analysis of variance was conducted in a

split plot factorial design, using General Linear Model in Genstat software with mean comparison achieved using Fisher's unprotected least significant difference test at $P \leq 0.05$.

5.1.3.3. Results and discussion

After 8 weeks of growth, explants of 'Little Pinkie' grown in culture vessels including ethephon in their medium (treatment B-2) produced ~ 2 times more shoots compared to the explants of all other treatments ($P \leq 0.001$) (Figure 5-6). Increased shoot formation induced by ethephon-containing medium was expected, as previously reported (refer to Chapter 3 and Appendix 13). However, aerial exposure of the explants to ethylene only, without direct contact of the tissue with ethephon in the medium (i.e. treatment B-1) did not result in any significant change in shoot formation in comparison with explants cultured without ethephon in the medium or ethylene in the atmosphere (treatments A-1 & A-2) (Figure 5-7). As such, this result corroborated those arising from exposing explants to ethylene gas (refer to Section 5.1.2).

Similar to all other experiments in this thesis, there was a position effect on the number of shoots developed, which increased as the distance of the position that the explant was taken from increased from the tip toward the base of the donor shoot ($P \leq 0.001$; Appendix 5). The interaction effect between treatments and position on the number of shoots was also significant ($P \leq 0.01$). This interaction was especially evident between the explants grown in the presence of ethephon compared to the absence of ethephon, and particularly between explants that originated from the lower part of the shoot (Node 2 and Base) compared to the upper part of the donor shoot (Tip and Node1). As such, application of ethephon in the medium encouraged explants originating from Node 2 and the Base to produce 2 times more shoots than the explants of equivalent positions in the absence of ethephon.



A-1: without exposure to ethylene either from the medium or the atmosphere

A-2: without any exposure to ethylene either from the medium or the atmosphere

B-1: only aerial exposure to ethylene

B-2: aerial exposure to ethylene in the headspace as well as absorption from the medium (refer to Figure 5-5)

Figure 5-6: Number of 2° shoots developed per explant of gentian 'Little Pinkie' (averaged across all positions): **A-1** without exposure to ethylene either from the medium or the atmosphere in the headspace, by being cultured in ethephon-free medium in a Petri dish and inserted into the ethephon-free medium in the vessel, **A-2** without any exposure to ethylene either from the medium or the atmosphere in the headspace, by being cultured in the vessel containing ethephon-free medium, **B-1** only aerial exposure to ethylene, by being cultured in a Petri dish containing ethephon-free medium but inserted into the vessel containing medium amended with 10 $mg.L^{-1}$ ethephon, **B-2** aerial exposure to ethylene in the headspace as well as absorption from the medium by being cultured in the culture vessel contained medium amended with 10 $mg.L^{-1}$ ethephon (refer to Figure 5-5). Data was square root-transformed and analysed with Fisher's unprotected least significant difference. Data are shown as the back transformed mean values. Means which do not share the same letter are significantly different at $P \leq 0.05$. Vertical lines represent ± standard error mean, n=8 vessels each containing 8 explants.

Ethephon also clearly delayed root production by reducing the root score; explants which were exposed to ethephon in their medium and produced the highest number of shoots had a lower root score (~23 to 30% lower) compared to other treatments ($P \leq 0.001$) (Figure 5-8). However, the root score of explants which were exposed to ethylene only in their atmosphere was not significantly reduced, corroborating those results from using ethylene gas (refer to Section 5.1.2). Root development decreased whereas total weight increased, as the distance of the position of explants increased from the tip ($P \leq 0.001$; Appendix 6).

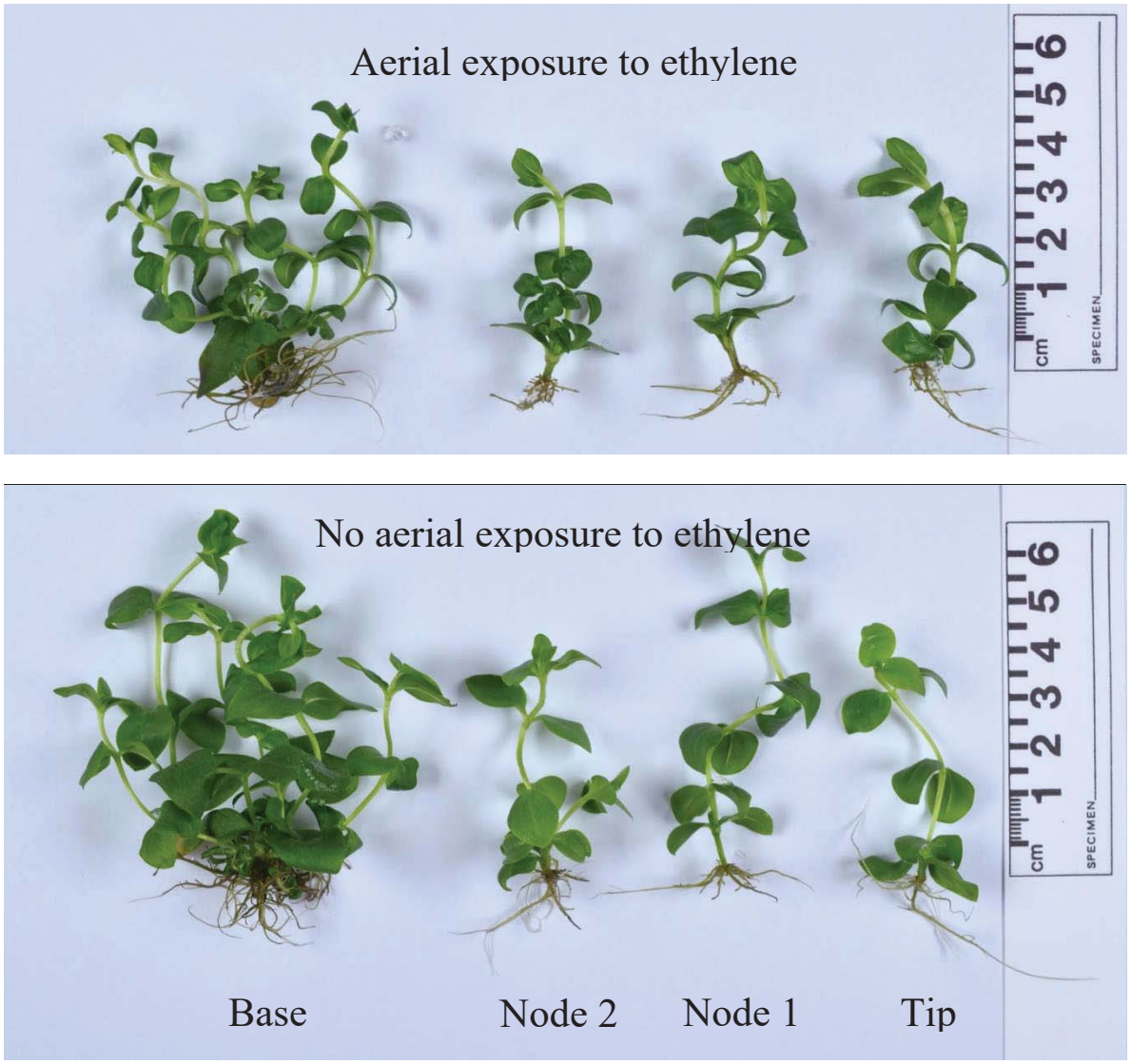
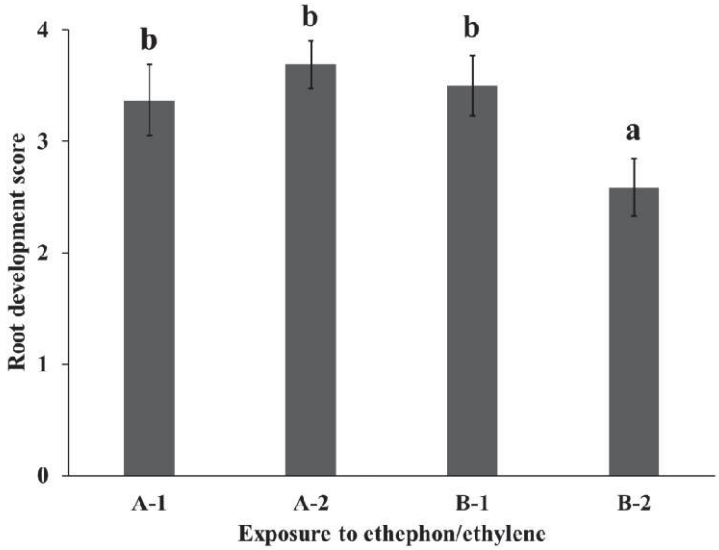


Figure 5-7: Side view of explants of gentian cultivar 'Little Pinkie' taken from four positions (Tip, Node 1, Node 2 and Base), after 8 weeks of growth *in vitro* with no ethephon in their medium but either with or without aerial exposure to ethylene. Explants without direct contact of the tissue with ethephon in the medium but been exposed to ethylene in their atmosphere did not result in any significant change in shoot formation in comparison with explants cultured without ethephon in the medium or ethylene in the atmosphere.



A-1: without exposure to ethylene either from the medium or the atmosphere

A-2: without any exposure to ethylene either from the medium or the atmosphere

B-1: only aerial exposure to ethylene

B-2: aerial exposure to ethylene in the headspace as well as absorption from the medium (refer to Figure 5-5)

Figure 5-8: Root growth from explants of gentian 'Little Pinkie' (from all positions) as influenced by treatments: **A-1** without any exposure to ethylene either from the medium or the atmosphere in the headspace, by being cultured in ethephon-free medium in a Petri dish and inserted in the ethephon-free medium in the vessel, **A-2** without any exposure to ethylene either from the medium or the atmosphere in the headspace, by being cultured in the vessel containing ethephon-free medium, **B-1** only aerial exposure to ethylene, by being cultured in a Petri dish containing ethephon-free medium but inserted into the vessel containing medium amended with 10 $mg.L^{-1}$ ethephon, **B-2** aerial exposure to ethylene in the headspace as well as absorption from the medium by being cultured into the culture vessel containing medium amended with 10 $mg.L^{-1}$ ethephon (refer to Figure 5-5). Mean comparison was done using Fisher's unprotected least significant difference. Different letters on mean values are significantly different at $P \leq 0.05$. Vertical lines represent ± standard error bars, n=8 vessels each containing 8 explants.

The results achieved in the current experiment on the effect of ethephon application, as well as the position, reaffirmed the results of similar experiments reported previously (refer to Chapter 3 and Appendix 13). However, there was a subtle difference between them. For example, the increase in number of shoots as affected by ethephon (~7 shoots) was lower than in previous experiments (e.g. 10 shoots; Chapter 3 and Appendix 13). In consideration of the efficacy of cultural conditions throughout the current thesis, it was likely that the explants used in the current experiment were not in their best vegetative condition (refer to Chapter 2, Section B). However, the effects of ethephon as well as nodal position on shoot formation, which had been observed in previous experiments, were still evident.

In the current experiment plant material originated from the tip (Tip), nodal positions (Node 1 and 2) as well as the rooted basal explants (Base). Explants taken from the rooted bases produced the most number of shoots and total weight. One of the possible explanations for such an increase in growth variables of explants from the Base, could be attributed to greater absorption of the medium's components, such as mineral nutrients and ethephon through the preformed roots. Nutrition and minerals would be needed for the growth and development of bud primordia which were reported previously to be formed at the node position (refer to

Chapter 3). Also, ethephon as a source of ethylene encourages bud release and accelerates bud development (Dimasi-Theriou et al., 1993; Mensualisodi et al., 1995). As detailed in earlier chapters; while Base explants included multiple bud primordia at the node position, Tip explants normally included the apical meristem which was a compact form of the stem, including several nodes together with two developed leaves. Node explants included a single node/metamer which contained two preformed axillary buds (refer to Chapter 3, Section 3.3.1). Hence Tip, rooted Base and Node explants were interpreted to be different physiological constructs from each other and, therefore, have physiological differences which are likely to have interacted with treatments to deliver the differing results observed. Given this inherent physiological difference, the statistical analysis was conducted for a second time, but using only the data from Node 1 and Node 2 positions (data not presented). This analysis confirmed the fact that inclusion of explants of different physiological structure was not placing any undue bias in interpreting the main treatment effect.

Atmospheric ethylene uptake, unlike absorption of ethephon from the medium, did not alter any growth variables in explants, and resulted in a response similar to those from the experiment presented in Section 5.1.2. Embryogenesis of *in vitro* callus of *Daucus carota* L. exposed to ethylene released from ethephon was not affected whereas direct contact of the callus with ethephon in the medium reduced it (Tisserat and Murashige, 1977). Although this research was not known to the author prior to planning and conducting the experiments of the current chapter, their results are in agreement with the results presented here. They also reported that exogenously supplied ethylene at a concentration of 5 $\mu L.L^{-1}$ over a 4-week culture period did not affect embryogenesis substantially, which is also in line with the lack of effect on shoot formation in explants exposed to aerial ethylene at 60 $nL.L^{-1}$ reported in Section 5.1.2 and within the current experiment. It is still not possible to tell from the results of either study whether ethephon within the media induces a much higher local concentration of ethylene inside the plant similar to that report which indicated penetration of a significant amounts of ^{14}C-labelled ethephon into the tissue of grapevine shoots (Nir and Lavee, 1981). However, it is noteworthy that the work with carrot callus used 5 $mg.L^{-1}$ gaseous ethylene, which was a very high concentration compare to that used in current research and therefore was expected to make a significant difference if a high concentration could enter the explant.

One of the other arguments for the observation of no change in the number of shoots raised from the explants exposed to only aerial ethylene as compared with the explants treated with

ethephon could possibly be due to the effect of ethephon on pH of the medium rather than ethylene. However, the *in vitro* growing medium which was utilised in all experiments in this thesis, and resulted in increased shoots in gentian plants, was buffered to be at pH 5.7 as part of the general lab protocol, with the ethephon then $mg.L^{-1}$ added (refer to Chapter 2). After application of ethephon the final pH of the medium was quantified in a sample before explants were cultured in each experiment, and was always found to be in the range 5.7 ± 0.1. Therefore adding ethephon, especially at such a small concentration utilised in the current experiments (10 $mg.L^{-1}$), did not make a significant change in the final pH of the medium. Hence the possibility of ethephon's effect on changing the pH of the medium was dismissed.

Another possibility for not observing any significant difference in shoot formation after exposing the explants to only aerial ethylene, could potentially result from a contribution by other products arising from ethephon's decomposition such as phosphate, chloride or any additional ethephon-metabolites other than ethylene. In their research with carrot callus (Tisserat and Murashige, 1977) reported that when autoclaving was used to remove ethylene from the medium amended with ethephon, the response was still significant as compared with control or ethephon-free medium. This therefore suggests that there was a contribution from non-volatile components, which in their case was attributed to phosphonate (Tisserat and Murashige, 1977). However ethephon has been reported to be metabolized to over 10 compounds in rubber tree (*Hevea brasiliensis* Willd. Ex A. Juss.) (Audley et al., 1976; Audley, 1979), with notable variation in the metabolism or decomposition of ethephon in the tissue of various crops (Yamaguchi et al., 1971). In contrast, the current study focused only on ethylene arising from decomposition of ethephon. Nevertheless, involvement of any metabolite or chemical component of ethephon on shoot formation response could be valid if ethylene did not make the expected response of increase in shoot formation.

Looking to the future, i.e. beyond the current thesis, so as to test the hypothesis that ethylene was the effective component from decomposition of ethephon on shoot formation in gentian, the effect of ethylene could be inhibited by application of ethylene inhibitors such as AVG, $CoCl_2$, STS, $AgNO_3$, 1-MCP, etc. AVG and $CoCl_2$ have been reported to inhibit ethylene biosynthesis, whereas STS, $AgNO_3$ and 1-MCP inhibit ethylene action (Yang and Hoffman, 1984; Purnhauser et al., 1987; Biondi et al., 1998; Khalafalla and Hattori, 2000; Arigita et al., 2003; Blankenship and Dole, 2003; Reis et al., 2003; Feng et al., 2004; Watkins, 2006). Except for 1-MCP which is a gas, other ethylene inhibitors could be applied within the *in*

vitro medium, in order to evaluate their effect on inhibition of ethylene within the current experimental system. However, preliminary trials would be needed on the concentration range of each compound in order to quantify the optimum concentration without phytotoxic effects on the explants of gentian. For 1-MCP however, due to its gaseous form a different methodology needs to be taken to expose the explants in a sealed vessel. Also, since 1-MCP inhibits ethylene action at the receptor level, it needs to be applied multiple times in order to guarantee that all the new receptors formed during explant development will be blocked and, therefore, its' efficacy is unpredictable. Recently a new water soluble ethylene antagonist, 3-cyclopropyl-1-enyl-propanoic acid sodium (CPAS) salt has been introduced which is considered as the soluble salt of 1-MCP (Huberman et al., 2014). Since it is a water soluble salt it could probably be dissolved in the medium and used for inhibition of ethylene's effect in such future *in vitro* studies. Since the results achieved in this thesis (refer to Section 5.1.1 and 5.1.3) indicated exposing explants to ethylene gas only from the atmosphere was not effective and ethephon absorption was needed through the culture medium to affect shoot formation, and also due to the limitation in time and resources aforementioned suggested ideas were not followed in current thesis.

5.1.3.4. Conclusion

Aerial supply of ethylene released from ethephon has been shown not to lead to changes in growth responses of explants of gentian 'Little Pinkie'. Only explants able to directly absorb ethephon from the medium showed the characteristic response of increased shoot formation and decreased root growth. It is therefore concluded that ethephon conversion to ethylene within the explant tissue (or exposure to breakdown products or other components of ethephon) is required for the shoot formation response.

Chapter 6. *In vitro* shoot formation in response to ethephon/ethylene, relation with auxin

6.1. General Introduction

While the mechanism by which ethephon/ethylene application in the culture medium increased shoot formation in gentian 'Little Pinkie' (refer to Chapter 3 and appendix 13) is not known, the effect of other plant growth regulators such as auxin and cytokinins on frequency of shoot formation should not be ignored (Hosokawa et al., 1996; Ivana et al., 1997; Morgan et al., 1997). As such, auxin: cytokinin ratio has been noted to be important on controlling development of shoots (Bangerth, 1994; Emery et al., 1998; Haver et al., 2002) and an optimum ratio has been reported to be required to optimise shoot regeneration (Skoog and Miller, 1957). Hence, as explored within the current as well as the next chapter, it was hypothesised that the effect of ethylene on increasing shoot formation in gentian might take place through influencing other plant growth regulators such as auxin and/or cytokinin, and consequently altering the combination of auxin: cytokinin toward the optimum.

Ethylene has been suggested previously to reduce the concentration of auxin by inhibiting polar auxin transport (PAT). The capacity of the petiole segments of *Phaseolus vulgaris* for transporting radiolabelled auxin was reduced following exposure to ethylene which was suggested to be due to the reduction of PAT in response to ethylene affecting bud formation *in vitro* (Osborne and Mullins, 1969). Given the contribution of adventitious buds to ethephon's effect on increasing shoot formation in gentian 'Little Pinkie', it is relevant to note that decreasing PAT in response to ethylene exposure was reported to be the mechanism underlying the formation of adventitious buds on bulb scale explants of *Lilium speciosum in vitro* (van Aartrijk et al., 1985a; van Aartrijk et al., 1985b). Following the reduction in concentration of auxin, the ratio of auxin to cytokinin in segments would likely be modified in favour of cytokinin.

In order to study the probable inhibitory effect of ethylene on PAT in explants of gentian cultured *in vitro*, it was proposed that auxin transport could be measured in explants when treated with or without ethephon in their medium, and compared with that for explants treated with a PAT inhibitor. Polar transport of auxin can be measured by comparing the concentration within the tissue extract of distal and proximal ends of the explant over time

(Osborne and Mullins, 1969; Hasenstein and Evans, 1988; Garrido et al., 2002). Auxin transport through plant tissue is a directional function which could be interrupted by application of PAT inhibitors such as N-1-naphthylphthalamic acid (NPA) and 2, 3, 5-triiodobenzoic acid (TIBA) (Thomson et al., 1973; Thomson and Leopold, 1974; Morris, 2000; Taiz and Zeiger, 2002). Synthetic substances such as TIBA inhibit auxin transport via competing with auxin for a membrane-bound site (Thomson et al., 1973; Geldner et al., 2001), while NPA specifically inhibits the auxin efflux transporter protein, PIN, possibly via a specific NPA-binding protein (Thomson and Leopold, 1974; Morris, 2000; Muday and Murphy, 2002). Also, anecdotally application of TIBA has been resulted in some other toxic side effects. Hence, in the current experiments NPA was selected to be applied as the PAT inhibitor.

Although auxin transport has been studied in an extensive range of plants *in vivo*, limited literature could be found with regard to its use in an *in vitro* system to be applied in current research. Hence, there was a need to modify the methodology used *in vivo* to make it suitable. The current chapter therefore consists of a series of experiments to first develop the methodology required for an *in vitro* system, and then, investigate the possibility of the effect of ethephon on PAT inhibition. Experiments 1 and 2 were preliminary experiments, with their results applied to the main experiment on transport of IAA using radiolabelled IAA in Experiment 3.

6.2. Experiment 1 - NPA dose response curve on shoot formation in gentian 'Little Pinkie' cultured *in vitro*

6.2.1. Introduction

To develop the methodology required for an *in vitro* system, a method of application and effective concentration of NPA needed to be determined. When ethephon was used *in vivo*, it has normally been applied in lanolin as a ring around the stem (Eklund and Little, 2000), and similarly applicable to NPA (van Hooijdonk et al., 2011). The total length of the stem used in an *in vivo* system by Eklund and Little (2000) for instance, was about 15 cm and the lanolin-treated section of the stem was 2 cm long, whereas the current experimental system for gentian *in vitro* comprised a total length of stem up to 2 cm (refer to Chapter 2, Section 2.2); the scale of the current system therefore was considered impracticable to apply as a ring around the stem in gentian explants. Given the experimental system under investigation, the

most convenient method of application of both ethephon and NPA was considered to be having it in the basal agar medium. However, given the lack of prior experience it was recognised that there might be a risk of phytotoxic effects on the explants of gentian by exposing them to a continuous source of NPA in the medium if the concentration were higher than required. As determined in previous studies for a range of physiological responses *in vitro*, for an array of plant genera, concentrations between 10 and 200 µM of NPA have been applied and various physiological responses achieved (Fischer et al., 1997; Petrasek et al., 2002; Vidal et al., 2003; Rahman et al., 2007). For the current study on gentian therefore, treatments comprised NPA applied at various concentrations to give media with final concentrations of 0, 2.5, 5, 10, 100, 500 and 1000 µM NPA. Experiment was undertaken twice trying different concentration ranges, wherein the second built on the findings of the first one. Results of the first experiment are presented here. The second repetition is presented in Appendix 7 and overall conclusions based on both experiments will be discussed.

Any morphological similarities (e.g. shoot formation) between explants of gentian cultured *in vitro* treated with ethephon compared to NPA, could be useful in supporting the hypothesis of an inhibitory effect of ethephon on PAT, i.e. before actually measuring PAT in treated explants. Therefore, objectives for this experiment on explants of gentian cultured *in vitro* were to:

- compare the effect of ethephon and NPA on growth variables
- quantify the effect of various concentrations of NPA on growth variables and,
- identify the concentration of NPA with the most similar effect to ethephon.

6.2.2. Material and methods

The base medium was PGR-free (refer to details within Chapter 2, Section 2.2). For the ethephon treatment, ethephon (Ethrel®; 480 $g.L^{-1}$ chlorethephon; Bayer CropScience, New Zealand) at a final concentration of 10 $mg.L^{-1}$, was added to the base medium as the comparative treatment after filter sterilisation (0.2 µm Minisart® filter; Sartorius Stedim Biotech, Germany) at 50°C (Purnhauser et al., 1987) (refer to Chapter 2). For NPA treatment, NPA (Naptalam, Assay (HPLC) 99.9%, Sigma-Aldrich, Germany) was added to the medium by microfiltration (0.2 µm Minisart®filter; Sartorius Stedim Biotech, Germany) after being autoclaved. Each culture vessel (refer to Chapter 2, Section 2.2) contained 50 mL of medium.

Plantlets of the gentian cultivars 'Little Pinkie' which previously had been grown and sub-cultured *in vitro* for several five-week growth cycles using a PGR-free medium in line with the general protocol for gentians within the tissue culture lab (Morgan et al., 1997) were used as donor plantlets to take explants (refer to chapter 2, Section 2.2).

Factors for the experiment comprised chemical treatments (Control, ethephon or a concentration range of NPA) and positions of Tip (apex and undeveloped leaves and nodes), Node 1 (first node below the tip) or Node 2 (the second node below the tip; Chapter 2, Figure 2-2). The range of concentrations of NPA within the final medium were 0, 2.5, 5, 10, 100, 500 or 1000 μM. For treatments which included ethephon, the final concentration of ethephon in the medium was 70 μM (10 $mg.L^{-1}$). There were three culture vessels as individual replications, each including nine explants from various positions distributed evenly. The number and type of shoots per explant were recorded after 8 weeks growth as either primary (1º) for Tip explants, secondary (2º) and tertiary (3º) for other explants (refer to Chapter 2, Figure 2-1). Also, shoot length was classified as short (less than 10 mm length), medium (10-20 mm) or long (20 mm or more), and was recorded as well as total fresh weight. Root development scores (overall number and length of roots for each explant scored out of 4) were also recorded as detailed in Section 5.1.2.2 and Chapter 2. Analysis of data was done on the basis of a split plot factorial design. The treatment factor was split into two contrasts: control versus NPA versus ethephon, and nested within that was each NPA concentration. The position factor was also split into two contrasts: Tip versus Nodes and, nested within that, Node 1 versus Node 2. Analysis of variance was conducted using the General Linear Model in Genstat software (version 17, 2014, VSNi Ltd, Hemel Hempstead, UK). Square root transformation on the data was done to make the variability more equal when needed. Means comparison was based on Fisher's protected least significant difference test, where in Genstat due to the limitation of the software, the Studentized Range statistic was used instead of Student's t test at $P \leq 0.05$, to make it equivalent to Tukey's test.

6.2.3. Results and discussion

6.2.3.1. NPA and ethephon on growth variables

In general, and for the growth variables recorded, application of ethephon produced similar results to that for application of NPA. Treatment with ethephon or NPA both resulted in plantlets which produced 1.5 times more shoots (2º) as compared with the control explants (P = 0.005; Table 6-1). However, number of tertiary shoots (3°) was not significantly affected

by the application of ethephon or NPA (Table 6-1). Because the tips responded significantly less than the nodal cuttings (data not shown), subsequent figures focus only on nodal cuttings (e.g. Figure 6-1A).

Table 6–1: *P* values resulting from the analysis of variance on a factorial design using chemical treatments (Control, ethephon or a concentration range of NPA) and positions (Tip, N1 (first node below the tip) or N2 (second node below the tip)) as factors. The treatment factor was split into two contrasts: control versus NPA versus ethephon, and nested within that NPA concentration. The position factor was also split into two contrasts: Tip versus Nodes, and nested within that N1 versus N2. Data on number of secondary (No. 2°) or tertiary shoots (No. 3°), long (No. long) or medium shoots (No. medium), root score and fresh weight were square root-transformed when needed. *P* values highlighted in red, were significantly equal or less than 0.05.

Factor	Sqrt No. 2° shoots	Sqrt No. 3° shoots	Sqrt No. long shoots	Sqrt No. medium shoots	Sqrt No. short shoots	Root score	Weight (mg)
Ctrl vs NPA vs Eth	0.050	0.436	<.001	0.861	0.012	0.008	0.34
NPA conc. Range	0.385	0.113	<.001	0.001	0.004	0.001	<.001
Tip vs Node	<.001	0.701	<.001	0.12	<.001	<.001	<.001
N1 vs N2	0.035	0.031	0.116	0.055	0.035	0.220	0.592
Tip vs Node × Ctrl vs NPA vs Eth	0.021	0.502	0.019	0.017	0.513	0.048	0.011
Tip vs Node × NPA conc. Range	0.317	0.794	0.841	0.509	0.922	0.681	0.385
Ctrl vs NPA vs Eth × N1 vs N2	0.576	0.219	0.685	0.439	0.347	0.495	0.434
NPA Dose × N1 vs N2	0.634	0.211	0.983	0.267	0.635	0.469	0.967

Application of NPA or ethephon both resulted in production of explants which were more compact in appearance than the control (Figure 6-2 & Figure 6-3), with 2.3 times reduction in the number of long shoots (20 mm or more) (Figure 6-1B). While there was no significant difference in the number of shoots of medium length (10-20 mm), exposing explants to either ethephon or NPA in the medium increased production of short (less than 10 mm) shoots in them by 3.2 or 1.6 times, respectively, as compared with control explants (Figure 6-1C). The results achieved here on the application of ethephon were similar to that reported in Chapter 1. Ethephon and NPA both decreased the development of roots (number and length) on the explants to ~ 2 times less than the control (Figure 6-3 and Figure 6-1D). Fresh weight of the explants increased by 25 and 30% compared to the control as a result of NPA application of up to 10 µM, and then started to decrease (20 and 55%) as the concentration of NPA in the medium increased beyond 10 µM ($P \leq 0.001$; Table 6-1, Figure 6-1E) probably due to toxicity. Concentration of 1000 µM NPA was considered supra-optimal, as all explants died and, therefore, this chemical treatment was removed from further statistical analysis. As

concentration of NPA increased in the medium, the number of long shoots developed by the explants decreased, but on the other hand the number of short shoots increased ($P \leq 0.001$; Figure 6-1B & Figure 6-1C). However, the number of short shoots developed by the explants treated with 100 µM NPA was 4.6 times more than the control ($P \leq 0.05$, Table 6-1 & Figure 6-1C). Among all concentrations of NPA applied, concentration of 100 µM produced the most similar effect to ethephon on growth variables of the explants. Low concentrations of NPA such as 2.5 and 5 µM did not affect any growth variables as compared to the control. Growth variables of the explants treated with NPA started to differ from the control as the concentration of NPA increased to 10 and 100 µM. High concentrations of NPA such as 1000 and 500 µM were also not similar to ethephon in their effect as they were supra-optimal on the explants, as evident by reduced total fresh weight and lack of root production (Figure 6-2 & Figure 6-3). Results of both repetitions of the experiments assisted to more confidently make the conclusion that NPA application produced a similar effect as ethephon on explants (refer to Appendix 7) and, among all concentrations of NPA, a concentration of 100 µM NPA produced explants in which their growth variables were the most similar to those from ethephon.

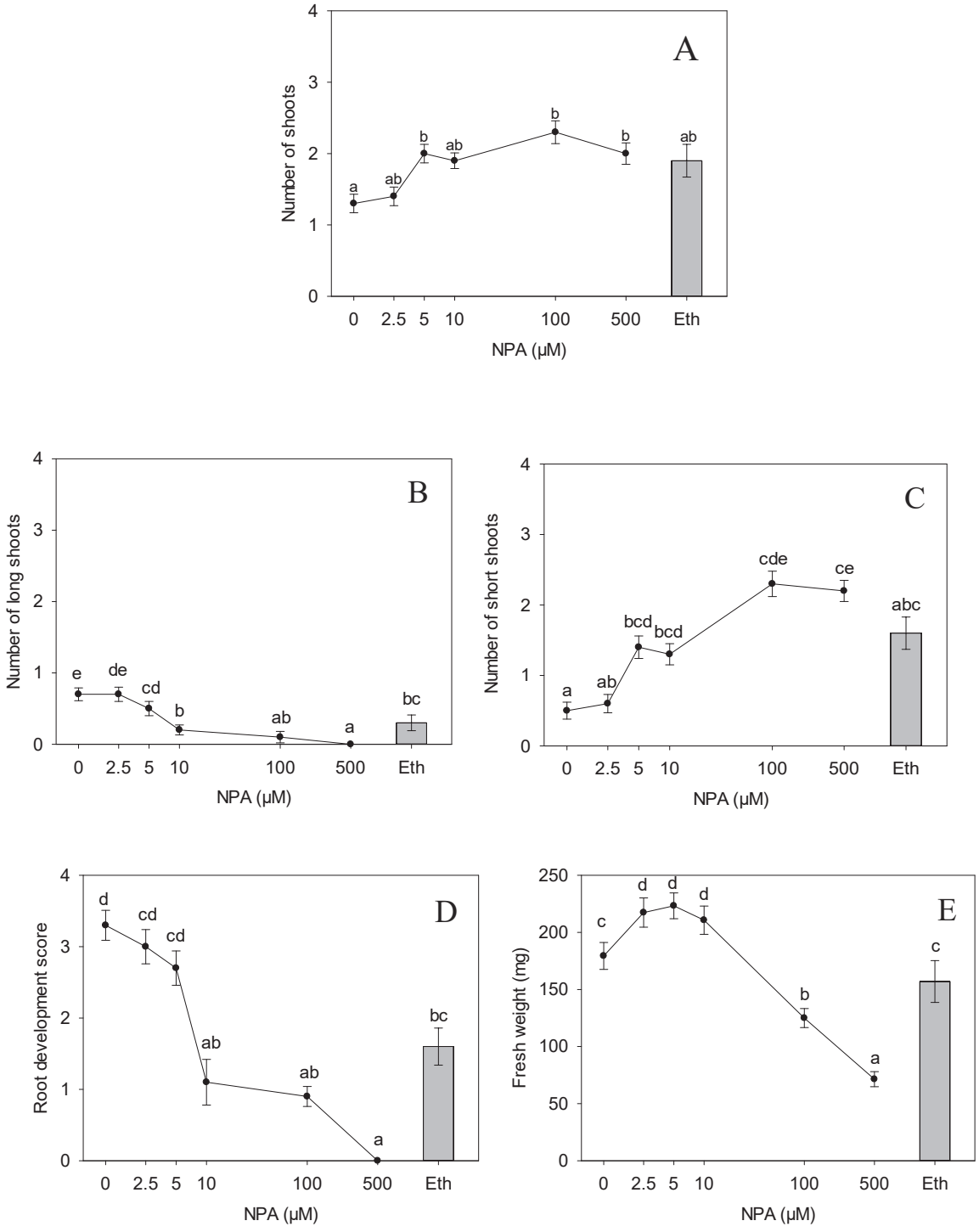


Figure 6-1: Number of **(A)** secondary (2°) shoots per explant, **(B)** long shoots and **(C)** short shoots developed from the explants; **(D)** root development score and **(E)** fresh weight of explants of gentian 'Little Pinkie' taken ONLY from nodal positions (Node 1 and 2) as affected by different chemical treatments (NPA at 0 (control), 2.5, 5, 10, 100 and 500 µM or 10 $mg.L^{-1}$ ethephon (Eth)) after 8 weeks growth *in vitro*. Data were square root-transformed and Mean comparison was done using the alternative LSD test where the Studentized Range statistic was used instead of Student's t test at $P \leq 0.05$. Vertical lines represented ± standard errors. Back-transformed mean values followed by different letters were significantly different at $P \leq 0.05$, n=3 vessels each containing 8 explants.

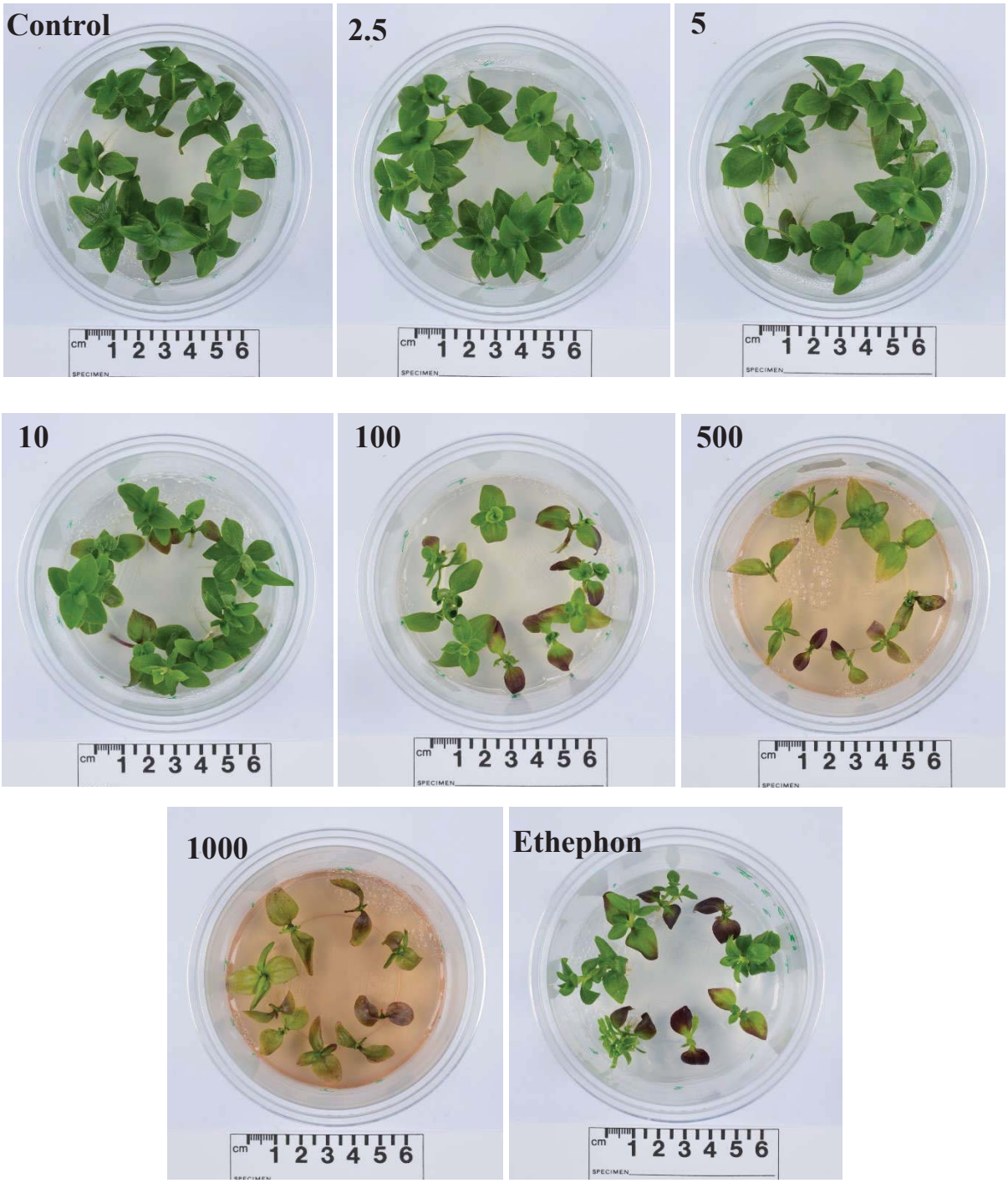


Figure 6-2: Aerial view of explants of gentian 'Little Pinkie' in preliminary repetition of the experiment taken from various positions (Tip, Node 1 and Node 2) distributed evenly in vessels, after 8 weeks of growth *in vitro* treated as control, having 10 mg.L^{-1} ethephon or different concentrations of NPA (0 (Control), 2.5, 5, 10, 100, 500 and 1000 µM) in the medium. Colour of the medium is a direct effect of NPA, not a biological response.

Figure 6-3: Side view representing the length of the explants of gentian 'Little Pinkie' in preliminary repetition of the experiment taken from different positions (Tip, Node 1 and Node 2) after 8 weeks growth in a medium amended with different concentrations of NPA (0, 2.5, 5, 10, 100, 500 and 1000 µM) *in vitro*.

The increased number of 2° shoots per explant resulting from NPA application has not been reported previously in gentian 'Little Pinkie' cultured *in vitro* (Figure 6-1A). This effect was very similar to that caused by ethephon and appears to indicate the outgrowth of a combination of various types of shoots, axillary, co-lateral and adventitious. As described in

Chapter 3, various types of bud primordia (axillary, co-lateral and/or adventitious) were microscopically identified at the node of nodal explants of 'Little Pinkie' cultured *in vitro*. These buds as well as apical buds in explants of the tip position, were the origin of shoots that arose from various types of explants in the current experiment. While explants of the tip position produced a maximum of one primary shoot, NPA (like ethephon) promoted outgrowth of 2° shoots from all types of buds in nodal explants. There have been some reports on the effect of NPA on promoting axillary bud outgrowth in other species such as pea plants and *Alstroemeria* (Nakajima et al., 2001; Pumisutapon et al., 2011). Therefore, it was hypothesised that a similar effect of NPA and ethephon could be due to a similar underlying mechanism on bud outgrowth, which was also evident by the similar increasing effect of ethephon and NPA on the number of short shoots (Figure 6-1C). An increasing effect of NPA on development of short shoots could be because of the increase in formation of 2° shoots from co-lateral and/or adventitious buds, similar to that described earlier for ethephon (refer to Chapter 3). In spite of the increase in the number of 2° shoots, a high concentration of NPA reduced other growth variables such as total fresh weight and root development, probably due to the possibility of toxicity of NPA at high concentrations (Figure 6-1D & E). The reduction in total fresh weight with increasing NPA concentration in the medium, was probably related with other growth variables. For example, reduction in root development as a result of the increase in concentration of NPA in the medium could affect nutrient absorption from the medium, and consequently decrease the vegetative vigour represented as length of shoots (reduction in the number of long shoots) and fresh weight of the explants. In a study on *Quercus robur* cultured *in vitro,* reduction in root growth and an increase in endogenous concentration of IAA resulted from application of NPA on shoots (Vidal et al., 2003). As, applicable to the current experiment, and supported by others, it was hypothesised that the effects of NPA on growth variables are attributed to inhibition of PAT (Thomson et al., 1973; Fischer et al., 1997; Vidal et al., 2003; DeMason and Chawla, 2004). Similar to NPA, ethylene pre-treatment also has been reported to inhibit transport of auxin in a range of crops such as tomato, cotton stem sections and segments isolated from seedlings of etiolated pea plants, depending on the dose of application (Beyer and Morgan, 1969; Suttle, 1988; Daniel et al., 1989). The similarity between the effect of NPA and ethephon observed in the current experiment therefore, would strengthen the hypothesis of an inhibitory effect of ethephon and/or ethylene released from ethephon on PAT and, as a result, increasing shoot formation. However, no literature was found on the inhibition of PAT in gentian by ethephon and/or ethylene and, therefore, future experiments needed to investigate that.

There was some inconsistency between the results of ethephon application on the explants in the current experiment, as compared to the experiment presented in other chapters (refer to Chapter 2, Section B), especially on the number of 2° shoots developed from the explants. The number of 2° shoots produced by explants treated with ethephon in two different experiments reported elsewhere in this thesis, was about 5 and 2 times more than the control (refer to Chapter 2), whereas in the current experiment was not statistically different from the control. Some of the possibilities causing such a difference could be related to the, technical procedure of making media and, vegetative vigour of explants used as the source material, etc. Hence, the experiment was repeated as detailed in Appendix 7. While results of the second repetition of experiment was in line with the first experiment, the effect of ethephon on growth variables, especially the number of 2° shoots, was improved in the second repetition. In first repetition the number of 2° shoots developed by the explants treated with ethephon was not significantly different from the control, in second repetition, treated explants produced 4.4 times more number of 2° shoots compared to the control, which was close to the results in other chapters (refer to Chapter 1, Section 1.1.3). The knowledge achieved in Chapter 2 in this thesis was therefore useful to restore the vegetative vigour of the donor plants before they were treated with ethephon.

6.2.3.2. Interaction of NPA and ethephon with position of the explant

While a similar trend to other chapters of this thesis (refer to Chapter 2, Section A) was observed on growth variables of the explants originating from different positions, there was also a significant interaction with the effect of NPA and ethephon ($P \leq 0.05$; Table 6-1). In general, the number of 2° shoots (mostly of short and medium length), and consequently total fresh weight of the explants, increased as the distance of the position that explants was taken from increased from the tip. The number of long shoots and the root development decreased. However, the number of 2° shoots was affected by an interaction between explant position with NPA or ethephon, which was clearer in the repeated experiment (Figure 6-4). As the position from which the explant was taken from was closer to the tip, more concentration of NPA was needed to produce the response. For example, the number of 2° shoots produced by explants from Node 3 increased as a result of application of 5 µM NPA. In comparison, explants of Node 1, which were closer to the tip, needed 100 µM NPA to produce the increase in number of 2° shoots (Figure 6-4). Differences between explants taken from various positions in response to the effective concentration of NPA as a PAT inhibitor on shoot formation enhancement, could be related to their difference in endogenous auxin

content (Goldsmith, 1977). It is likely that the quantity of endogenous auxin would decrease as the distance of the explant position would increase from the tip. Therefore, less concentration of NPA would be needed to inhibit the transport of auxin as the distance of the explant increases from the tip. Ethephon at the concentration applied in the current experiment (10 mg.L^{-1}) also increased the number of 2° shoots produced by the explants, as the distance of the position increased from the tip (Figure 6-4).

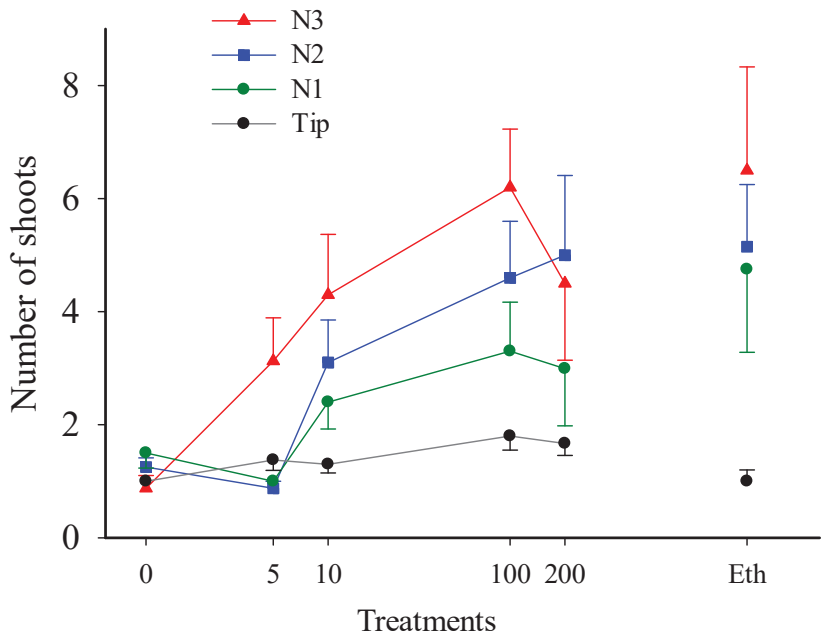


Figure 6-4: Interaction effect of different chemical treatments (NPA at concentrations of 0, 2.5, 5, 10, 100 and 200 µM and ethephon (Eth)) with the position that explants of gentian 'Little Pinkie' were taken from (tip (Tip), node 1 (N1), node 2 (N2) or node 3 (N3)) on the number of shoots (1º for Tip explants or 2º for other explants) developed from the explants after 8 weeks growth *in vitro*. Data were square root-transformed and Mean comparison was done using the alternative LSD test where the Studentized Range statistic was used instead of Student's t test at $P \leq 0.05$. Vertical lines represented either + or - standard errors for clarity on back-transformed mean values, n=3-6 vessels each containing 8 explants.

6.2.4. Conclusion

Explants treated with NPA as a PAT inhibitor presented similar effects on growth variables as those treated with ethephon, which built up evidence for the possible inhibitory effect of ethephon on PAT. As a result of either ethephon or NPA application, explants of gentian 'Little Pinkie' developed additional short and medium 2º shoots compared to the control and, therefore, shoot formation increased. Among all concentrations of NPA applied in the medium, concentration of 100 µM was determined as the concentration with the most similar effect on growth variables to ethephon. A higher concentration than 100 µM had toxic effects on explants.

6.3. Experiment 2 - Duration of ethephon exposure on shoot formation *in vitro*

6.3.1. Introduction

Following the results achieved in Experiment 1, in which ethephon produced similar effects to NPA on growth variables of the explants, and thereby building up evidence for the possible inhibitory effect of ethephon on PAT, the current experiment was carried out to further investigate this possibility. The possibility of an inhibitory effect of ethephon on PAT, and consequently increase in shoot formation, could be studied by application of radiolabelled auxin on explants treated with or without ethephon (Goldsmith, 1977; van Aartrijk et al., 1985a). Before application of radiolabelled auxin however, explants would first need to be exposed to ethephon for a duration necessary to make the effect (refer to Experiment 3). Hence, in order to investigate how long explants needed to be exposed to ethephon to achieve the increasing response in shoot formation, the current experiment was carried out.

Results of the experiments presented in earlier chapters of this thesis were utilized in order to determine the time range (treatments) for the application of ethephon/ethylene on the explants in current experiment. Data presented in Chapter 3 and Appendix 13, illustrated explants of gentian 'Little Pinkie' treated with ethephon in their medium produced additional shoots after 8 weeks of culture *in vitro*. Additional shoots observed after 8 weeks of culture could be seen, microscopically, to originate from additional bud primordia on the explants which were evident 4 weeks after being exposed to ethephon in their medium (refer to Chapter 3, Figure 3-12). While the effect of ethephon on increasing shoot formation was evident after 4 or 8 weeks, it was likely that any inhibition of ethephon on PAT would have happened earlier. In order to investigate the efficacy of shorter durations of exposure than 4 weeks, information gained in previous chapters were utilized. The concentration of ethylene first quantified at 4 hours after adding ethephon to the medium, showed a slight peak after 48 hours which remained relatively unchanged over a two weeks of monitoring (refer to Chapter 4, Figure 4-7). Hence, durations of explant exposure to ethephon/ethylene proposed for the current experiment were 0, 4, 24 and 48 hours, 1, 2, 4 and 8 weeks. The current experiment in this chapter was therefore planned to:

- determine the effective duration of ethephon exposure on increasing shoot formation in explants of gentian 'Little Pinkie'

6.3.2. Materials and methods

The base medium (PGR-free) was prepared as detailed earlier (refer to Chapter 2, Section 2.2). For the chemical treatment with inclusion of ethephon, micro-filtered ethephon was added to the autoclaved medium to give a final concentration of 10 $mg.L^{-1}$ (refer to Chapter 3). Fifty mL of the medium was inserted into the same plastic culture vessels as previously utilized in all experiments. Plantlets of gentian 'Little Pinkie', which previously had been taken from nodal explants (node with the short internodes and a pair of developed leaves) (Chapter 1, Figure 1-3) grown and sub-cultured *in vitro* for several five week cycles, were used as the source material of explants for this experiment.

Treatments were made up of factorial combination of three factors;

1) duration of explant exposure (0, 4, 24 and 48 hours, 1, 2, 4 and 8 weeks) to,

2) medium amendment (with 10 $mg.L^{-1}$ or without ethephon) and,

3) position (tip (Tip), first node (Node 1) and second node (Node 2; refer to Chapter 2, Figure 2.2) from which explants were taken.

In order to create different durations of ethephon exposure, at each duration explants were transplanted from a culture vessel containing medium with ethephon to a culture vessel containing medium without ethephon. Also, to capture the possible effect of transplanting on shoot formation, at the same time the same process was repeated by transferring another batch of explants from the medium without ethephon to a fresh medium without ethephon at each time interval as well. Four replications of vessels for each treatment were utilized. Nine explants were cultured in each culture vessel and grown under standard *in vitro* growth condition, as described in Chapter 2. The number of shoots (1º, 2º or 3º) shoots were recorded separately after 8 weeks. The design was a split-plot design, with culture vessels considered as plots and individual explants as sub-plots. Analysis of variance was conducted using the General Linear Model in Genstat software (version 17, 2014, VSNi Ltd, Hemel Hempstead, UK). Means comparison was based on Fisher's protected least significant difference test, where the Studentized Range statistic is used instead of Student's t test at $P \leq 0.05$, and is equivalent to Tukey's test.

6.3.3. Results and discussion

Depending on the duration of exposure, explants cultured in the medium with inclusion of ethephon produced average of 1.5 times more 2° shoots than the explants exposed to medium without ethephon ($P \leq 0.05$) (Table 6-2). Explants exposed to ethephon for four weeks produced about twice the number of 2° shoots compared to the explants in medium without ethephon. By increasing the duration of exposure to ethephon from four to six and eight weeks, the number of 2° shoots did not significantly differ from that at four weeks (Figure 6-5). However, a minimum duration of two weeks exposure to ethephon was probably required in order to establish the significant increase in number of 2° shoots evident at four weeks (started emerging from third week), as compared with those in medium without ethephon.

Table 6–2: Probability (*P* values) from the analysis of variance for a split plot factorial design. Every culture vessel considered as a plot and each individual explant as a sub-plot. Factors comprised the duration of explant exposure, type of medium and the position of the explants.

Factor	**Number of 2° shoots**
Medium	<.001
Time	0.012
Position	<.001
Medium × time	0.037
Time × Position	0.262
Medium × Position	<.001
Medium × Time × Position	0.339

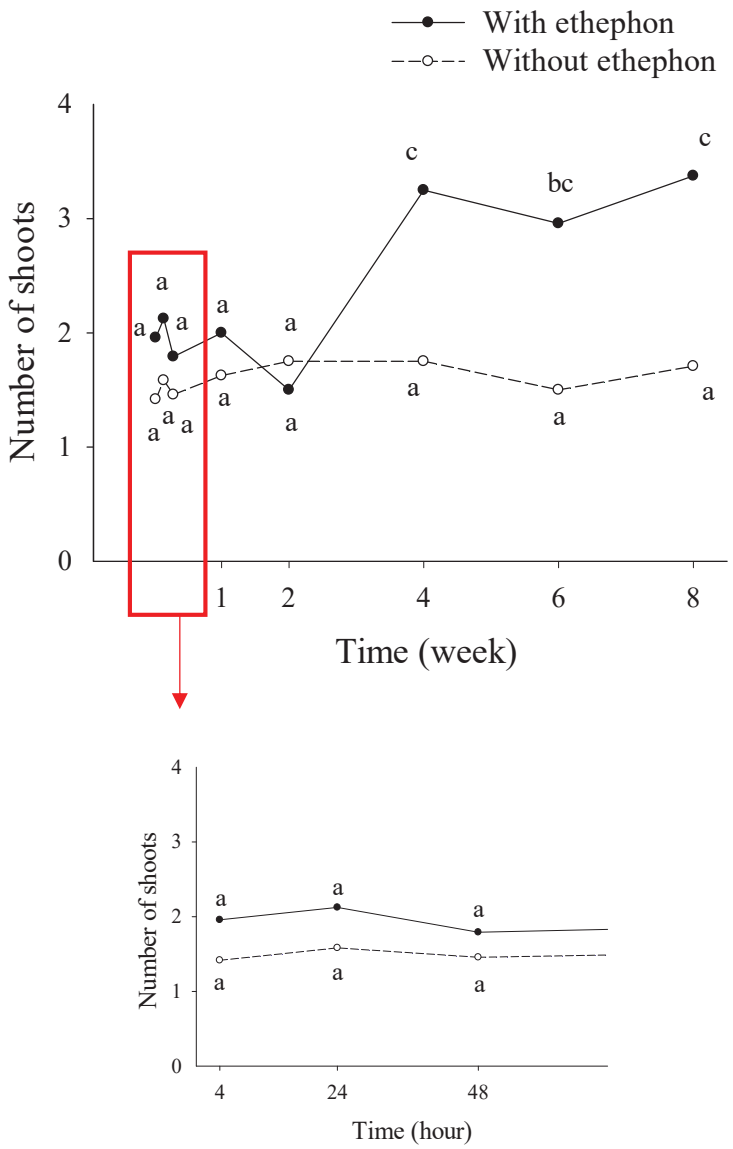


Figure 6-5: Number of 2° shoots produced by the explants of gentian 'Little Pinkie' after 8 weeks growth *in vitro* when exposed to different medium with or without ethephon for different durations (**A:** 1, 2, 4, 6 and 8 weeks, red box represent data before week 1) and (**B:** 0, 4, 24 and 48 hours). Mean comparison was done using the alternative LSD test where the Studentized Range statistic was used instead of Student's t test.. Means followed by different letters within each graph and between graphs were significantly different at $P \leq 0.05$, n = 4 vessels each containing 8 explants.

There are some reports in the literature that indicated the importance of the timing and duration of ethylene/ethephon application on the physiological effect, which is in line with the results achieved in the current experiment. The effective duration of exposure to ethephon/ethylene has been ranged between 3 days of culture to enhance the production of adventitious buds on bulb-scale explants of *Lilium* (van Aartrijk et al., 1985b) to about two weeks on *Petunia hybrida* L. (Dimasi-Theriou et al., 1993) and *Pinus radiata* (Kumar et al., 1987). It is likely that the duration of exposure and/or concentration of ethephon/ethylene applied in various studies might differ depending on the plant species, tissue and culture system.

Similar to all previous experiments in this thesis, which included explants from various positions along the donor shoot, regardless of application of ethephon, the number of shoots produced by the explants was also affected by the position ($P \leq 0.05$) (Table 6-2). As the distance of the position increased from the tip, the number of 2° shoots produced by the explants increased (Figure 6-6). Explants of Node 2 position produced 3.4 times more 2° shoots compared to the Tip explants, which produced a single primary shoot in the absence of ethephon. Likewise, in the presence of ethephon there was a 7.7-fold increase in the number of 2° shoots developed from the explants of the Node 2 position compared to the Tip explants. Furthermore apart from the explants of the Tip position, explants of all other nodal positions produced more 2° shoots in the presence of ethephon compared to the absence of ethephon (Figure 6-6).

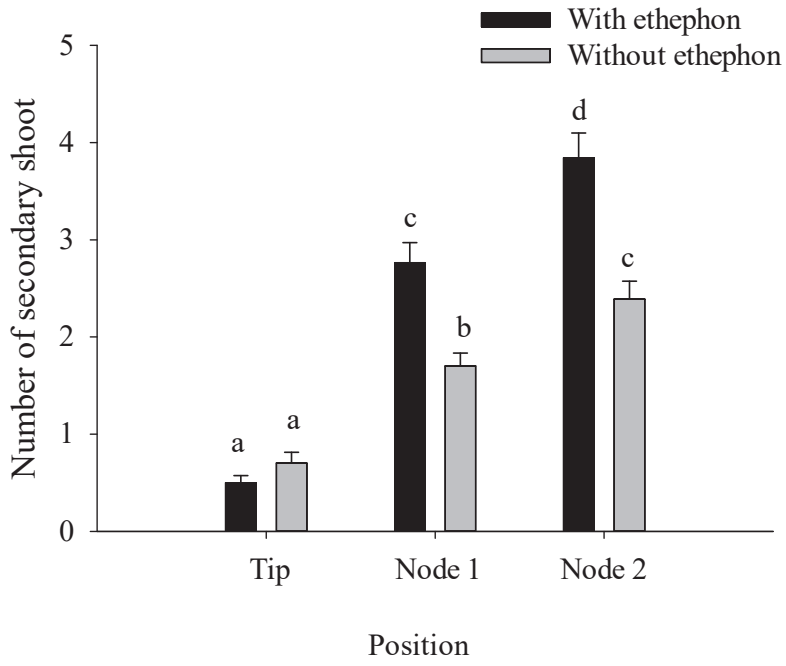


Figure 6-6: Number of shoots (1º for Tip or 2º for Node 1 and Node 2) produced by the explants of gentian 'Little Pinkie' originating from different positions along the shoots of the donor plantlet: tip (Tip), first node below the tip (Node 1) and second node below the tip (Node 2), when exposed to medium either with or without 10 $mg.L^{-1}$ ethephon after 8 weeks growth *in vitro*. Mean comparison was done using the alternative LSD test where the Studentized Range statistic was used instead of Student's t test. Vertical lines represent + standard error of means. Means followed by different letters between bars were significantly different at $P \leq 0.05$, n = 4 vessels each containing 8 explants.

6.3.4. Conclusion

Numbers of 2° shoots developed from the explants of gentian 'Little Pinkie' in the absence or presence of ethephon started to significantly differ from each other at four weeks of culture. Therefore, minimum duration which explants needed to be exposed to ethephon in order to

establish changes in PAT was probably two weeks to then show the significant increase in number of 2° shoots, as compared with those in medium without ethephon at four weeks.

6.4. Experiment 3 - Ethephon/ethylene on transport and metabolism of auxin in explants of two gentian cultivars cultured *in vitro*

6.4.1. Introduction

The effect of ethylene, as the most probable effective component from the decomposition of ethephon (Yang, 1969; Goudey et al, 1987a; Biddington, 1992; Marin et al., 2006), on shoot formation in explants of gentian 'Little Pinkie', seem to be not directly but through a cross-talk with other plant growth regulators especially auxin. Inhibition of ethylene on auxin transport has been suggested to be the mechanism by which ethylene affects the concentration of auxin in plants (Goldsmith, 1977). As an example, bud formation in bulb scale explants of *Lilium speciosum* increased following treatment with ethylene and was suggested to be due to the inhibition of auxin transport and probably changing auxin concentration (van Aartrijk and Blom-Barnhoorn, 1983; van Aartrijk and Blom-Barnhoorn, 1984; van Aartrijk et al., 1985a; van Aartrijk et al., 1985b).

Transport of auxin has been reported in the past to be polar from tip toward the base of the plant (basipetal) (Went, 1941; Goldsmith, 1977; Hasenstein and Evans, 1988; Luthen, 2015). Polar auxin transport (PAT) has been investigated by comparing transport in segments provided with radiolabelled auxin in an acropetal (base toward the tip) versus basipetal (tip toward the base) direction (Goldsmith, 1977; Marks et al., 2002; Luthen, 2015). If applicable to the current plant system, it was considered likely that ethylene would affect shoot formation through metabolising auxin to other auxin metabolites such as indoleacetylaspartic acid and many other metabolites in the tissue (Beyer and Morgan, 1970; Ernest and Valdovinos, 1971; Vijayaraghavan and Pengelly, 1986). However, evidence for the effect of ethylene on auxin concentration, metabolism or movement *in vitro* is limited and, especially, there was no report within the literature on the transport and metabolism of auxin in explants of gentian. Hence, Experiment 3 presented in this chapter combined refined methodologies from above so as to study the hypothesis that ethephon/ethylene increased 2° shoot in explants of gentian 'Little Pinkie' cultured *in vitro* via affecting auxin transport and/or metabolism.

The difference in shoot formation response between the cultivars 'Little Pinkie' and 'Showtime Diva' (refer to Chapters 3 and Appendix 13) could also be attributed to their differences in auxin transport, if the hypothesis that ethephon/ethylene would affect shoot formation through the inhibition of auxin transport was correct. As one of the necessary factors controlling the cell differentiation and development of any plant organ, is a proper and timely supply of auxin to the tissue (Rubery and Sheldrake, 1974; Friml, 2003; Petrasek et al., 2006), then not only metabolism, but also transport of auxin to target tissues may be involved. Hence, it was hypothesised that the difference in the response between the two cultivars to ethephon was related to their difference in the metabolism and/or transport of auxin to the target tissue (i.e. nodes).

In the current experiment, feeding explants of both gentian cultivars with radiolabelled auxin (2-^{14}C-indole-3-acetic acid; ^{14}C-IAA) was used to test the hypothesis (refer to Section 6.3.2). Prior to the application of radiolabelled auxin explants were treated with either ethephon or the auxin transport inhibitor NPA, so as to inhibit PAT. Both chemicals were applied within the agar medium at the concentration (NPA at 100 µM or ethephon at 70 µM) and duration (two weeks) reported to be effective in the previous experiments (refer to Chapter 1 and Section 6.1 and 6.2; Experiments 1 and 2). ^{14}C-IAA was supplied to the apical or basal ends of plant segments, and the basipetal and acropetal transport through the plant segment and into the receiver agar block was determined (Refer to Section 6.3.2). Additionally however, in order to determine what if any metabolism of IAA had occurred within the plant, reverse phase, high pressure liquid chromatography (RP-HPLC) was used to separate and quantify IAA and IAA metabolites (Barkawi et al., 2008; van Hooijdonk, 2009; Nakurte et al., 2012). For this experiment, therefore, the specific objectives were to quantify the metabolism and transport of auxin in explants:

- pre-treated with ethephon or NPA compared to no pre-treatment (control) and,
- of two cultivars of gentian; 'Little Pinkie' versus 'Showtime Diva'.

6.4.2. Materials and methods

6.4.2.1. Pre-treatments before application of ^{14}C-IAA

The base medium (PGR-free) was prepared as detailed in Chapter 2. Additional pre-treatment media were created by either ethephon (10 mg.L^{-1} = 70 µM) or NPA (100 µM), being added

to the medium by microfiltration, after being autoclaved. Each culture vessel contained 50 mL of these pre-treatment medium.

Plantlets of the gentian 'Little Pinkie' and 'Showtime Diva', previously grown and sub-cultured *in vitro* over several five-week cycles, were used as the source material of explants for this experiment. Considering the influence of nodal position on vegetative growth (refer to Chapter 2, Section A), all explants for this experiment were taken from the same position i.e. second node below the tip (Figure 6-7), with an internode length a little longer than previous experiments (at least 1.5 cm) to facilitate the application of radioactivity.

Chemical pre-treatments comprised the control (i.e. base medium), final concentrations of 70 µM ethephon or 100 µM NPA. There were six vessels of each chemical treatment containing eight explants each, so as to allow for possible losses due to contamination (refer to Section 6.1). A final number of four vessels were used to determine auxin transport and metabolism, after explants had been exposed to chemical pre-treatments for two weeks (refer Section 5.2; Experiment 2).

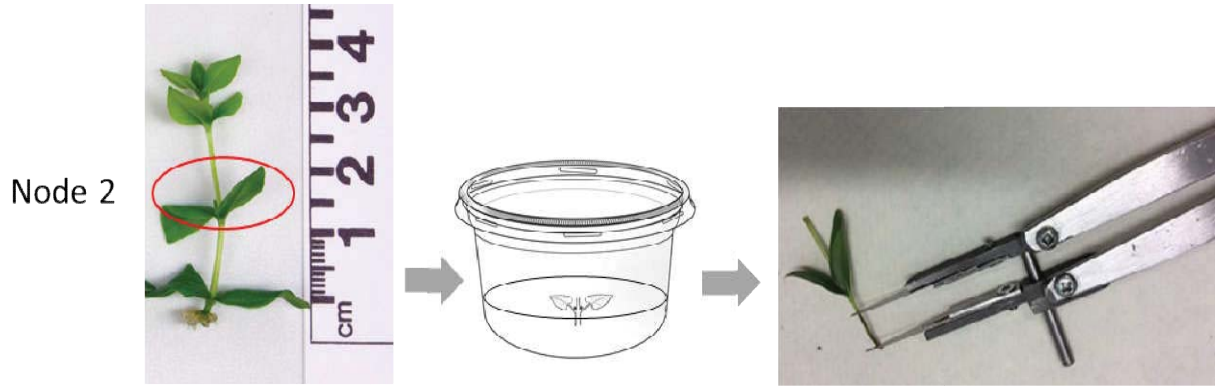


Figure 6-7: Explants were all taken from only Node 2 (second node below the tip), cultured in pre-treatment medium for two weeks and then before application of radioactivity, 10 mm length of bottom internode was separated by means of a double blade cutter to achieve uniform length.

6.4.2.2. Designing the system for application of radioactive auxin

The system designed for application of ^{14}C-IAA (250 uCi total radioactivity, specific activity 55 mCi/mMol, 0.1 mCi/mL, MW = 175.19) comprised a donor and receiver agar block placed at either end of the explant (Figure 6-8; (Burg and Burg, 1967; Goldsmith, 1977; Daniel et al., 1989)). Based on the general protocol described by Burg and Burg (1967) for determining basipetal transport, ^{14}C-IAA in the donor agar would enter the distal (true apical end; furthest from the stem-root junction of the plant) end of the stem, travel upwards against gravity through the stem, and finally reach to the receiver agar at the proximal (nearest to the

stem-root junction of the plant) end of the stem. As detailed within Figure 6-8, modifications to the basic method were done to develop a system usable for small explants cultured *in vitro*.

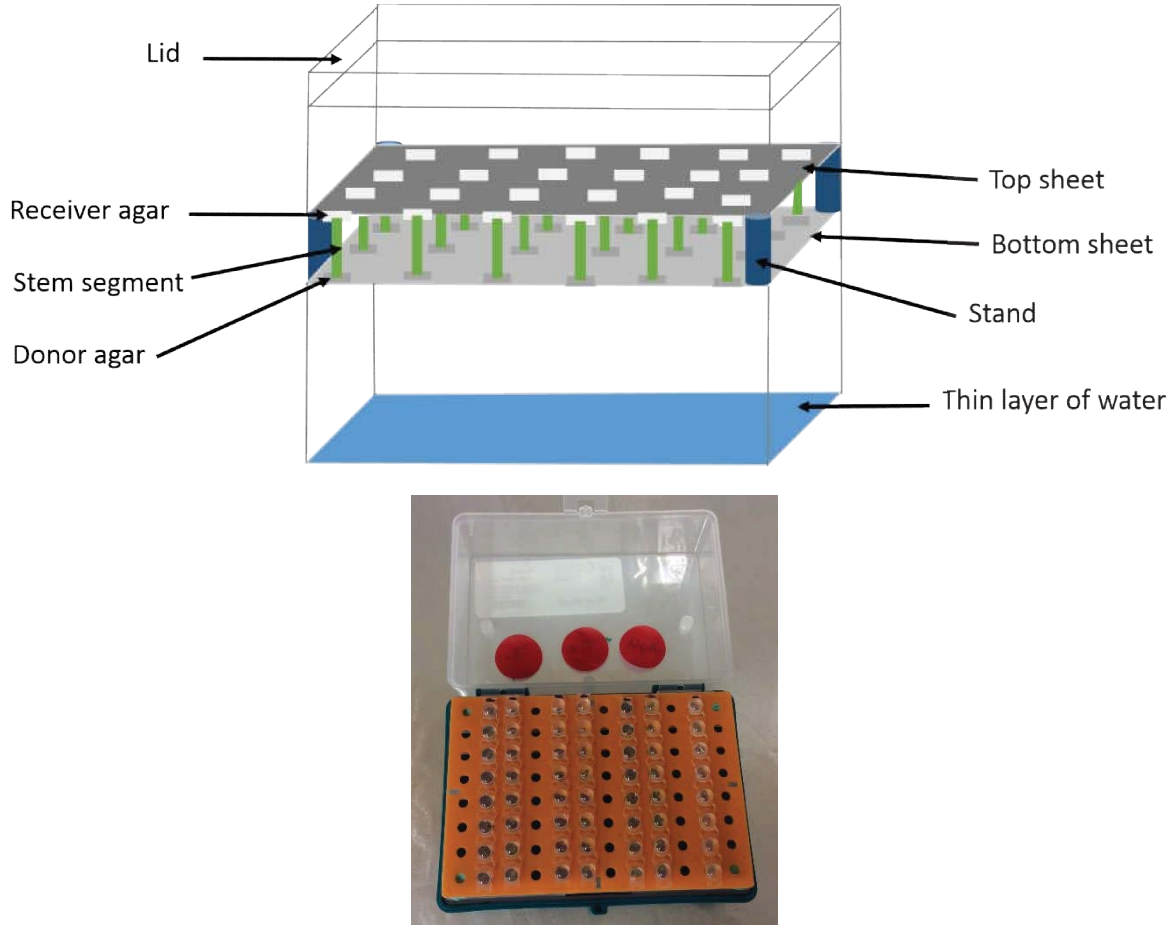


Figure 6-8: System designed and used for ^{14}C-IAA transport in stem segments of gentian cultured *in vitro*. Diagrammatic illustration at top and photograph below. The system was included within a plastic box (150×100×50 mm) with lid. A thin layer of water at the bottom of the box provided moisture so as to minimise desiccation. Plastic sheets (top and bottom) were installed in the middle of the box (8 mm away from each other) to make the stand for holding the receiver and donor agar blocks in position. Stem segments located between two sheets, with their proximal end inserted into the receiver agar and the distal end into the donor agar. The top sheet was a sheet with holes in it which stem segments could pass through to make contact with the blocks of donor and receiver agar (see also Figure 6-9).

6.4.2.3. Application and extraction of ^{14}C-IAA

The purity of the radiolabelled auxin was checked chromatographically prior to use and contained only one very small contaminant peak, so was not further purified. To prepare the donor blocks the agar solution (3%) was kept warm at 50 °C on a heater with magnetic stirrer to prevent solidification. The indented lids of 1.5 mL Eppendorfs, with a maximum capacity of 70 µl, were utilised to produce blocks of donor and receiver agar. Fifty µl of liquid agar was pipetted into each small Eppendorf lid. To prepare the donor agar; for each cultivar a 4 mL solution containing 26000,000 dpms (disintegrations per minute) of ^{14}C-IAA was mixed into warm (30 ºC) liquid solution of agar (9 mL). Fifty µl of the agar solution pipetted into the small eppendorf lids to make the donor block of agar, resulting in each block of donor

agar containing ~ 100,000 dpm. Eppendorf lids containing donor agar blocks were fixed on the bottom sheet installed in the middle of the container (Figure 6-8). The second sheet which was fixed above the first one was a sheet with holes in through which stem segments could pass to contact with the receiver agar. This orientation resulted in basipetal transport occurring against gravity, so as to prevent gravity-driven contamination. The distance between the top and bottom sheet was about 8 mm, which was a little less than the total length of the stem segment (10 mm), to allow the tip and base of the stem segment to make good contact with both the donor and receiver agar blocks. The top sheet held the stem vertically after being inserted into the agar block. In addition to the orientation described above, with the apical end at the bottom of the system, some stem segments were also inverted to determine the amount of acropetal transport occurring in the system.

Once the system setup was ready to start the application of ^{14}C-IAA to the explants, which had been pre-treated either with ethephon or NPA for two weeks, explants were initially taken out of their pre-treatment culture vessels. In order to use in the transport study, the lower internode stem segment (10 mm length) was separated from the rest of the explant (node and leaves together with the upper internode segment) to be used for radioactive treatment. A fresh re-cut was done at the bottom of the stem which had been in contact with the original pre-treatment medium during the period of cultivation prior to treatment with ^{14}C-IAA. In order to cut the stems and achieve stem segments of uniform length, a double blade cutter was used (Figure 6-7). Since auxin transport is directional, and in order to orientate stem segments in the right direction, the proximal end of the stem segment (closer to the base of original explant, which would be in contact with receiver agar) was marked before the segment was separated from the rest of the explant. Cutting the segments was done only at the time of ^{14}C-IAA application, in order to avoid developing a dry surface at the top and bottom of the cut stem. In addition, moisturized paper towels were used to keep the stem segments moist before being inserted into the transport system. Between establishing each treatment, razor blades were wiped using ethanol.

As mentioned, stem segments were orientated so as transport was always against gravity. For determining basipetal transport, the distal end of the stem for each cultivar treated with each chemical treatment was inserted into an individual block of the donor agar installed at the bottom sheet and pushed in a little to ensure good contact with the agar. In order to calculate the non-specific acropetal transport of IAA, some stem segments of each cultivar and each chemical treatment were placed in the reverse direction, with their proximal end inserted into

the donor agar at the bottom, instead of their distal end. Individual receiver blocks were then pressed on the other end (upper with respect to gravity). Therefore, for samples orientated in basipetal direction, bottom part was the distal end of the stem (closer to the true apex) and top part was the proximal end of the stem (further from the true apex). For samples in acropetal direction, bottom part was the proximal end of the stem and the top part was the distal end of the stem.

The treatment associated with the position of each group of 16 stem segments in basipetal direction in each of three containers in a split plot design was recorded. Eight stem segments for each treatment in acropetal direction were also included in each container. All stem segments were checked again for good contact with the agar blocks. Container lids were closed and wrapped with polyethylene cling film (Glad Wrap™) in order to maintain humidity inside the container, so as to minimise desiccation of the stem segments. Since IAA would be destroyed by light (Leopold, 1964), all three containers were covered using tin foil and kept in a dark incubator at 28 °C during the period of IAA transport .

The likely duration needed for transport of radioactivity was investigated in a test experiment prior to the main experiment, with a duration of four hours determined to be satisfactory for the radioactive transport in the main experiment (Appendix 8). Once the transport time (four hours) finished, stems were taken out and blotted dry so as to remove potential ^{14}C-IAA in the agar attaching to the outside of stem tissue (especially the part which had been in contact with the donor agar). A new piece of paper tissue was used for cleaning each time between samples in order to prevent cross contamination with ^{14}C-IAA. Every four blocks of agar (either receiver or donor) from the same treatment were combined into individual scintillation vials. Stem segments were cut into upper and lower 5 mm segments using a razor blade as illustrated in Figure 6-9. Razorblades were changed for each treatment and cleaned by wiping from sample to sample using paper tissue and a diluted solution (2%) of DECON 90 (Decon laboratories Ltd., England) that contains 0.5 to 2% potassium hydroxide (KOH) (MSDS specifications). Different parts of the stem segments went into different scintillation vials (e.g. four pieces of the stem taken from the most distal end into one vial).

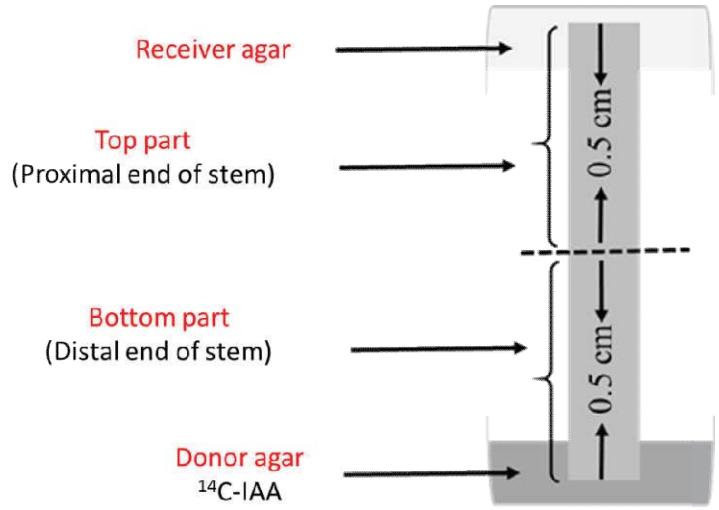


Figure 6-9: Schematic illustration representing the system used for transport of radiolabelled auxin including donor agar at the bottom, receiver agar on the top, stem segment with 10 mm length. Stem segment located in the middle in a reversed direction to gravity, with the proximal end on the top part and distal end at the bottom part of the transport system. Radiolabelled auxin was applied in donor agar. Dashed line represent where the cut was done to divide the stem to two segments each 5 mm length. For acropetal transport the orientation of the segment was reversed.

Radiolabelled auxin, or its metabolites, which had been transferred into the receiver agar, accumulated into the plant segments or remained in the donor agar were extracted by adding 3 mL 80% methanol to each vial. Vials were sealed and placed on a shaker at 20°C in darkness for 24 h to extract the radioactive compounds. As shown in a pilot experiment to optimise the efficiency of extraction (Appendix 9), there was no difference between extracting intact piece of stem tissue 1 mm in diameter, as compared to first grinding the tissue; intact tissue was used for the extraction step. From the total volume of the extract (three mL) one mL was used to investigate metabolism and the rest (two mL) for the transport study.

6.4.2.4. Metabolism of ^{14}C-IAA

A gradient of HPLC grade acetonitrile and trimethylamine (TEA) (40 mmol L^{-1} acetic acid solution adjusted to pH 3.38 with TEA) was used for separation of IAA and its various metabolites as previously reported by (van Hooijdonk, 2009). Solvents were prepared prior to each sample run, filtered through a 0.2 µm Teflon filter (Phenomenex®, PHENEX™, NZ.) and degassed in a sonicator (S 100H Elmasonic, Total Lab Systems Ltd. NZ.) for 30 minutes degassed. Delivery of the solvent to the column (220 x 4.6 mm octadecyl silica (ODS) column (Biosystems Inc., Brownlee (part no.0711-0023, Spehri-5), RP-18, C18) was by using a Waters™ 501 and a Waters™ 510 HPLC pump controlled by a Waters™ automated gradient controller.

Samples (one mL) which had been taken from the original extract of receiver agar, as well as the upper and lower segments of the stem and donor agar after the transport period, were prepared to be injected into the RP-HPLC. Samples of each of the two replications were combined together to achieve two mL of extract. Extracts then were filtered and reduced to dryness in vacuo, re-suspended in 150 µL of 80% TEA: 20% acetonitrile, shaken for 15 seconds to be fully dissolved, and then degassed by sonicating for 5 minutes. Before injection of any samples to HPLC, the retention time of IAA was obtained by injecting 20 µl of a standard solution of IAA containing100 ng IAA (Sigma-Aldrich, Germany).

Fractions of each 50 µL sample injected into the RP-HPLC were collected in vials, starting at 48 seconds after injection. Determination of suitable time intervals between fractions was investigated as detailed in Appendix 10. After extraction, 10 mL of scintillation fluid (Ultima Gold, PerkinElmer, USA) was added to each vial, shaken well to give a clear solution and stored in darkness at 21 °C for 24 hours before the measurement. A scintillation counter (Tri-Carb 2900TR, PerkinElmer, USA) was used to determine the amount of radioactivity as disintegrations per minute (dpm) in each sample. The scintillation counter detected and measured beta radiation derived from the ^{14}C-IAA. It was important to prevent the scintillant solution including samples, from being exposed to visible light for at least 12 hours before measurement, in order to increase the accuracy in measurements by allowing chemiluminescence to decay. The percentage of total radioactivity of each collected fraction was calculated from the total amount of radioactivity recovered and counted from each sample injected into HPLC, plotted against retention time and compared with the retention time of IAA, in order to investigate the metabolism of ^{14}C-IAA in various parts of the system.

6.4.2.5. Transport of ^{14}C-IAA

10 mL of scintillant was added to the extract in each vial, shaken, stored in the dark overnight and then counted. There were six and two replications (each comprised of 4 segments), respectively, used for determining basipetal and acropetal transport of each chemical treatment, for each cultivar. Number of dpm per 2 mL of the extract for each replication were counted for various parts of the transport system (receiver agar, top part, bottom part and donor agar) in both directions (basipetal and acropetal) for each cultivar, and calculated for 3 mL to achieve the amount of dpm in each 4 segments. Recorded variables were the radioactivity derived from ^{14}C-IAA that:

- had been transported and counted in the receiver agar,

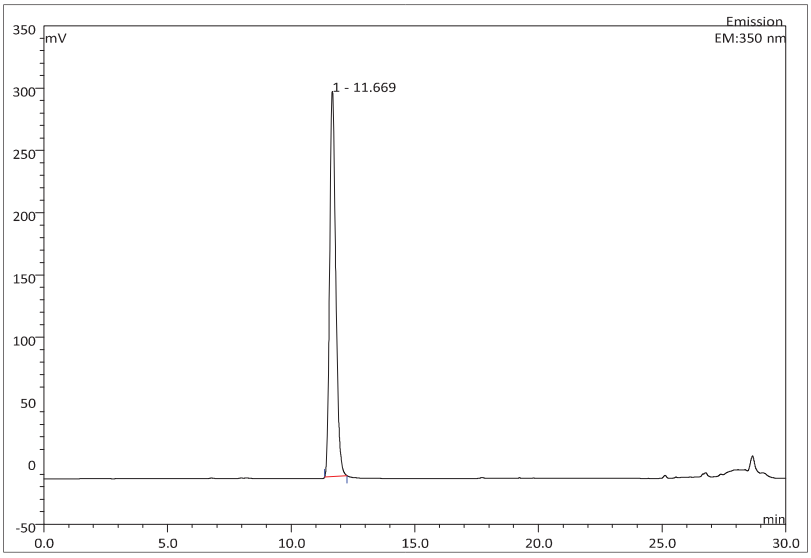
- had been transported in the upper and lower segments of the stem and,
- remained in the donor agar after the transport period.

The quantity of radioactivity which remained associated with IAA was calculated from the amount of radioactivity retrieved and counted for each transport part of each cultivar treated with various chemical treatments in each direction. To calculate the radioactivity remaining associated with IAA, the data achieved from the peak at retention time of IAA for each sample was used (refer to Section 6.4.2.4). The quantity of IAA metabolites for each transport part of each cultivar treated with various chemical treatments in each direction was calculated from the difference between total radioactivity retrieved, and the radioactivity remaining associated with IAA.

Analysis of variance on the data was done using general linear model in Genstat software (version 17, 2014, VSNi Ltd, Hemel Hempstead, UK). Factors used in the analysis were cultivar ('Showtime Diva' and 'Little Pinkie'), chemical treatment (control, inclusion of ethephon or NPA in the medium) and the transport (acropetal or basipetal). A split plot design was used for the analysis, with individual plants as the main plots, and parts as the subplots; cultivar, chemical treatment and the transport were main-plot factors, plant stem parts and the interactions with cultivar and chemical treatment were subplot factors. Log transformation was done on the data in order to make the variability of the data from various treatment combinations more equal. Means comparison was based on Fisher's protected least significant difference test where the Studentized Range statistic is used instead of Student's t test at $P \leq 0.05$ and is equivalent to Tukey's test.

6.4.3. Results

Retention time for a standard solution of IAA injected to HPLC required to study the metabolism of ^{14}C-IAA was 11 minutes and 48 seconds (11:48) after the injection, as represented by the peak in Figure 6-10. The peak for IAA in this standard solution of IAA was used as described next, to compare with the results of the metabolism of radioactivity as affected by the direction of transport and chemical treatments.



No.	Ret.Time	Peak Name	Height	Area	Rel.Area	Amount
	min		mV	mV*min	%	
1	11.67	n.a.	309.151	88.120	100.00	n.a.
Total:			309.151	88.120	100.00	0.000

Figure 6-10: Retention time of IAA in a 20µl injection of a standard solution of IAA represented as a peak.

The distribution of fractions over their retention time for each part of the transport system (receiver and donor agar as well as top and bottom parts) illustrated in Figures 6-11 to 6-14, showed IAA and IAA metabolites which were used to study the direction of radioactivity transport. The peak at retention time of 11:48 which was the similar retention time for IAA, illustrated by red colour in Figures 6-11 to 6-14, represented IAA peak. Other peaks illustrated in black, represented other metabolites of IAA. The peak at 2:48 min. which was labelled for its percentage of the total radioactivity in the following figures of this section and discussed later, was an example of the metabolites (referred to as metabolite X here) which constantly appeared in all samples and, therefore, was considered likely to be involved in the different shoot formation response of cultivars (Figures 6-11 to 6-14). Compare to the standard solution of IAA, control samples of 'Little Pinkie' oriented to study basipetal transport, contained IAA peak in all parts of the system including receiver and donor agar and stem segments (top and bottom parts). Therefore, while some radioactivity in the form of IAA still remained in the donor agar, it also was transported through the stem segment to the receiver agar as expected. In contrast, in control samples oriented to study acropetal transport

in 'Little Pinkie', transport of radioactive IAA was not expected and did not occur as evident by very small IAA peaks in all parts of the transport system except for the donor agar (top and bottom parts and receiver agar; Figure 6-12). IAA metabolites including metabolite X were observed in both basipetal and acropetal directions.

The percentage of total radioactivity that resided in IAA was 36% for the receiver blocks, but only 4% for the top part for basipetal transport. In contrast for acropetal transport IAA was only 3% in the receiver block and 3% in the top part. Respective figures for an unidentified metabolite (metabolite X), were 5% and 35% in the basipetal direction, and 34% and 15% for the acropetal direction, showing huge contrasts with both the IAA figures and with direction of transport.

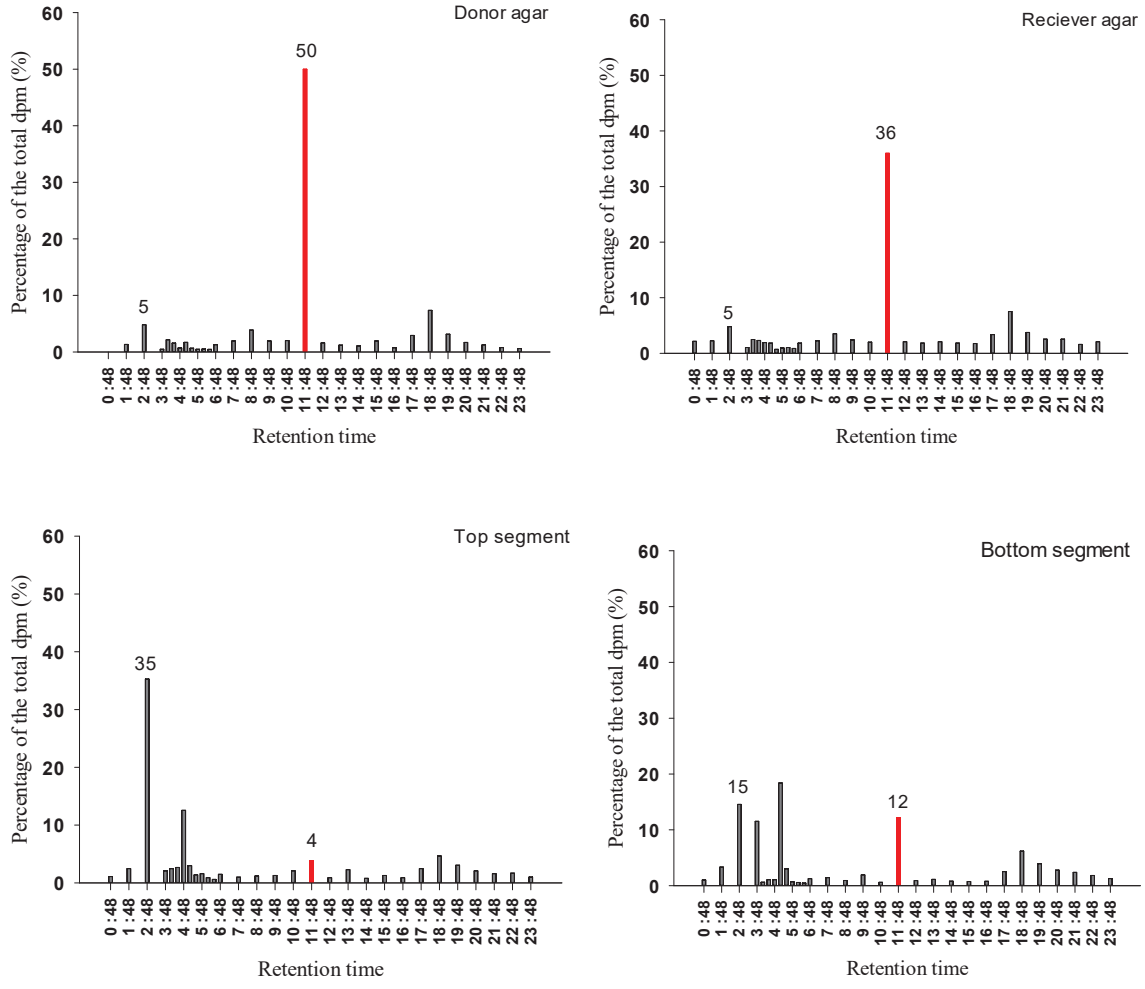


Figure 6-11: Collected fractions (percentage of each fraction from the total radioactivity recovered and quantified) of a 50 µl solution injected into HPLC, contained ^{14}C-IAA extracted from samples been exposed to radioactive IAA for 4 hours, taken from various parts of the **basipetal** transport system i.e. donor and the receiver agar, top part and bottom part for control explants of **'Little Pinkie'** over their retention time. Major red peak represented the percentage of total ^{14}C-IAA which was in the form of IAA as it was at the similar retention time as the standard solution of IAA. Black peaks represented IAA metabolites, with metabolite X labelled to show its percentage of the total radioactivity.

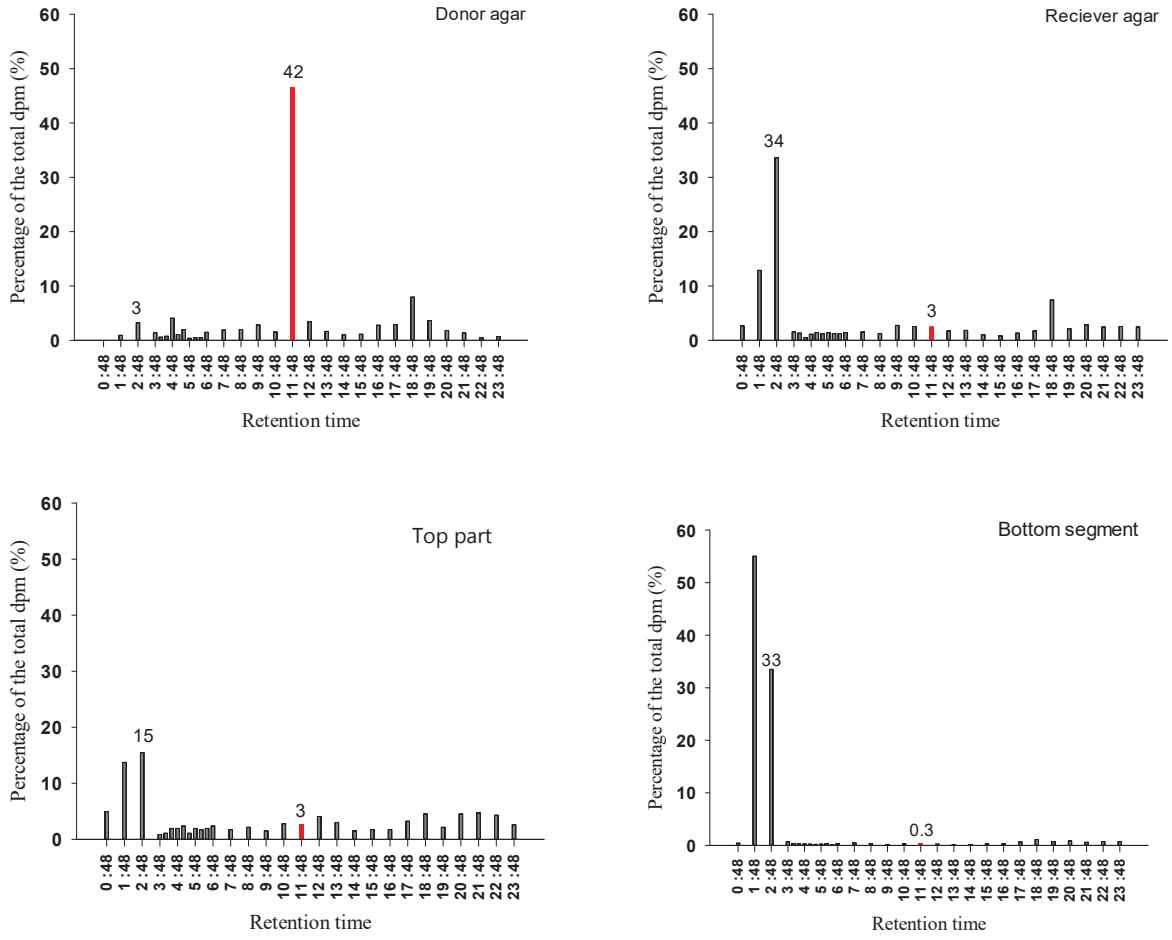


Figure 6-12: Collected fractions (percentage of each fraction from the total radioactivity recovered and quantified) of a 50 µl solution injected into HPLC, containing ^{14}C-IAA extracted from samples been exposed to radioactive IAA for 4 hours, taken from various parts of the **acropetal** transport system i.e. donor and the receiver agar, top part and bottom part for control explants of **'Little Pinkie'** over their retention time. Major red peak represented the percentage of total ^{14}C-IAA which was in the form of IAA as it was at the similar retention time as the standard solution of IAA. Black peaks represented IAA metabolites with metabolite X labelled to show its percentage of the total radioactivity.

The amount of IAA present and the extent to which it was metabolized showed differences with both cultivar ('Little Pinkie' or 'Showtime Diva') and chemical treatment (control, ethephon or NPA) (Figures 6-13 & 6-14). Those differences associated with IAA are most easily compared in receiver blocks, and as expected for basipetal direction was always more than that for acropetal direction (Table 6-3). IAA was a low percentage of total radioactivity in the top and bottom parts for 'Little Pinkie' with NPA but unexpectedly not with 'Showtime Diva'. However, ethephon, greatly reduced the percentage of IAA in the bottom part for 'Little Pinkie' (12% to 4%) but not in 'Showtime Diva' (26% and 30%). It was interpreted that Metabolite X, might be involved in shoot formation as evident by the differences noted in association with the treatments and cultivars. Metabolite X was high in the top part for 'Little Pinkie' control but decreased with ethephon treatment and even more with NPA treatment (35, 23 and 9% respectively). In contrast Metabolite X was greatly

reduced in the top part for 'Showtime Diva' compared to 'Little Pinkie' (Figure 6-13 & Figure 6-14).

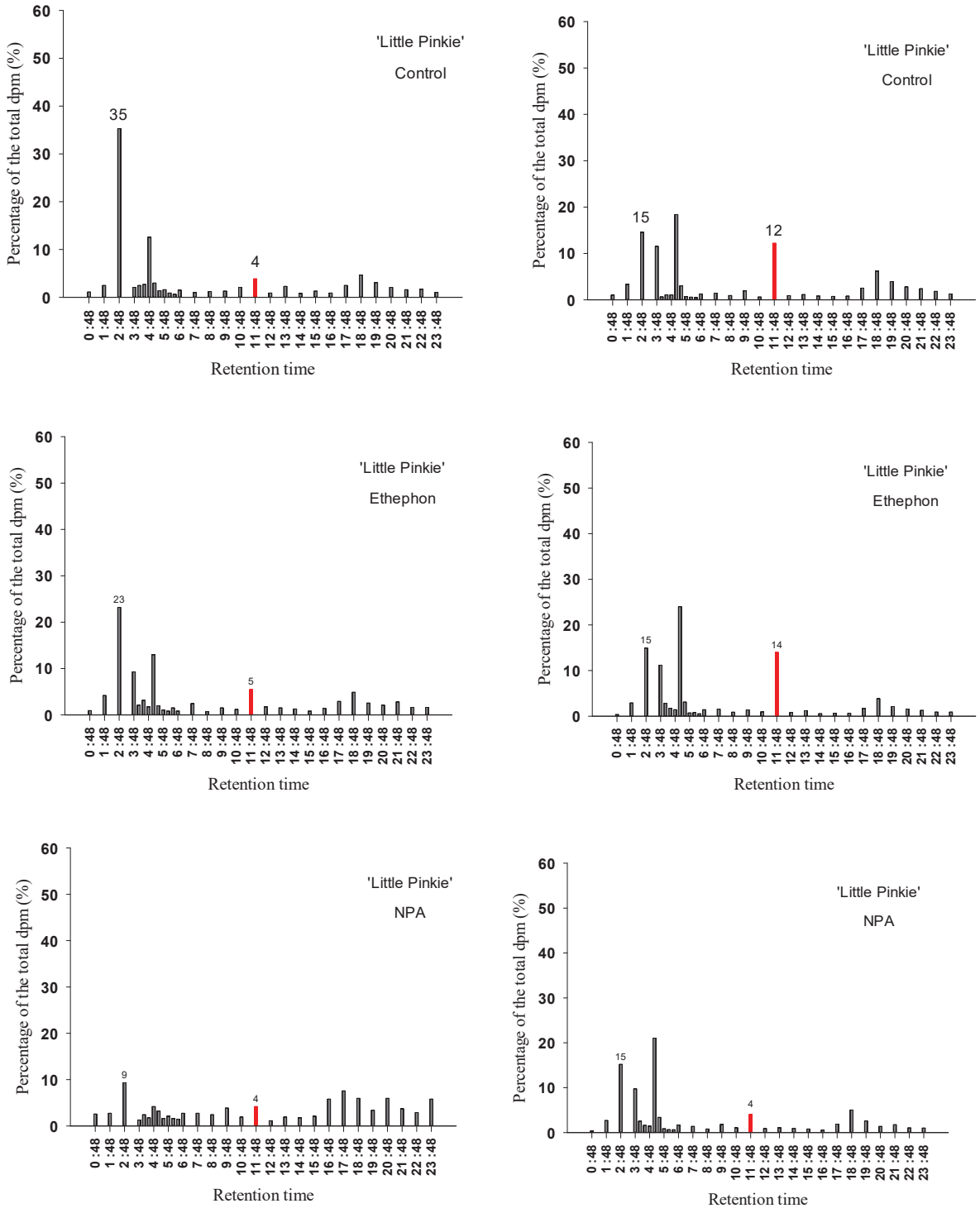


Figure 6-13: Collected fractions (percentage of each fraction from the total) of a 50 µl solution injected into HPLC, contained ^{14}C-IAA extracted from samples exposed to radioactive IAA for 4 hours, taken from top and bottom parts for 'Little Pinkie' either as control, treated with ethephon or NPA over the retention time. Black peaks represent IAA metabolites.

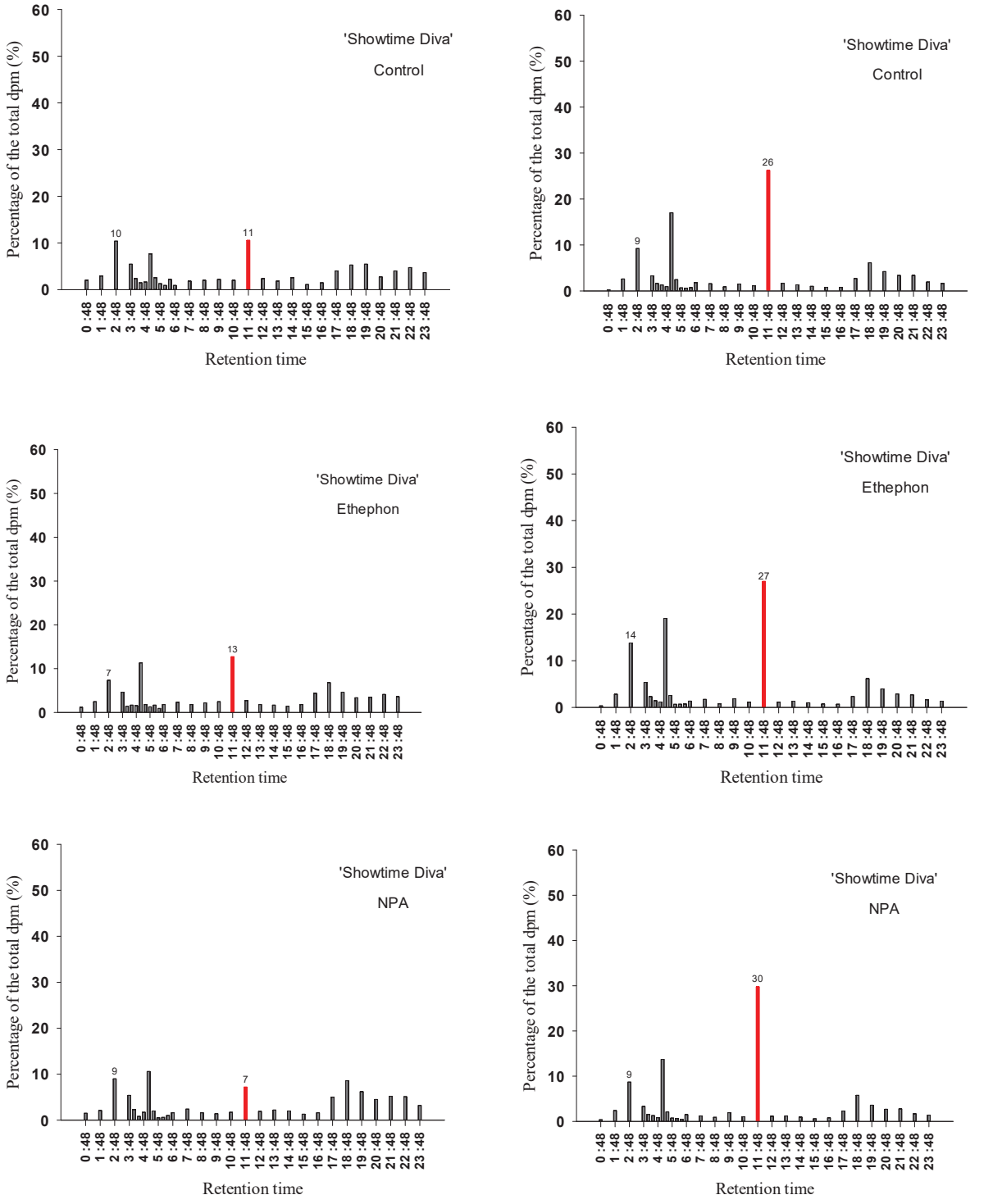


Figure 6-14: Collected fractions (percentage of each fraction from the total) of a 50 µl solution injected into HPLC, contained ^{14}C-IAA extracted from samples exposed to radioactive IAA for 4 hours, taken from the top and bottom parts for 'Showtime Diva' either as control, treated with ethephon or NPA over the retention time. Black peaks represented IAA metabolites, with Metabolite X percentage shown above its retention time bar.

Table 6–3: Percentages of the ^{14}C-IAA from the total radioactivityfound in each plant segment or agar blocks (receiver agar, top part, bottom part and donor agar) after 4 hours of application at basipetal or acropetal directions, for two cultivars of gentian ('Showtime Diva' and 'Little Pinkie') pre-treated with various chemical treatments (ethephon or NPA in their medium or control).

Chemical treatment	**Transport part**	**'Showtime Diva'**		**'Little Pinkie'**	
		IAA basipetal (%)	**IAA acropetal (%)**	**IAA basipetal (%)**	**IAA acropetal (%)**
Control	Receiver agar	47	8	36	2
Control	Top part	13	9	4	3
Control	Bottom	26	1	12	2
Control	Donor agar	48	42	50	47
Ethephon	Receiver agar	41	1	36	1
Ethephon	Top part	11	5	4	2
Ethephon	Bottom part	17	1	14	1
Ethephon	Donor agar	42	52	48	65
NPA	Receiver agar	47	1	35	1
NPA	Top part	7	2	5	3
NPA	Bottom part	30	2	15	1
NPA	Donor agar	56	43	49	61

With regard to IAA transported into the receiver blocks (Table 6-3), in the basipetal direction 47% of the radioactivity was still IAA in the control treatment of 'Showtime Diva', and neither ethephon nor NPA seemed to have much effect on this percentage. Radioactivity in the form of IAA for 'Little Pinkie' was less than for 'Showtime Diva' and, in acropetal direction (2 and 8%), far less than in basipetal direction (36 and 47%) for both cultivars.

After four hours of transport, there was a trend in the quantity of radioactivity decreasing from donor agar toward the bottom and top parts (Table 6-4). The amount of transported radioactivity in both directions into the receiver agar was interestingly more than that transported into the top parts for both cultivars, which could be due to accumulation over time. Total radioactivity lost in acropetal and basipetal directions with 'Showtime Diva' was respectively 1.5 and 1.2 times less than in 'Little Pinkie'. Chemical treatment did not seem to affect the radioactivity lost.

Table 6–4: Radioactivity transported and total retrieved or lost after 4 hours of application from initial amount of ~ 400,000 dpm ^{14}C-IAA in various parts of the transport system (receiver agar, top part and bottom part and donor agar) in basipetal or acropetal direction, for two cultivars of gentian ('Showtime Diva' and 'Little Pinkie') pre-treated with various chemical treatments (ethephon or NPA in their medium or control) n=6 (basipetal) or 2 (acropetal).

Chemical treatment	Transport part	'Little Pinkie'		'Showtime Diva'	
		Transported dpm basipetal	Transported dpm Acropetal	Transported dpm Basipetal	Transported dpm Acropetal
Control	Receiver agar	3312±443	2786±206	4765±614	6845±171
Control	Top part	1913±87	503.25±59	1133 ±170	539±61
Control	Bottom part	15823±811	19989±1484	12693±832	10429±1378
Control	Donor agar	223807±2055	220182±1985	253376±3088	276433±6034
Total retrieved		**244856 ± 2458**	**243460 ± 236**	**271968 ± 3253**	**294246 ± 7644**
Total lost		**155144 ± 2458**	**156540 ± 236**	**128033 ± 3253**	**105754 ± 7644**
Ethephon	Receiver agar	5601±1676	8170±1126	3543±564	3849±573
Ethephon	Top part	2382±455	1075±301	1262±180	283±93
Ethephon	Bottom part	15131±658	20845±2895	13266±290	11750±568
Ethephon	Donor agar	220403±4633	218577±4182	263750±3181	249962±3665
Total retrieved		**243517 ± 6303**	**248668 ±2111**	**281821 ± 3055**	**265845 ± 2432**
Total lost		**156483 ± 6303**	**151332 ± 2111**	**118179 ± 3055**	**134155 ± 2432**
NPA	Receiver agar	1999±623	6207±328	5310±431	4752±318
NPA	Top part	1549±101	1223±179	704±61	415±11
NPA	Bottom part	15833±968	19852±251	12794±647	7526±367
NPA	Donor agar	219055±3940	242026±4123	254775±1833	250813±3412
Total retrieved		**238344 ± 4581**	**268309.3 ± 3968**	**273584 ± 1682**	**263506 ± 3968**
Total lost		**161656 ± 4581**	**131691 ± 3968**	**126416 ± 1682**	**136494 ± 3968**

Data in Table 6-5 showed the radioactivity from the initial amount of ~ 400,000 dpm ^{14}C-IAA remaining associated with IAA after being transported in various parts of the transport system (receiver agar, top part and bottom part and donor agar) after 4 hours of application in basipetal or acropetal direction, for two cultivars of gentian ('Showtime Diva' and 'Little Pinkie') pre-treated with various chemical treatments (ethephon or NPA in their medium or control). Using the vertical bar for LSD values, radioactivity which remained associated with IAA, found in receiver agar of 'Little Pinkie' in the basipetal direction, was significantly more than that in the acropetal direction (1192 vs 55.7), and less than 'Showtime Diva' (2239 vs 547.6) ($P \leq 0.05$; Figure 6-15). NPA did not significantly change the basipetal transport of radioactive IAA to receiver agar in 'Showtime Diva' (2239 to 2496) but significantly decreased it in 'Little Pinkie' by 1.4 times (1192 to 830). Once 'Little Pinkie' was compared

to 'Showtime Diva', NPA decreased basipetal transport of radioactive IAA to receiver agar by 3 times (2496 to 830) (Figure 6-15 & Table 6-5). In comparison with the control, ethephon treatment did not significantly change radioactive IAA found in the receiver agar for both cultivars after being transported basipetaly. In top parts in the basipetal direction neither ethephon nor NPA significantly affected the quantity of IAA for 'Showtime Diva'. In 'Little Pinkie' however, while the effect of ethephon was not significant, NPA reduced the quantity of IAA by 3.6 times (from 76.5 to 21).

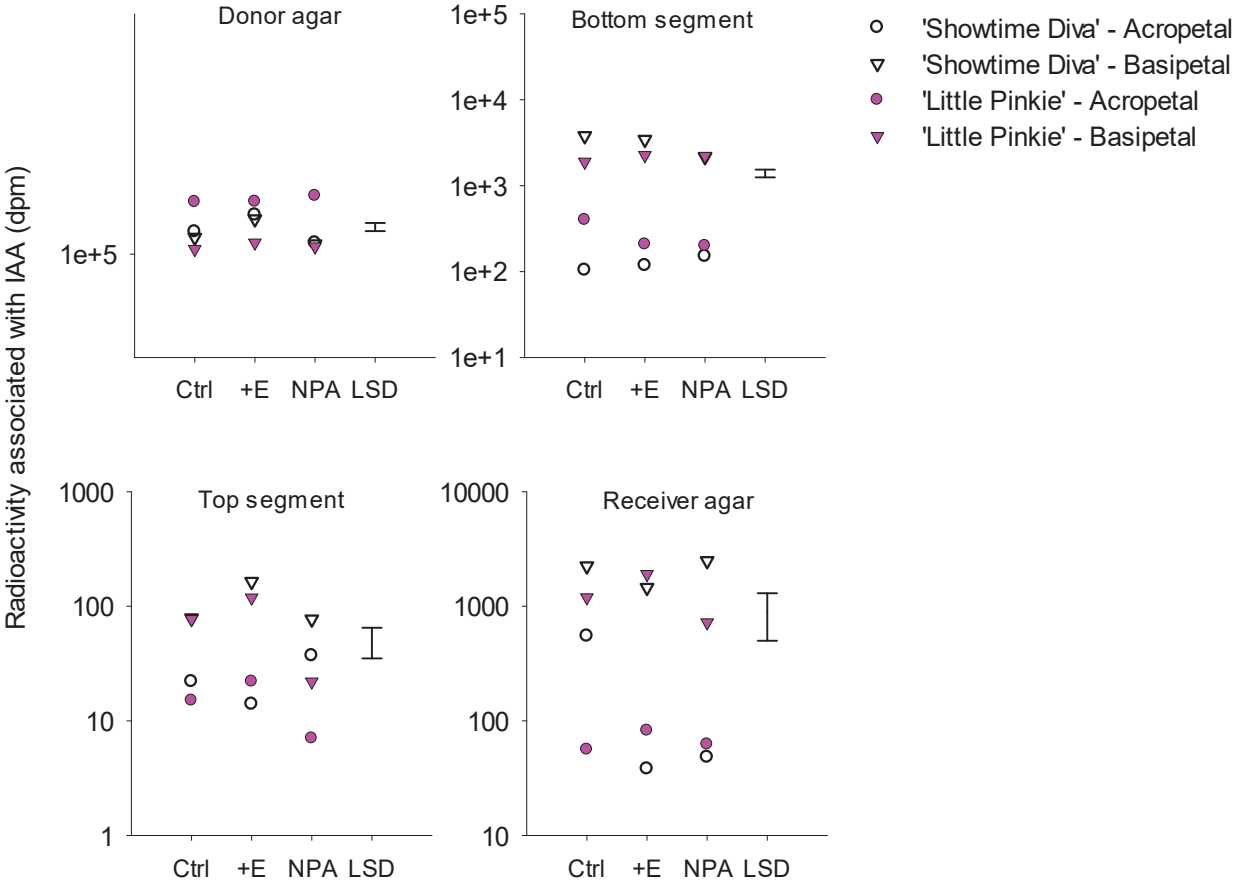


Figure 6-15: Distribution of radioactivity associated with IAA (dpm) in various parts of the transport system (receiver agar, top and bottom parts and donor agar) after 4 hours of application at basipetal or acropetal direction, in two cultivars of gentian ('Showtime Diva' and 'Little Pinkie') pre-treated with various chemical treatments (control (Ctrl), ethephon (+E) or NPA in their medium). Vertical bar represents Fisher's LSD between average values at $P \leq 0.05$, n=6 (basipetal) or 2 (acropetal).

Similar to the total transported radioactivity and the radioactivity associated with IAA, there was a decreasing trend in the IAA-metabolites (i.e. difference between total transported radioactivity and the radioactivity in the form of IAA in Table 6-5) found in the donor agar to the top part. Receiver agar however, contained more IAA metabolites than the top part in all treatment combinations. IAA metabolites accumulated in bottom part for 'Little Pinkie' in

both acropetal and basipetal directions were, respectively 1.9 and 1.6 times (19589 vs 10325 and 13924 vs 8885) more than for 'Showtime Diva' ($P \leq 0.05$). Treatment with NPA compared to the control, did not affect the amount of IAA metabolites found within the receiver agar of both cultivars, but decreased it in the top part of 'Little Pinkie' in both directions. Ethephon compared to the control did not affect the amount of IAA metabolites found in basipetal direction in any part, of any cultivar, but increased it in receiver agar of 'Little Pinkie' in the acropetal direction by 3 times (8088 vs 2730), compared to the control, with no significant influence on 'Showtime Diva'.

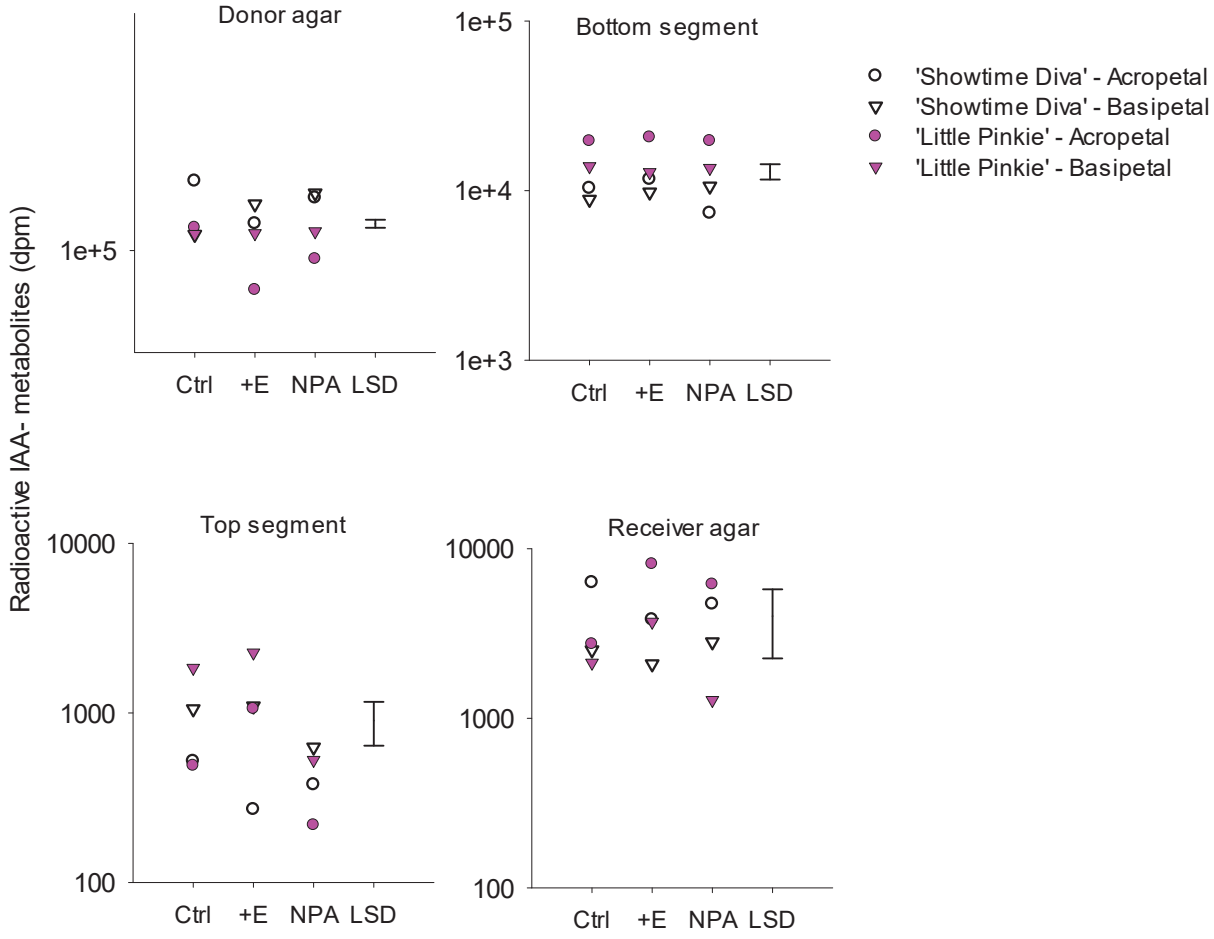


Figure 6-16 Distribution of radioactivity in the form of IAA metabolites (dpm) in various parts of the transport system (receiver agar, top and bottom parts and donor agar) after 4 hours of application at basipetal or acropetal direction, in two cultivars of gentian ('Showtime Diva' and 'Little Pinkie') pre-treated with various chemical treatments (control (Ctrl), ethephon (+E) or NPA in their medium). Vertical bar represent Fisher's LSD between average values at $P \leq 0.05$, n=6 (basipetal) or 2 (acropetal).

Table 6–5: Radioactivity remaining associated with IAA and radioactivity in the form of IAA-metabolites, from the initial amount of ~ 400,000 dpm ^{14}C-IAA, after being transported in various parts of the transport system (receiver agar, top and bottom parts and donor agar) after 4 hours of application in basipetal or acropetal direction, for two cultivars of gentian ('Showtime Diva' and 'Little Pinkie') pre-treated with various chemical treatments (ethephon or NPA in their medium or control). n=6 (basipetal) or 2 (acropetal). Radioactivity in the form of IAA metabolites was calculated from the difference between total radioactive retrieved and the radioactivity remained associated with IAA.

Chemical treatment	Transport part	'Little Pinkie'				'Showtime Diva'			
		IAA-associated basipetal	Metabolites basipetal	IAA-associated acropetal	Metabolised acropetal	IAA-associated basipetal	Metabolised basipetal	IAA-associated acropetal	Metabolised acropetal
Control	Receiver agar	1192 ± 159	2120 ± 283	55.7 ± 4.1	2730 ± 201	2239 ± 289	2525 ± 325	547.6 ±13.7	6297 ± 157
Control	Top part	76.5 ± 3.5	1836.7 ± 83.8	15.1 ± 1.8	488.2 ± 57.5	79.3 ± 11.9	1054 ± 158	21.6 ± 2.4	517.7 ± 58.3
Control	Bottom part	1898.8 ± 97.3	13924 ± 714	399.8 ± 29.7	19589 ± 1454	3808 ± 250	8885 ± 582	104.3 ± 13.8	10325 ± 1365
Control	Donor agar	111904 ± 933	111904 ± 1027	103486 ± 933	116696 ± 1052	141891 ± 1729	111486 ± 1359	116102 ± 2534	160331 ± 3500
Ethephon	Receiver agar	1904 ± 570	3697 ± 1106	81.7 ± 11.3	8088 ± 1114	1452 ± 231	2090 ± 333	38.5 ± 5.7	3811 ± 567
Ethephon	Top part	119.1 ± 21.4	2262 ± 406	21.5 ± 6	1054 ± 295	164.1 ± 23.4	1098 ± 156	14.2 ± 4.6	269.3 ± 88.4
Ethephon	Bottom part	2269.7 ± 98.7	12861 ±559	208.5 ± 29	20637 ± 2866	3449.2 ± 75.4	9817 ±215	117.5 ± 5.7	11633 ± 562
Ethephon	Donor agar	107998 ± 2270	112406 ± 2363	142075 ± 2718	76502 ± 1464	126600 ± 1527	137150 ± 1654	129980 ± 1906	119982 ± 1759
NPA	Receiver agar	830 ± 224	1079 ± 399	62 ± 3.3	6145 ± 324	2496 ± 203	2814 ± 229	47.5 ± 3.2	4704 ± 315
NPA	Top part	21 ± 4.03	1526.8 ± 196.8	6.7 ± 2.4	1216.6 ± 176	77.5 ± 6.8	626.8 ± 54.7	37.3 ± 1	377.4 ± 10.2
NPA	Bottom part	2217 ± 136	13616 ± 832	198.5 ± 2.5	19653 ± 249	2175 ± 110	10619 ± 537	150.5 ± 7.3	7376 ± 359
NPA	Donor agar	105146 ± 1891	113909 ± 2049	147636 ± 2515	94390 ± 1608	107006 ± 770	147770 ± 1063	107850 ±1467	142964 ± 1945

6.4.4. Discussion

6.4.4.1. Direction of IAA transport

As a result of the comparison between the total transported radioactivity and the radioactivity transported in the form of IAA, it was concluded that the majority of the radioactive IAA transport was in the basipetal direction (Tables 6-4 & 6-5). Total radioactivity initially quantified, seemed to be transported in both acropetal and basipetal directions, which was not expected (Table 6-4). However, the distribution of fractions over their retention time for samples taken from the receiver agar, as well as stem tissue in the acropetal direction, showed very small IAA peaks (refer to Figures 6-11 & 6-12). Therefore, it was likely that the radioactivity which was transported in the acropetal direction was not in the form of IAA. Once the amount of radioactivity which had been transported to the receiver agar as well as in the stem tissue in the form of IAA was calculated and analysed, it was found to be significantly lower in the acropetal direction than the basipetal direction (Table 6-5 and Figures 6-11 & 6-12). For instance, radioactivity associated with IAA transported acropetally to the receiver agar of control samples of 'Little Pinkie' was about 18 times (from 36% to 2%) smaller than the radioactive IAA in the basipetal direction (Table 6-3). Therefore, acropetal transport of IAA was considered negligible, and there was no need to subtract the acropetal from basipetal transport in order to calculate the transported IAA.

Transport of auxin in basipetal direction, similar to that has been noted previously (Went, 1941; Goldsmith, 1977; Hasenstein and Evans, 1988; Luthen, 2015) occurred against gravity in this bioassay, from tip of the stem segment which had been provided with the radiolabelled auxin in donor agar toward the base. Observation of acropetal transport of auxin here as well as in some studies in the past, (Goldsmith, 1966; Leopold, 1967; Hasenstein and Evans, 1988) could be attributed to either simple diffusion (Goldsmith, 1966; Leopold, 1967) that might only occur when radioactivity move from the donor agar into the bottom cells of the lower stem segments which the diffusion gradient would be large, but after that it would have to be transported actively in basipetal direction via the action of both influx and efflux carriers located in cell membrane (Galweiler et al., 1998; Muday, 2000; Ljung et al., 2002).As found out in current study and detailed in following sections, the possible explanation for the observation of such an unusual acropetal direction reported by (Goldsmith, 1966; Leopold, 1967) and others, but not investigated further in the past, was related to the movement of metabolite/s of IAA, not IAA (Figure 6-11 to 6-14 & Table 6-5).

However, type of those metabolites which have been observed in current study by the evidence of multiple peaks at various retention time as for the IAA peak, remained to be identified in future.

There was a decreasing trend in the quantity of total transported radioactivity, with radioactivity remaining associated with IAA and IAA metabolites from donor agar toward the bottom part and top part (Tables 6-4 & 6-5). However, in receiver agar, total radioactivity, radioactivity remaining associated with IAA and IAA metabolites retrieved, were more than that in the top part, indicating accumulation in the receiver agar over time with probably little or no back diffusion from the agar into the tissue. Donor agar in all treatment combinations contained the most quantity of total radioactivity, radioactivity associated with IAA and IAA metabolites. Such difference between different parts of the transport system in their radioactivity content could not be due to any variation between blocks of donor agar in their radioactivity content because no significant difference was found between different replications of the transport system (receiver agar + top part + bottom part + donor agar) for both cultivars in their total amount of radioactivity ($P = 0.98$). It was therefore likely that the difference in the amount of radioactivity within the receiver agar and stem segments were due to the difference between cultivars in immobilising and/or transport of IAA, as detailed in following sections. This is an important result as the increased metabolism occurred in the cultivar that produces shoots more freely. From the initial amount of radioactive IAA provided through donor agar, only ~ 40% to 65% remained in the form of IAA in donor agar (Table 6-3 & Figure 6-11 & 6-12). Therefore, ~ 35% to 60% was metabolised in the tissue, which presumably back-diffused into the donor agar after being metabolised in stem segments, since metabolites were found in the donor agar.

6.4.4.2. Effect of ethephon and NPA on IAA transport in different cultivars

To investigate the hypothesis of increasing shoot formation in ‘Little Pinkie’ due to the inhibition of PAT, the amount of radioactive IAA which was transported in a basipetal direction and retrieved from the total known amount of radioactivity provided within the donor agar of control samples were compared with that treated with ethephon and NPA. Control (-E) samples of ‘Little Pinkie’ seem to accumulate radioactive IAA in their tissue more than ‘Showtime Diva’ (e.g. ~ two times in bottom part (3808 vs 1898.8)), whereas ‘Showtime Diva’ seems to transport significantly more IAA to the receiver agar (1192 vs 2239) (Table 6-5 & Figure 6-15). Such difference between cultivars in their transport or

immobilisation of IAA in their tissue might be related to their difference in natural shoot formation. Although cultivars differed also in acropetal movement of IAA, radioactive IAA that had been transported acropetally in both cultivars were always less than the basipetal direction (Figure 6-15), which was in line with other researcher's reports (Went, 1941; Goldsmith, 1977; Hasenstein and Evans, 1988; Luthen, 2015).

Application of both ethephon and NPA as PAT inhibitor increased shoot formation in 'Little Pinkie', which provided an evidence to support the hypothesised inhibitory effect of ethephon on PAT. However, result of ethephon application on the radioactivity transported in the form of IAA, compared to the control did not support the hypothesised inhibitory effect of ethephon on PAT, compared to the results for NPA. While radioactive IAA measured in either cultivar in basipetal direction was not significantly affected by ethephon treatment, NPA decreased it in the top part of 'Little Pinkie' compared to the control, but unexpectedly did not significantly change it in 'Showtime Diva' (Figure 6-15 & Table 6-5). NPA was expected to inhibit the transport in both cultivars as it has been well-known to inhibit IAA transport in a basipetal direction through specifically inhibiting the auxin efflux transporter protein, PIN, possibly via a specific NPA-binding protein located on the basal plasma membrane (Thomson and Leopold, 1974; Morris, 2000; Muday and Murphy, 2002). One of the possibilities for observation of no significant response by 'Showtime Diva' to NPA could be due to the insufficient concentration and/or duration of NPA pre-treatment to affect this cultivar, compared to 'Little Pinkie'. Therefore, this experiment could be repeated in the future on 'Showtime Diva' by application of NPA pre-treatments at various concentrations, preferably higher than 100 µM which was applied in current research (n.b. determining rate to use was developed in preliminary experiments based on 'Little Pinkie'; refer to Section 6.1). Also, as hypothesised, ethephon was expected to inhibit PAT as evident by similar increased shoot formation response to NPA in 'Little Pinkie' reported earlier (refer to Experiment 1 of current chapter), but it didn't. One possibility for the lack of ethephon's effect on basipetal transport of IAA was that ethephon/ethylene's may have been affecting auxin synthesis (Burg and Burg, 1966b; Hansen and Grossmann, 2000) and/or metabolism (Ernest and Valdovinos, 1971) rather than the transport. The possibility of increased shoot formation response to ethephon application, through IAA metabolism detailed in following section. Another possibility could be due to non-availability of ethylene during the IAA transport assay due to volatilisation. As such it was possible that ethephon was metabolised to ethylene in the pre-treatment period, but was therefore unavailable once explants were

transferred to fresh radioactive medium for the IAA transport study. Although it would be technically challenging, application of an effective concentration of gaseous ethylene during the IAA transport bioassay could be considered in future experiments. Also, it was possible that the duration of two weeks ethephon pre-treatment applied on the explants, to then show the increase in shoot formation in 3 to 4 weeks, was probably not adequate to inhibit the transport. Therefore, in future repetition of the current experiment, a longer duration than two weeks or alternatively, continuous exposure to ethephon by application in the donor agar might assist in determining whether-or not- ethephon inhibits basipetal transport of IAA in gentian. Lack of a response to ethephon in the transport experiment could also result from low vegetative vigour of the explants due to flowering, which might have made the explants non-responsive to ethephon, as detailed in Chapter 2. Therefore, including some additional replicate vessels including explants of all treatments would be suggested in future to check the increased shoot formation in response to the ethephon and NPA treatments.

6.4.4.3. IAA metabolites in cultivars as affected by ethephon and NPA

As illustrated by Figures 6-11 to 6-14, apart from the IAA peak, within the top and bottom parts of the transport system for both cultivars there were several visible peaks which indicated the existence of IAA metabolites or degradation products (Figures 6-11 to 6-14). Peaks at retention times other than IAA were also found in the receiver agar of acropetal transport. Therefore, while not identified in the current research for their type, IAA metabolites were probably capable of being transported in both directions.

It has been noted by (Normanly and Bartelt, 1999) that metabolism of auxin which results in losing its biological activity, could be in the form of an oxidation and/or conjugation process which occur through changing the indole ring or auxin's side-chain (Figure 6-17). Two groups of conjugates (ester-type and amide-type) have been described in a variety of plant species which some of them cannot be hydrolysed back to the free, active hormone (Ljung et al., 2002). The peak that was observed in current study at retention time other than the IAA peak, could be related to one of those ester-type or amide-type compounds. The difference between total retrieved radioactivity and the radioactivity remaining associated with IAA represents metabolism plus radioactive IAA lost during the assay in the form of labelled CO_2 (Table 6-5).

Cultivars of gentian which naturally differ in shoot formation, differed also in their IAA metabolism. Control samples of 'Little Pinkie' (cultivar with high frequency of shoot

formation) seem to metabolise IAA quite a lot, as evident by significantly more IAA metabolites in their bottom part transported in both acropetal and basipetal directions compared to 'Showtime Diva' (cultivar with low frequency of shoot formation) (Table 6-5 & Figure 6-16). In donor agar, which was the source of radioactive IAA, and no IAA metabolite was expected to be found, 'Showtime Diva' contained 1.4 times (160331 vs 116696) more IAA metabolites in acropetal direction than in 'Little Pinkie'. Therefore, it seems that significant amount of radioactive IAA which had been transported acropetally by 'Showtime Diva', got metabolised in the tissue and back diffused into the donor agar whereas in 'Little Pinkie' radioactive IAA been transported into the tissue acropetally, got immobilised in the bottom part after being metabolised.

While available literature on IAA metabolism has been mostly focused on the catabolic pathway, de-carboxylative process, identification and classification of IAA metabolites in plants (Sasaki et al., 1994; Tuominen et al., 1994; Ljung et al., 2002) examples of some reports could be found on the difference between various plant species in IAA metabolism (Feung et al., 1975; Nakamura et al., 1985; Kohler et al., 2004) which supported the observations in current study on the difference in the IAA metabolism between cultivars of gentian. Metabolism of 2, 4-dichlorophenoxyacetic acid (2, 4-D), which is a synthetic auxin, has been reported to differ among various plant species as well as within a single plant species even when cultured at different locations (Feung et al., 1975; Nakamura et al., 1985; Kohler et al., 2004). For instance, metabolites of 2, 4-D have been reported to differ among five different plant species under investigation, with carrot having the minimum percentage of water soluble metabolites (13.2%) to corn which contained up to (64.6%) (Feung et al., 1975). Within a single species, metabolism of 2, 4-D, differed between cultivated genotype of tobacco, compare to other two wild genotypes (Nakamura et al., 1985). Even two populations of ground ivy plants from the same genotype which cultured in two different locations also differed in their foliar uptake and acropetal translocation of 2, 4-D which affected their sensitivity toward the application of 2, 4-D. The susceptible population of ivy absorbed 37% more ^{14}C 2, 4-D through foliar application than the tolerant population (Kohler et al., 2004). However, the difference between cultivars of gentian in the metabolism of auxin was found in current study for the first time.

Ethephon treatment did not change the amount of IAA metabolites found in basipetal direction but increased it in acropetal direction in the top part and receiver agar in 'Little Pinkie' compared to the control. Therefore, while the effect of ethephon on basipetal

transport of IAA was not significant, the metabolism of IAA transported acropetally in 'Little Pinkie' was increased which could be possibly related to the increased shoot formation response of this cultivar to ethephon application. In contrast, in 'Showtime Diva' with no increase in shoot formation in response to ethephon, the amount of IAA metabolites found in top part did not significantly change in either direction compared to the control. NPA which resulted in increased shoot formation in Little Pinkie' (refer to Experiment 1), decreased the amount of IAA metabolites transported to the top part compare to the control that could be in turn due to the decrease in basipetal IAA transport. In contrast, in 'Showtime Diva' with no significant difference on basipetal IAA transport (Figure 6-16 & Table 6-5), no change occurred in IAA metabolites and probably no increase in shoot formation was expected as a result of NPA application.

Application of ethephon in the current research increased the metabolism of IAA in 'Little Pinkie' (although only in in acropetal direction), which was in line with other's reports on the effect of ethylene on IAA metabolism (Beyer and Morgan, 1969; Ernest and Valdovinos, 1971; Wood, 1985; Sagee & Goren, 1990). Also, there have been evidences of inhibitory effect of ethephon/ethylene on PAT in the past (Went, 1941; Ernest and Valdovinos, 1971; Goldsmith, 1977; Luthen, 2015), which together with other mechanisms such as conjugation of IAA to inactive products (Riov and Yang, 1982), auxin synthesis (Burg and Burg, 1966b; Hansen and Grossmann, 2000) and IAA catabolism (Beyer and Morgan, 1970; Wood, 1985; Sagee & Goren, 1990; Winer et al., 2000) as well as the increase in IAA metabolism (Ernest and Valdovinos, 1971) resulted in reducing endogenous IAA concentration. It also has been reported that in stem segments of *Coleus blumei* pre-treated with ethylene, metabolism of ^{14}C-IAA to indoleacetylaspartic acid increased (Ernest and Valdovinos, 1971). While some other investigators (Goren, Bukovac & Flore, 1974) did not detect any effect of ethylene on IAA conjugation with aspartic acid, the data presented here (Table 6-5 & Figure 6-15) also indicated that ethylene pre-treatment enhanced IAA metabolism in gentian 'Little Pinkie'. However, in current research, application of ethephon did not inhibit PAT in gentian due to the possibilities detailed in previous section such as: unavailability of ethephon/ethylene to the explants during the transportation of auxin and/or non-responsive plant material suggesting the need for a repeating the experiment with a revised methodology.

6.4.4.3.1. Metabolite X in relation with shoot formation

The peak at 2:48 min. was an example of the metabolites (referred to as metabolite X here) which constantly appeared in all samples, varied in amount with treatment and cultivar and, therefore, was considered to be possibly involved in the different shoot formation response of cultivars (Figures 6-11 to 6-14). Metabolite X was observed in the stem segments oriented to study basipetal direction but in a lower percentage than the acropetal samples. In contrast to IAA which was transported in a basipetal direction, metabolite X might have been transported regardless of direction. Therefore in circumstances that transport may not be possible in the form of IAA, it possibly could occur through conversion to some intermediate metabolites such as metabolite X. Although identification of metabolite X was not attempted in current experiment, it represented retention time close to that has been chromatographically characterized as indole-acetyl-aspartic acid in the past (Ernest and Valdovinos, 1971). It was also possible that cultivars might differ in the type and, therefore, movement of particular metabolite/s such as metabolite X (Figures 6-13 & 6-14). For instance, the quantity of the peak illustrating percentage of metabolite X in top and bottom parts of 'Little Pinkie' control were higher (respectively 35% and 15%) compared to 'Showtime Diva' (10% and 9%). However, because of lack of further information on identification and quantification of metabolite X, the influence of this metabolite remained just as a possibility, not more. Therefore, in addition to the difference between cultivars in transporting IAA, the differences between IAA metabolites and probably particular metabolites (e.g. metabolite X) could be another source of the difference in natural shoot formation between cultivars.

As illustrated in Figure 6-13, the percentage of the peak illustrating metabolite X in the top part for 'Little Pinkie' treated with ethephon, decreased from 35% to 23% in control. Although when compared with the decreasing effect of NPA on the same peak (9% vs 35% in control) the effect of ethephon did not seem to be strong, but even a small decrease might be effective depending on the sensitivity of the tissue (Trewavas, 1982; Firn, 1986). Also, while NPA and ethephon decreased the relative percentage of metabolite X in top part for 'Little Pinkie' as compared with the control (9% and 23% respectively compared to 35%), there was little difference in 'Showtime Diva' (9% and 7% respectively compared to 10%) which could be another reason for the different response of cultivars to various chemical treatments (Figure 6-13 & 6-14).

As a result of the current research, the difference between cultivars of gentian in their natural shoot formation could be explained probably by the differences in transport, synthesis and/or the metabolism of auxin in relation with the ratio of auxin to cytokinin. Higher metabolism (more metabolite X) of IAA in 'Little Pinkie' might result in a lower quantity of IAA and perhaps lower auxin: cytokinin ratio at the node, and consequently more shoot formation compared to 'Showtime Diva'. The decrease in the quantity of IAA at the node of the explants of 'Little Pinkie' as a result of PAT inhibition by NPA, probably would also lower the auxin: cytokinin ratio and increase in shoot formation (Christianson and Warnick, 1983; Coenen and Lomax, 1997) (refer to Chapter 1, Section 1.1.3). However, data achieved here did not support the hypothesised inhibitory effect of ethephon on PAT in any of cultivars. While future methodological changes in the current experiment is suggested to investigate the hypothesis of PAT inhibition by ethephon/ethylene more, the influence of ethephon on inhibition of auxin synthesis (Burg and Burg, 1966b; Hansen and Grossmann, 2000) as well as auxin metabolism on reducing auxin: cytokinin ratio and increasing shoot formation response should not be ignored. Also, it is possible that ethephon was involved in changing the ratio of auxin: cytokinin by influencing the quantity of cytokinin, as covered in next chapter.

6.4.5. Conclusions

In explants of 'Little Pinkie', NPA inhibited basipetal transport of auxin, which perhaps consequently reduced IAA metabolism and, as found in Experiment 1 of this chapter, increased shoot number. As a result of NPA application, the quantity of non-metabolised IAA was hypothesised to be reduced, which would lower the ratio of auxin: cytokinin at the node and result in increased shoot formation. However, findings within this experiment did not support the hypothesised inhibitory effect of ethephon on basipetal IAA transport in 'Little Pinkie', due possibly to an inadequate duration of ethephon application as well as variation in vegetative growth of plant material. Also, ethephon did not affect IAA metabolism in basipetal direction, but enhanced it in acropetal direction only in 'Little Pinkie'. However, the effect of ethephon on increasing shoot formation could be in relation with reducing auxin synthesis and as a result lowering the ratio of auxin: cytokinin at the node. Reducing the concentration of a specific IAA metabolite such as metabolite X as a result of ethephon or NPA application also might have played a role in shoot formation. Reduction in Metabolite X following the application of ethephon which also occurred as a result of NPA application was hypothesised to play a role in the increased shoot formation response in 'Little Pinkie' and

therefore is suggested to be tested in future. In contrast to 'Little Pinkie', in 'Showtime Diva' shoot formation was not affected by ethephon nor probably by NPA, because there was no significant change in PAT and metabolism of auxin, and no reduction in metabolite X.

Differences reported previously throughout this thesis between cultivars of gentian in their natural shoot formation could also be explained by their difference in quantity of IAA and IAA metabolites. The high shoot formation response in 'Little Pinkie' compared to 'Showtime Diva' could be related to more IAA metabolism that was hypothesised to lower the endogenous concentration of IAA at the node and decrease auxin: cytokinin ratio.

Chapter 7. Ethephon/ethylene effects on *in vitro* shoot formation in relation with cytokinin

7.1. Introduction

Cytokinins are another class of phytohormones that can be provided through the culture medium *in vitro*, and influence growth and development of plants through their interaction with various factors including other phytohormones/PGRs. Cytokinins are involved in root and shoot apical dominance, axillary bud growth, and other developmental processes in plants typically occurs through promoting cell division, growth and differentiation (Kieber, 2002). However, like other phytohormones, cytokinins do not affect plants through a linear pathway. The final outcome of application of an individual phytohormone is the result of cross-talk between the hormonal pathways of various phytohormones (Vanstraelen and Benkova, 2012). Synergetic, antagonistic and additive interactions between cytokinin and auxin have been demonstrated (Coenen and Lomax, 1998). The auxin: cytokinin ratio appears to be one of the commonly reported important means of expressing the interaction of cytokinins with auxin in the regeneration response towards a specific *in vitro* morphogenic process (Christianson and Warnick, 1983; Coenen and Lomax, 1997).

Any factor that might influence and modify the auxin: cytokinin ratio has been reported to control *in vitro* organogenesis, wherein a high ratio resulted in root formation, whereas a low ratio promoted formation of adventitious (Kim and Ernst, 1994; Peres and Kerbauy, 1999; Huang et al., 2000) or presumably adventitious and axillary shoots (Sudha et al., 1998). A decrease in auxin supply could result in either increasing the synthesis or exporting cytokinins from roots to shoots, and consequently lowering the ratio of endogenous auxin: cytokinin (Coenen and Lomax, 1997; Turnbull et al., 1997). In a natural plant system (*in vivo*), increasing the endogenous concentration of cytokinin as a result of synthesis in roots (Bangerth, 1994) or de novo in the stem (Tanaka et al., 2006) or, any decrease in auxin supply, would result in a low ratio of auxin: cytokinin and an increase in shoot formation. The promoting effect of cytokinins on formation of multiple shoots has also been reported following the exogenous application of cytokinin *in vitro* (Sudha et al., 1998; Huang et al., 2000), which was probably due to the increase in endogenous level of cytokinins and, consequently, lowering the ratio of auxin: cytokinin (Turnbull et al., 1997; Aremu et al.,

2014; Cosic et al., 2015). Hence, with the current *in vitro* system in mind, as it has been already well established for other cultivars of gentian (Sharma et al., 1993; Morgan et al., 1997; Fiuk and Rybczynski, 2008), it was hypothesised that exogenous application of cytokinin would also increase shoot formation in explants of 'Little Pinkie'. If the hypothesis was true, it could be concluded that the endogenous concentration of cytokinin would probably have increased and, consequently, the ratio of auxin: cytokinin would have declined (refer to Chapter 6).

In the current research the mechanism by which ethephon applied in the culture medium resulted in increased shoot formation within explants of gentian 'Little Pinkie', was hypothesised to be through changing the ratio of auxin: cytokinin. Support for this hypothesis is evident by application of ethephon on young petunia seedlings which increased the number and length of their lateral shoots (Haver and Schuch, 2001). The author reported that lateral shoot growth was accompanied by a decrease in the auxin: cytokinin ratio, as a result of ethylene's effect on IAA level in the shoot which resulted in an increase in the percentage bud break, formation of lateral shoots, and suppression of internode elongation (Haver and Schuch, 2001). In current research endogenous quantification of phytohormones (especially auxin and cytokinin) could assist investigating the hypothesis more directly. However, it would be a resource intensive process and, therefore, was not pursued in the current thesis. Alternatively, as utilised in the research presented in this chapter, amending the growing medium with PGRs within an *in vitro* system could provide an opportunity to investigate the relationship between these two phytohormones and their effect on shoot formation. This research approach was based on the hypothesis that the effect of cytokinins as a PGR exogenously applied in the culture medium would be similar to the effect of ethephon and/or ethylene on increasing shoot formation. As a result, any similarity between the effect of exogenous application of cytokinins and ethephon through the culture medium on shoot formation in gentian, together with the results presented in Chapter 6) on transport and metabolism of auxin, could provide evidence to support the hypothesis of ethephon/ethylene's effect on lowering the auxin: cytokinin ratio.

Cytokinins are either of the adenine-type, such as Kinetin, Zeatin, and 6-benzylaminopurine, or the Phenylurea-type, such as diphenylurea and thidiazuron (TDZ) (Chen et al., 1985; Mok and Mok, 2001). Cambium and other actively dividing tissues synthesize cytokinins in roots (Chen et al., 1985). Aerial parts of the plant also have the capacity to synthesize its own cytokinin, independent of long-range transport from the root system (Nordström et al., 2004).

For the purpose of inducing multiple shoots *in vitro*, various types of cytokinins have been applied through the culture medium at various concentrations depending on the plant species and the segment used as an explant (Seelye et al., 1994; Hosokawa et al., 1996; Sudha et al., 1998; Huang et al., 2000). BAP, as an adenine-type, and TDZ, as a Phenylurea-type, have been the most commonly used cytokinins *in vitro.* Using callus of *Gentianella scabra*, TDZ at concentrations between 0.5 and 1 $mg.L^{-1}$ were more efficient (100 to 80% calli produced shoots) in shoot regeneration compared to BAP (2 to 5.6% calli produced shoots) been applied at a similar concentration range (Jomori et al., 1995). However, in another study out of various types of cytokinin on 5 cultivars of *Gentianella* leaf explants, applied BAP the most effective at improving the percentage of somatic embryogenesis (Fiuk and Rybczynski, 2008). Hence, TDZ and BAP were both selected as comparative sources of cytokinin within the current experiment. Based on previous reports (Hosokawa et al., 1996; Morgan et al., 1997; Zhang, 2008) BAP and TDZ at various concentration rage was considered in order to achieve a dose response curve and possibly creating various ratios of auxin: cytokinin (refer to Appendix 11). Therefore it was hypothesised that the mechanism by which ethylene released from ethephon increased shoot formation in explants of gentian 'Little Pinkie' cultured *in vitro* was through affecting cytokinins. Hence, in a series of experiments the following comparisons were undertaken with regard to the number of shoots arising from explants cultivated *in vitro*, i.e.:

- comparison between various concentrations of cytokinin in the medium,
- comparison of two different forms of cytokinins (i.e. TDZ and BAP),
- comparison of various concentrations of cytokinins with ethephon.

7.2. Materials and methods:

Axenic plantlets of gentian 'Little Pinkie', which previously had been grown and sub-cultured *in vitro* for several four-week growth cycles were used as donor plantlets to take explants in line with the general protocol for gentians within the tissue culture lab (Morgan et al., 1997). Explants had been previously sub-cultured using a modified medium (PGR-free and MS macro-salts at half strength) detailed in with modifications detailed in Chapter 2. Culture vessels were disposable 290 mL clear plastic (Alto Packaging, Hamilton, New Zealand) tubs with snap-on lids which allowed gas exchange (refer to Chapter 4, Figure 4-2). Each culture vessel contained 50 mL of medium and explants were cultured *in vitro* under the standard growth condition described previously in Chapter 2.

In order to create treatments; PGR-free medium was amended with filter sterilised (0.2 µm Minisart ® filter; Sartorius Stedim Biotech) ethephon (Ethrel®; 480 $g.L^{-1}$ chlorethephon; Bayer CropScience, New Zealand) at a final concentration of 10 $mg.L^{-1}$, TDZ (490 $g.kg^{-1}$ Thidiazuron, Dropp®, N.S.W, Australia) or BAP (6-benzylaminopurine, B3403-5G Sigma Aldrich, New Zealand). Both TDZ and BAP were applied separately at concentrations of 0, 0.3, 0.6, 0.9, 3, 6, 9 or 18 $mg.L^{-1}$. Shoots of plantlets were divided into explants, each 10 mm in length as either: Tip (apex and undeveloped leaves and nodes), Node 1 (first node below the tip), Node 2 (the second node below the tip) or rooted base (Base) Chapter 2, Figure 2-2), and positioned with the nodes above the surface of the medium for eight weeks under standard growth conditions (refer to Chapter 2, Section 2.2). Shoot formation, as represented by the number of shoots per explant (i.e. 1º and/or 2º shoots; Chapter 2; Figure 2-1), was recorded after 8 weeks of growth.

The experiment comprised a factorial arrangement of two treatment factors; position along the shoot from which explants were derived (i.e. Tip, Node 1, Node 2 or Base) and growth medium (ethephon or 0, 0.3, 0.6, 0.9, 3, 6, 9 or 18 $mg.L^{-1}$ of either TDZ or BAP). Each treatment comprised four to six culture vessels as individual replicates, with each culture vessel containing eight explants. Analysis of variance was conducted using the general linear model on square root transformed data using Genstat software (version 17, 2014, VSNi Ltd, Hemel Hempstead, UK). Mean separation was achieved using Fisher's protected least significant difference test where the Studentized Range statistic was used instead of Student's t test at $P \leq 0.05$.

7.3. Results and discussion

7.3.1. Interaction of cytokinin with position of the explant

The number of shoots from explants treated with cytokinins or ethephon was also affected by the position that explants originated from. There was an increasing trend in the number of shoots from explants treated with either cytokinin as the distance of the position increased from the tip ($P \leq 0.05$). Depending on the type and concentration of cytokinin applied, explants taken from the Base position were capable of producing up to 15.5 shoots as compared with the explants from the Tip position, which produced a maximum of 7 shoots (Figure 7-1). While distinction between the type of shoot arising from the base position was not possible, due to their small size, they were considered to be a mixture of axillary, co-lateral and/or adventitious origin as evident by microscopic investigations in Chapter 3.

However, as a result of cytokinin application to tip explants, some of the extra shoots in addition to the single primary shoot which was expected to arise from the explant, were almost certainly evidence for production of some shoots adventitiously. While the effect of cytokinins on stimulation of axillary shoot formation is well known, the effect of cytokinins on adventitious bud formation and their subsequent growth here is interesting and requires more investigation in future.

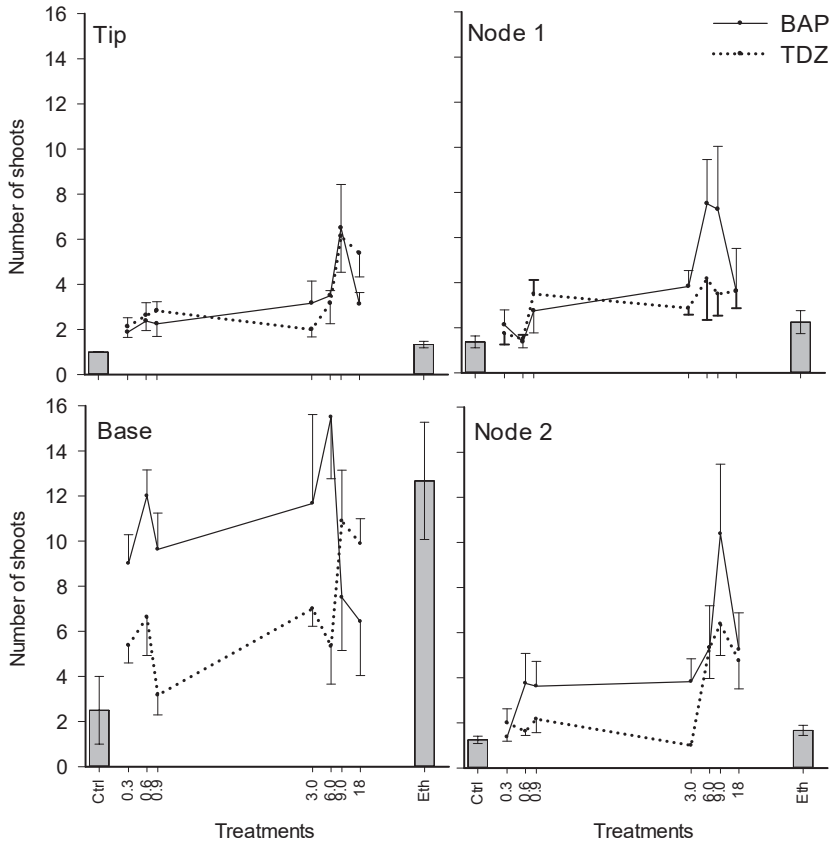


Figure 7-1: Number of shoots (1º and/or 2º) developed from the explants of gentian 'Little Pinkie' originated from tip (Tip), first or second node below the tip (Node 1 or 2) or base (Base) after 8 weeks growth in the medium amended with 0.3, 0.6, 0.9, 3, 6, 9 or 18 mg.L^{-1} of BAP (solid line) or TDZ (dashed line) presented on a logarithmic scale, as compared with control (Ctrl) or 10 mg.L^{-1} ethephon (Eth) (bars). Data were square root transformed and the mean comparison was based on Fisher's protected least significant difference test, where the Studentized Range statistic is used instead of Student's t test at $P \leq 0.05$ and is equivalent to Tukey's test. For clarity, vertical lines represent either + or - standard error bars on the actual (back-transformed) mean values, N = 4-6 vessels each containing 8 explants.

In addition, the difference in the number of shoots on explants treated with BAP as compared with TDZ increased as the distance of the position that explant derived from increased from the tip toward the base. Explants exposed to the medium amended with BAP produced on average ~ 1.5 times more shoots as compared with TDZ when taken from the Base position. In comparison, in explants taken from the Tip, the number of shoots were similar to that for explants treated with both types of cytokinins (Figure 7-1). Similar to the effect of cytokinins, for explants treated with ethephon the maximum number of shoots (up to ~ 13) was produced

by the explants taken from the Base position, as compared to the maximum number of up to ~ 2 shoots produced by the explants taken from all other positions. While as presented in the earlier chapters of this thesis (refer to Chapter 2, Section A) explants from the Base position were naturally capable of producing more shoots compared to other positions, application of either ethephon or cytokinin resulted in developing even more shoots compare to the absence of ethephon or cytokinins ($P \leq 0.05$).

One of the reasons for production of significantly more shoots by the explants taken from the Base compared to other positions, was presumably related to extra bud primordia which developed adventitiously at the node position (refer to Chapter 3, Figure 3-7). As detailed in Chapter 3, there was an increasing trend in the number of bud primordia at the node, as the distance of the position which explants were taken from increased from the Tip. Hence, while microscopic investigation was not undertaken within the current study, it was likely that explants from the Base position would contain the most number of bud primordia among the explants from all positions. Bud primordia initiated at the node, might remain dormant or develop to shoots depending on the apical dominance or growth promotors such as cytokinins (Turnbull et al., 1997; Shimizu-Sato and Mori, 2001; Shimizu-Sato et al., 2009). Similar to the effect of ethephon, cytokinins also encouraged development of bud primordia (axillary, co-lateral and/or adventitious) to shoots, providing an evidence to support the hypothesis of ethephon's effect on increasing shoot formation through increasing endogenous cytokinin level.

7.3.2. Type of cytokinin versus ethephon on shoot formation

Application of cytokinin in the form of BAP in the culture medium increased the number of shoots, on average, by 3.5 (Tip) and 10.2 (Base) times and with TDZ, by 3.3 (Tip) and 6.9 (Base) times more, than the control (PGR-free medium; $P \leq 0.001$) (Figure 7-1). While not necessarily expressed as auxin: cytokinin ratio, the increasing effect of cytokinins on shoot formation observed was similar to what was previously reported on *Gentiana* as well as other plant genera (Sharma et al., 1993; Seelye et al., 1994; Jomori et al., 1995; Kim et al., 1997). Similar to the effect of cytokinins, application of ethephon also enhanced the number of shoots by almost 1.3 (tip) and 12.7 (Base) times (on average) compared to the control with 1 (Tip) and 2.5 (Base) shoots ($P \leq 0.001$) (Figure 7-1), similar to that reported in petunia (Haver and Schuch, 2001). Such similarity between the effect of ethephon and cytokinins on the number of shoots, supported the hypothesis that ethephon and/or ethylene increased shoot

formation in gentian through lowering auxin: cytokinin ratio. Increased number of shoots in petunia treated with ethephon was reported to be due to lowering auxin: cytokinin ratio resulted from an increase in cytokinin level inside the plant (Haver et al., 2002).

As explored below, the production of more shoots by explants exposed to either cytokinins or ethephon, presumably resulted from extra bud primordia which developed either accessorily and/or adventitiously at the node position. In the absence of PGRs (Control), explants of 'Little Pinkie' produced a maximum of two shoots which, as detailed in Chapter 3, arose from axillary buds. This is compared to explants exposed to ethephon and cytokinins which produced more than 2 shoots. Distinction between shoots of different origin (axillary, co-lateral and/or adventitious) was almost impossible. However, both microscopic and macroscopic evidence presented in Chapter 3 illustrated that ethephon had its effect on increasing shoot formation by stimulating axillary, co-lateral and/or adventitious buds. While in the current experiment microscopic examination was not undertaken on the node position of explants exposed to cytokinins, given both the number of shoots (more than two) and location of shoots (all around the node; associated and non-associated with the location of axillary buds), it was considered most likely that the extra shoots were derived either accessorily or from adventitious buds. Similarly, effect of cytokinins on stimulation of explants to produce adventitious shoots has been reported by others previously (Kim and Ernst, 1994; Peres and Kerbauy, 1999; Huang et al., 2000). In another study also, encouraging effect of cytokinins on formation of multiple (more than two) shoots has been noted, which was interpreted to be from a combination of adventitious and axillary buds (Sudha et al., 1998).

BAP at any concentrations applied in current experiment on increasing shoot formation was either more effective than TDZ at that concentration or statistically similar (Figure 7-1). Also, at the point of assessment explants treated with BAP were visually differed from those treated with TDZ (Figure 7-2). Explants treated with BAP produced extra shoots with longer internodes compare to the explants treated with TDZ. Current experiment primarily focussed on the number of shoots per explant and, therefore, quantitative data were not recorded to describe shoot size. However, following the application of cytokinins shoot were shorter in length but more in number, compare to the control. These visual characteristics in explants been exposed to cytokinins were similar to the explants exposed to ethephon, both within the current experiment and in previous chapters (Chapter 1; Figure 1-4). In contrast to the resulting shoot system, for the explants treated with TDZ the root system was visually

stronger (more number and length) than those treated with BAP or ethephon. However, quantified data on the size of shoots and roots were not collected within the current experiment and, therefore, was considered worth quantifying in future experiments involving the application of cytokinins.

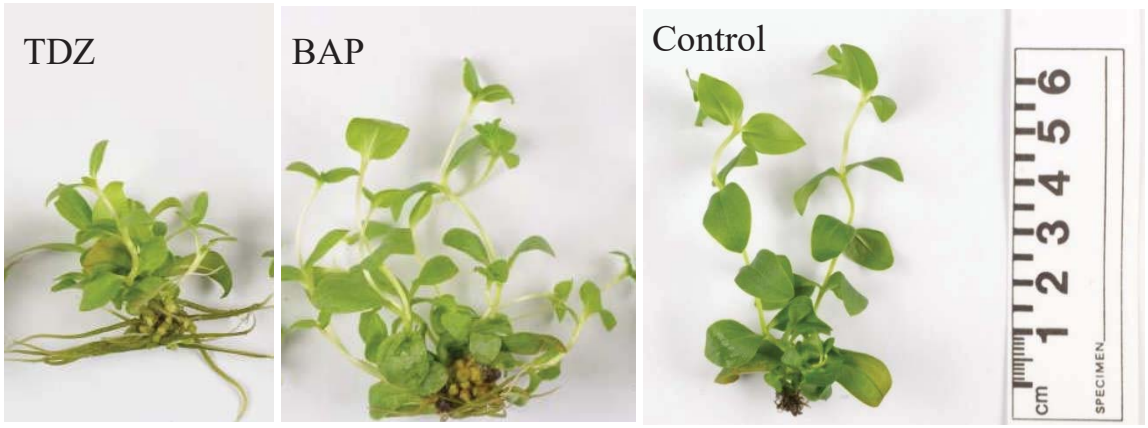


Figure 7-2: Visual appearance of nodal explants of gentian 'Little Pinkie' after 8 weeks growth in an *in vitro* medium containing 9 mg.L^{-1} of (A) TDZ or (B) BAP.

7.3.3. Concentration range of cytokinin

There was an increasing trend in the number of shoots as the concentration of BAP or TDZ in the medium increased and, therefore, presumably the ratio of auxin: cytokinin decreased (Figure 7-1). The number of shoots produced by explants exposed to both cytokinins followed a similar pattern with increasing concentration. However, at almost all the concentrations, BAP seem to be more effective than TDZ on shoot formation in explants. Only at concentrations more than 3 mg.L^{-1} did TDZ, increase the number of shoots compared to the control, whereas BAP at a concentration 10 times less (i.e. 0.3 mg.L^{-1}), resulted in an increase ($P \leq 0.001$). The maximum number of shoots was produced at a concentration of 9 mg.L^{-1} by explants treated with both types of cytokinins (Figure 7-1). Application of both sources of cytokinin, at higher concentrations (18 mg.L^{-1}) resulted in a reduction in the development of shoots. The decrease in the number of shoots at a supra-optimal concentration had been also reported in another study on *Gentiana corymbifera* and *Gentiana cerina* (Morgan et al., 1997). However, there was a big difference between the optimum concentration of BAP reported in that study (0.2 mg.L^{-1}), compared to the current study (9 mg.L^{-1}). Similarly, with either BAP or TDZ, *Gentiana* has previously been reported to have an optimum concentration of up to 5.0 mg.L^{-1} or 0.75 mg.L^{-1} respectively for shoot regeneration (Sharma et al., 1993; Jomori et al., 1995), which was less than the optimum 9 mg.L^{-1} reported in the current study. The difference between various studies in effective

concentration of cytokinins on explants, could probably be related to differences in genotype, absence or presence of other PGRs in the culture medium, and type of plant tissue used as the explant (Cacho et al., 1991; Pellegrineschi and Tepfer, 1993; Fiuk and Rybczynski, 2008). Therefore, the sensitivity of cells to endogenous phytohormones and hormonal receptor interaction might be different between explants in various studies (Firn, 1986; Trewavas, 1983). It was likely that in each specific genotype a certain level of cytokinin together with auxin would produce an optimum ratio of auxin: cytokinin required to optimise shoot formation in explants. Also, in some of those studies callus needed to be developed first from the explant so as to subsequently produce adventitious buds, whereas in the current study there were already some pre-existing bud primordia at the node plus adventitious buds which developed within the treatment medium (refer to Chapter 3 In the current study, concentrations higher than the optimum might be surplus to the plant's requirement imposing negative effects on the growth of explants, including the number of shoots (Sahoo et al., 1997; Saha et al., 2016). Nevertheless, in the current study while 18 $mg.L^{-1}$ of both sources of cytokinins (highest concentration applied) was not the optimum concentration, explants still produced more shoots (3.1 and 3.8 times more) than those within the control ($P \leq 0.001$) (Figure 7-1).

While not quantified in the current study, one of the reasons underlying the difference in the number of shoots produced by the explants taken from the Base as compared with other positions was probably attributed to the difference between explants in their root system. As illustrated in Chapter 2, Figure 2-1, explants from the basal position were already rooted (from previous growth cycle) at the time of being inserted into the culture vessel, whereas explants of other positions did not include roots and, therefore, had to produce roots during the 8 week period of culture. Roots may have provided the shoots with an increased supply of cytokinins (Bangerth, 1994) which, together with the cytokinin supply from the medium (Seelye et al., 1994; Jomori et al., 1995), could be responsible for bud outgrowth and shoot development.

The increase in the number of shoots developed by the explant treated with ethephon, and increased the distance of their position from the tip, could be related to the apical dominance and the ratio of auxin: cytokinin (Shimizu-Sato and Mori, 2001; Kalousek et al., 2010a). During bud outgrowth cytokinins contribute to the outgrowth of dormant buds which are under apical dominance by triggering buds to export auxin and, therefore, develop to shoots (Tanaka et al., 2006; Kalousek et al., 2010a). In addition, the ratios of auxin: cytokinin which

has been reported to affect the growth of plants (Ferguson and Beveridge, 2009), in a study on *Lupinus angustifolius* L. was quantified to be low at the basal nodes with rapid growing axillary buds, whereas the ratio was high in the slow growing axillary buds located at the nodes closer to the tip (Emery et al., 1998). In their research it was concluded that rapid bud outgrowth occurs only when a higher amount of cytokinin compared to auxin is accumulated in the tissue and, therefore, auxin: cytokinin ratio is in the favour of cytokinin. While cytokinin content of the explants was not quantified in the current study, shoot outgrowth of the explants been exposed to cytokinin was similar to the explants treated with ethephon in their medium. Therefore, it was hypothesised that the increased shoot outgrowth following the application of ethephon could result from the possible increase in cytokinin content (Bangerth, 1994; Haver et al., 2002) and, consequently, decrease in auxin: cytokinin ratio within the explant. However, future measurement of cytokinin concentration in explants of gentian treated with ethephon would test this hypothesis more directly. With the knowledge that ethephon had possibly an effect on the auxin: cytokinin ratio, future strategies could be developed to improve the results of ethephon treatment by application of a range of concentrations and/or promote the number of shoots by treating explants with cytokinin before or after ethephon application. It might even be possible to substitute ethephon treatment with cytokinin application.

7.4. Conclusions

Similar to ethephon, application of two types of cytokinins in the medium at concentrations up to 18 mg.L^{-1} increased shoot regeneration in explants of 'Little Pinkie' cultured *in vitro*. However, the maximum number of shoots was achieved at 9 mg. L^{-1}. Application of BAP was more efficient than TDZ for increasing the number of shoots in explants. Increased shoot number as a result of cytokinin application, similar to that achieved with ethephon, provided an evidence to support the hypothesis that lowering the ratio of auxin: cytokinin was the mechanism by which ethephon increased branching in 'Little Pinkie'. Ethephon reduced auxin: cytokinin ratio probably by reducing auxin concentration, increasing cytokinin concentration or both. However, future measurement of cytokinin concentration in explants of gentian treated with ethephon was suggested to test this hypothesis more directly.

Chapter 8. General discussion

8.1. Introduction

The mechanism of ethephon's effect on increasing number of shoots in gentian 'Little Pinkie' was investigated in this research. As summarised diagrammatically in Figure 8-1, shoot formation and in general, growth and development of an explant in an in vitro culture system, is affected by environmental factors as well as plant-related factors. Ethephon and/or ethylene released from ethephon in the in vitro culture medium, as one of the environmental factors, increased shoot formation in explants of gentian 'Little Pinkie', especially in basal node (refer to Chapter 3, Figure 3-13), as a result of stimulating axillary, co-lateral and/or adventitious buds as well as development of new adventitious bud primordia (refer to Chapter 3). However, the effect of ethephon application in the medium on increasing shoots in explants of 'Showtime Diva' was much smaller (refer to Appendix 13). In contrast to the application of ethephon in the medium, application of gaseous ethylene in the atmosphere around the explant, at a concentration similar to that released from ethephon in the culture vessels, did not affect shoot formation in node 2 or node 3 cuttings; nor did ethylene released from ethephon in culture medium if cuttings were held in medium without ethephon (refer to Chapters 3 & 4 & 5). From a developmental perspective the mechanism by which ethephon increased shoot formation was through stimulating the development of pre-existing bud primordia at the node of the explants (axillary) as well as initiation and development of new bud primordia (adventitious) (refer to Chapter 3, Figure 3-11).

As illustrated in Figure 8-1, changing the ratio of auxin: cytokinin was hypothesised to be the mechanism by which ethephon affected shoot formation in 'Little Pinkie'. Ethephon and/or ethylene could have reduced the endogenous ratio of auxin to cytokinin through the reduction in auxin and/or increase in cytokinin level at the node. The following sections provide more detail on the mechanism of ethephon's effect on shoot formation of gentian mentioned above.

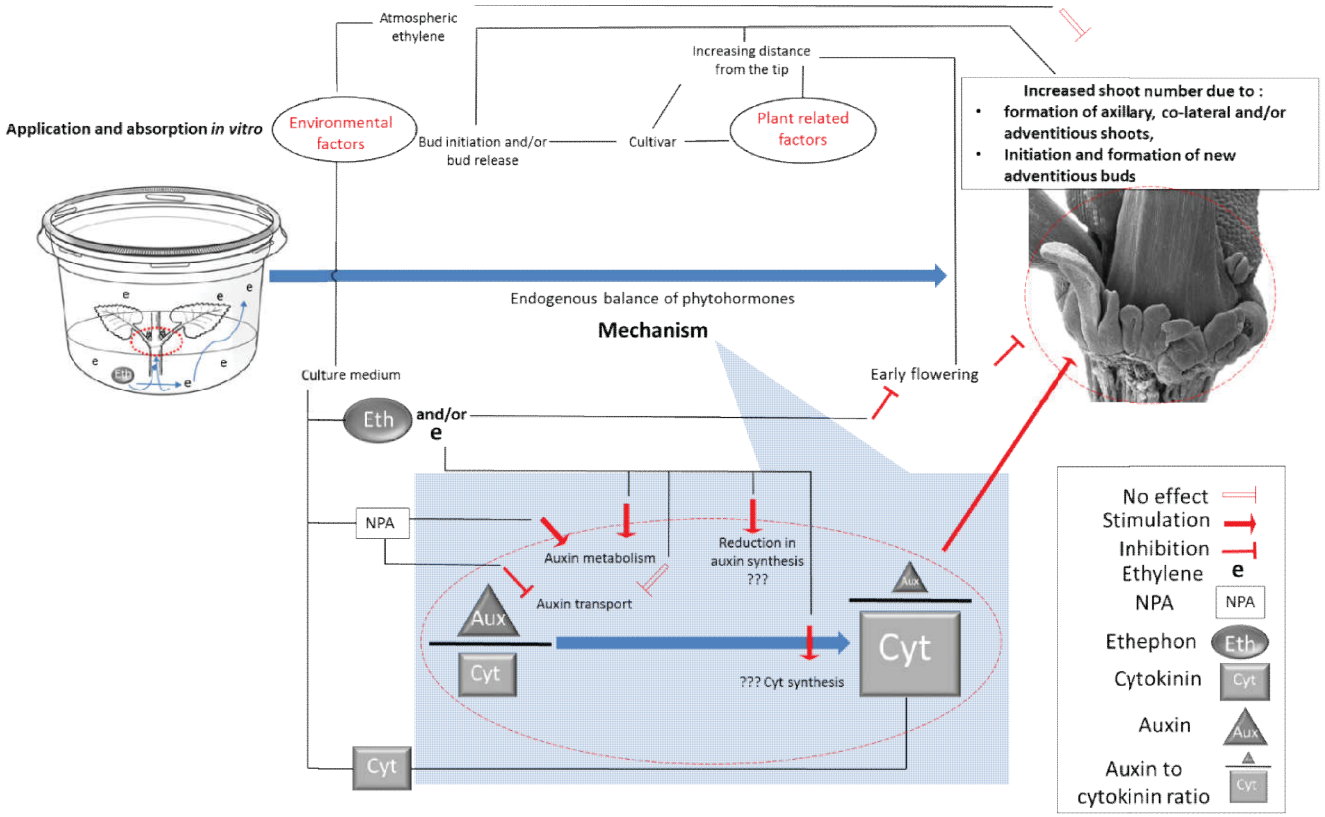


Figure 8- 1 Diagram of the proposed model for the mechanism by which ethephon/ethylene (e), released from the decomposition of ethephon within the culture medium stimulates shoot formation at the node position (red dotted circle) in explants of gentian 'Little Pinkie'. Ethephon and/or ethylene is proposed to reduce the endogenous ratio of auxin to cytokinin which could have occurred through the reduction in auxin and/or increase in cytokinin level at the node. Ethephon and NPA both resulted in an increase in shoot formation in 'Little Pinkie', which had originally been hypothesised to be through the inhibition of PAT. However, while NPA as a PAT inhibitor inhibited the basipetal transport of auxin, ethephon unexpectedly did not. However, rather than the inhibition of PAT, reducing endogenous concentration of auxin which result in lowering the ratio of auxin: cytokinin as a result of ethephon application might have occurred due to reducing auxin synthesis by ethylene as noted in the past, as well as changes in the metabolism of auxin as evident by the decrease in metabolite X that also occurred following NPA application. Exogenous application of cytokinins also increased shoot formation in 'Little Pinkie' which provided the evidence to support the hypothesis that lowering auxin: cytokinin ratio resulted in increased shoot formation. Ethephon/ethylene therefore was hypothesised to increase the endogenous level of cytokinin and consequently reduce the ratio of auxin: cytokinin at the node of the explants. Increase in shoot number following ethephon application resulted from, stimulating pre-existing axillary buds and development to shoots, perhaps sequentially followed by formation of co-lateral shoots and/or adventitious shoots, as well as development of new adventitious buds. Alteration in the endogenous balance of phytohormones toward lowering the ratio of auxin: cytokinin by ethephon was hypothesised to be the mechanism to stimulate buds and increase shoot number. The difference on shoot formation between cultivars of gentian as well as between explants taken from positions with various distance from the tip, were also hypothesised to be attributed at least partly to the difference in their endogenous balance of phytohormones such as auxin: cytokinin ratio.

8.2. Ethephon and shoot formation - initiation, release and outgrowth of various bud primordia

In the current research, ethephon stimulated additional shoots (up to 20 to 25; refer to Appendix 12) to develop, while a maximum of two shoots are normally expected to develop from the two pre-existing axillary buds within a typical metamer of gentian (refer to Chapters 2 & 3) (Ho, 2001; Struwe & Albert, 2002). Additional shoots that developed following the application of ethephon resulted from stimulating and accelerating the release of existing axillary buds and/or formation and/or release of accessory bud primordia as well as formation and/or development of adventitious bud primordia, at the node of 'Little Pinkie' (refer to Chapter 3, Figure 3-11). Pre-existing axillary buds were located in the axil of leaves or associated with it, probably co-lateral buds in close association with axillary buds, and adventitious buds developed at other locations around the node not associated with axillary and co-lateral buds. Therefore, different mechanisms might be expected to be involved in formation of the various types of shoots (co-lateral and/or adventitious), and development of new adventitious buds as well as pre-existing axillary buds to shoots. It is likely that there might be a chronological sequence of bud/shoot formation, as previously was reported in a study on crown bud formation in gentian (Samarakoon et al., 2016). In gentian cultured *in vivo*, with multiple crown bud primordia Samarakoon et al. (2014) the first accessory bud probably originated from the axillary bud, and the second accessory bud from the first one (Shah and Unnikrishnan, 1969; Shah and Unnikrishnan, 1971). Therefore, in gentian cultured *in vitro*, pre-existing axillary buds were probably the first buds to release and develop shoots following by formation of co-lateral shoots from buds which were probably located close to axillary buds. Development of adventitious shoots compared to other types of shoot probably required more time for firstly, initiation and development of bud followed by bud release and making vascular connection to the main stem. Therefore, adventitious shoots were probably the last type of shoots to develop. Ethephon application seem to stimulate all bud types and their development, as evident by developing up to about 20 to 25 shoots while only two were expected (Figure 8-1; refer to Chapter 3). Also, in addition to the increase in the number of shoots developed from the node (2° shoots), regardless of their origin, ethephon application also increased development of 3°shoots (from subsequent axillary buds on 2° shoots) in 'Little Pinkie' (refer to Chapter 6, Table 6-1). Therefore, it was suggested that development of shoots from all bud types as a result of ethephon application was probably due to the involvement of similar mechanism for all. Also, number of shoots in 'Little Pinkie' increased

even in the absence of ethephon, as the distance of the position that explants were taken from increased from the tip. Observation of up to 15 shoots by some individual explants taken from the basal node compare to only one in the explants from the tip, even in the absence of ethephon (refer to Chapter 2, Section B), was attributed to a low auxin level and low ratio of auxin: cytokinin, which therefore provided a clue for the mechanism of ethephon's effect. Hence, as detailed below, alteration in the endogenous balance of phytohormones such as auxin and cytokinin via lowering the ratio of auxin: cytokinin was hypothesised to be the mechanism by which ethephon resulted in the morphological change of the increased number of shoots as well as new bud primordia.

Integral to the hypothesis presented in Figure 8-1, low auxin: cytokinin ratio is required for bud initiation and release (Leyser, 2009). In nodal explants of 'Little Pinkie' cultured *in vitro*, initiation of additional buds accessorily (associated with pre-existing axillary buds) as well as adventitiously (individual buds far from axillary buds) as a result of ethephon application was probably due to the effect of ethephon on lowering auxin: cytokinin ratio. Subsequently, during bud outgrowth, buds produce their own auxin which would make the vascular connection to the stem (Aloni et al., 1995; Levyadun and Aloni, 1995; Aloni et al., 2000). At the time of insertion of the explants into culture medium, no vascular traces connecting the pre-existing axillary bud primordia to the vasculature of the main stem were found probably because they were not developed enough at that stage (refer to Chapter 3, Figure 3-4). However, at the end of growth cycle of the explants *in vitro*, axillary shoots developed and their vascular traces located in close association with where the leaf vascular traces left the vascular ring of the stem at the axil of leaves. Therefore, bud primordia after initiation and release by ethephon, produced their own auxin which developed vascular connection to the main stem for axillary buds and in case of co-lateral buds to the vascular system of axillary buds. There are reports on positive but indirect effect of ethephon on regeneration of xylem and tracheid in other plants (Miller et al., 1984; Eklund and Little, 2000; Pramod et al., 2013), been suggested to be through auxin and/or cytokinin that supports the main hypothesis of this research. However, some aspects of the complete mechanism may differ between various pathways of shoot formation from buds of different origin which requires future investigation (refer to Chapters 6, 7 & 3).

8.2.1. Cultivar difference

As illustrated in Figure 8-1, cultivars of gentian differed in number of shoots that arose from their node in response to ethephon. This difference was morphologically due to the difference in capability of these cultivars to produce different number of bud primordia at their node, which was hypothesised to result from the difference in endogenous hormonal balance and sensitivity of their cells at the node. As evident by the microscopic images taken from the node region of nodal explants of 'Little Pinkie' at the end of their culture cycle in vitro, bud primordia (axillary, co-lateral and/or adventitious) were located anywhere around the node in the absence of ethephon, developed to shoots and/or increased in number, in the presence of ethephon. Therefore, although differentiated parenchymal cells of the stem at the node, perhaps were not expected to actively divide (meristematic) and initiate adventitious and/or co-lateral buds, the node region of 'Little Pinkie' was meristematically active (refer to Chapter 3, Figure 3-7). In contrast, in 'Showtime Diva', regardless of ethephon application, apart from two axillary bud primordia at the node, no other meristematic activity (bud) was evident (refer to Chapter 3, Figure 3-15). Such a unique characteristic of having meristematic activity all around the node (not only associated with axillary buds) presumably resulted from the difference in sensitivity of cells in 'Little Pinkie' to endogenous phytohormones, compare to 'Showtime Diva'. Application of ethephon was hypothesized therefore to affect shoot formation through alteration of the endogenous balance of phytohormones, and as described in the next section, particularly auxin: cytokinin ratio within the cells, at the node region of explants. However, the effect of ethephon application in the medium on increasing shoots in explants of 'Showtime Diva' was much smaller (refer to Appendix 13).

8.2.2. Position of the explant- distance from the tip

Ethephon and/or ethylene released from ethephon in the *in vitro* culture medium, as one of the environmental factors, increased shoot formation (axillary, probably co-lateral and/or adventitious) in explants of gentian 'Little Pinkie', especially in basal explants, as well as development of new adventitious bud primordia (refer to Chapter 3, Figure 3-11). As shown in Figure 8-1, in 'Little Pinkie' in the absence of ethephon, there were clearly more bud primordia in the explant as the distance of their position increased from the tip (refer to Chapter 2, Figure 3-13). Some individual explants from the base produced up to 15 shoots which was probably due to the age of those explants making their leaves less efficient to produce auxin and consequently low level of auxin at their node. In presence of ethephon

positional difference in the number of shoots arising from node was still observed and even magnified depending on the concentration of ethephon (up to 20 to 25 shoots in basal cuttings; Appendix 12). In tip cuttings however, generally fewer than two shoots formed as a result of ethephon application, suggesting that any shoots that did grow out were axillary. While not quantified in this thesis, it is likely that the difference between positions in development of shoots arised from the node (2º shoots) was probably due to diverse physiological conditions prevailing in the explant used, such as endogenous levels of phytohormones (Emery et al., 1998), difference in the sensitivity of cells to endogenous phytohormones and interaction with hormonal receptor (Trewavas, 1983; Firn, 1986), and differences in tissue age (Santana-Buzzy et al., 2005; D'Onofrio and Morini, 2006). Differences in shoot formation with increasing distance of the explant's position from the tip along the donor shoot, is also consistent with previous reports by others on a controlling mechanism through alteration of the endogenous hormonal balance (Emery et al., 1998; Santana-Buzzy et al., 2005). Explants taken from the position closer to the tip produced less number of shoots probably because their cells were less differentiated than the explants taken from the positions close to the base (Marcelis-Van Acker, 1994). However, explants taken from the tip position were more committed to produce early flowering *in vitro* compare to the explants taken from the basal positions due to being physiologically 'less juvenile' (Fortanier and Jonkers, 1975)(refer to Chapter 2, Section B). Therefore, strategies such as rejuvenation of explants, by taking them from 2º shoots which had been developed from the base position and sub culturing explants regularly at durations not longer than 5 weeks, reduced flowering in explants. As illustrated in Figure 8-1, the increase in the number of 2º shoots developed by ethephon and by increasing the distance of the position of the explant from the tip, was consistent with the hypothesis previously stated in removing apical dominance as well as lowering the endogenous ratio of auxin: cytokinin, and supports the background hypothesis for how ethephon might be working (Shimizu-Sato and Mori, 2001; Kalousek et al., 2010b) (refer to Chapter 1, Section 1.2.5).

8.3. Hormonal mechanism of increased shoot formation by ethephon

Increasing effect of ethephon on number of shoots raised from the node (axillary, co-lateral and/or adventitious) of 'little Pinkie' was hypothesised to result from alteration of endogenous balance of phytohormones and in particular lowering the ratio of auxin: cytokinin (Skoog and Miller, 1957). Low ratio of auxin: cytokinin could have either resulted from low concentration of auxin or high level of cytokinins. Ethephon's effect on increasing

number of shoots therefore was hypothesized to be through reduction of auxin level by inhibition in polar auxin transport and/or increase in cytokinin level by stimulating cytokinin synthesis as discussed below.

8.3.1. Ethephon on transport and metabolism of auxin

There has been extensive evidence from the past to support the hypothesis of inhibitory effect of ethephon/ethylene on PAT (Went, 1941; Ernest and Valdovinos, 1971; Goldsmith, 1977; Luthen, 2015), which together with other mechanisms such as conjugation of IAA to inactive products (Riov and Yang, 1982) and IAA catabolism (Beyer and Morgan, 1970; Wood, 1985; Sagee, 1990; Winer et al., 2000) as well as the increase in IAA metabolism (Ernest and Valdovinos, 1971) resulted in reducing endogenous IAA concentration following by lowering the ratio of auxin: cytokinin. In the current study, ethephon and NPA both increased shoot formation in 'Little Pinkie', which was an evidence to support the hypothesised effect of ethephon on reducing auxin level through the inhibition of PAT (refer to Chapter 6, Figure 6-1). However, once basipetal transport of auxin was quantified, NPA as a PAT inhibitor inhibited the basipetal transport of auxin whereas ethephon did not, which was unexpected (refer to Chapter 6, Figure 6-15 & Table 6-5). Several possibilities could be responsible for the results achieved here which are strongly suggested to be considered in future investigations on the effect of ethephon on auxin level. The effect of ethylene released from ethephon on reducing endogenous auxin synthesis has been previously noted (Burg and Burg, 1966b; Hansen and Grossmann, 2000) that provided strong probability of an alternative mechanism in current study for ethephon's effect on increasing shoot formation through lowering the ratio of auxin: cytokinin. However, some possibilities required further investigations in future before the hypothesised inhibitory effect of ethephon on PAT was rejected. First possibility was due to not enough concentration of ethylene during auxin transport, in the system to inhibit the transport of auxin. Inadequate concentration of ethylene probably resulted from inadequate duration of ethephon pre-treatment (2 weeks). Also, it was possible that concentration of ethylene reduced due to being volatised and became unavailable soon after taking away from the source of ethephon/ethylene (agar medium). Hence, a continuous exposure to the source of ethylene (ethephon in the medium) was probably required in order to inhibit PAT (refer to Experiment 2 of chapter 6). Another possibility was related to utilising non-responsive plant material due to being physiologically at their flowering stage, which presumably made them different in their endogenous balance of phytohormones compare to that in vegetative stage (refer to Chapter 2, Section B).

Therefore, it is recommended in future repetition of this experiment to include ethylene/ethephon in the system (e.g. gaseous ethylene or ethephon in donor agar) during the auxin transport and increase the number of replicates to keep some plant material until the end of *in vitro* growth cycle to quantify and confirm the explant's response to ethephon.

Another observation was made in current thesis which was in line with the possibility of inadequate concentration of ethylene being responsible for not affecting the PAT. While ethylene had been hypothesised to be the most influential component of ethephon decomposition on shoot formation in 'Little Pinkie', it did not affect shoot formation once applied on the explants through the atmosphere, at the concentration similar to that quantified in vessels containing ethephon (refer to Chapters 4 & 5) (Figure 8-1). In addition, exposing explants to ethylene released from ethephon, without being in direct contact with ethephon-containing medium did not also affect shoot formation in explants (refer to Chapter 5, Figure 5-6). Only explants been able to directly absorb ethephon from the medium showed the characteristic response of increased shoot formation. Therefore, it was likely that higher concentration of ethylene than that used through the atmosphere was required to make the influence. Also, while quantitative measurements was not done in current research on the endogenous concentration of ethylene in explants, and as it was expected for vegetative tissue, application of ethephon did not cause autocatalytic ethylene production in explants of gentian, in contrast to that reported in fruit tissue (Burg and Burg, 1965; Rhodes, 1980; Hoffman and Yang, 1982) (refer to Chapter 5, Section 5.1.1), it was hypothesized that endogenous concentration of ethylene released from continuous exposure of ethephon in the culture medium was higher than that at the atmosphere to make the response. Therefore, it is likely that concentration of endogenous ethylene in explants been treated with ethephon in their medium for only two weeks and then been taken away from that, was not adequate to inhibit the PAT. Future experiments is needed to generate further light on this possibility.

While most of the previous studies on the application of ethephon on various crops have been focused on only atmospheric ethylene (Domir and Foy, 1978; Kong and Yeung, 1994; Haver et al., 2002), findings of current research indicated that application of atmospheric ethylene did not increase the shoot number. Utilizing ethylene inhibitors such as AVG, 1-MCP, etc could be used to investigate the involvement of ethylene released from ethephon on shoot formation. However, findings of current research indicated that increasing shoot response occurred only if tissue was in direct contact with the medium amended with ethephon (Chapter 5, Section 5.1.2). Therefore, direct absorption of ethephon by the tissue was needed

to make the effect. Effect of ethephon could be related to the metabolites of ethephon within the plant tissue as it broke down to ethylene over the long-term (Foster et al., 1992).

In current research, application of ethephon did not seem to affect auxin metabolism in basipetal direction but resulted in a slight increase in the metabolites in acropetal direction in 'Little Pinkie', which was in line with other's reports in the past (Beyer and Morgan, 1969; Ernest and Valdovinos, 1971; Wood, 1985; Sagee & Goren, 1990) (refer to Chapter 6, Figure 6-15). However, ethephon reduced the quantity of a specific IAA metabolite such as metabolite X which also occurred as a result of NPA application and therefore was hypothesised to play a role in the increased shoot formation response in 'Little Pinkie'. Future investigation on identification of this metabolite as well as quantification in presence and absence of ethephon would assist to test this hypothesis.

As illustrated in Figure 8-1, cultivars of gentian cultured *in vitro* probably differed from each other in their endogenous balance of phytohormones. Ethephon was unlikely to cause autocatalytic ethylene production in explants (refer to Chapter 5). Also, findings of the current research did not support the hypothesis that ethephon increased shoot formation in cultivars of gentian following the inhibition of PAT. However, there was a difference between cultivars of gentian in the metabolism of auxin (refer to Chapter 6, Figure 6-16). Control explants of 'Little Pinkie', as a cultivar with high number of shoots, had more IAA metabolites in their bottom segments and more metabolite X in top segments compared to 'Showtime Diva' as a cultivar with low number of shoots. Metabolite X in 'Little Pinkie' decreased as a result of both ethephon and NPA application and therefore hypothesized to play a role in the increased shoot formation response. In contrast to 'Little Pinkie', 'Showtime Diva' did not show significant change in the metabolism of auxin and no reduction in metabolite X, which was hypothesised to be the reason for the difference between cultivars in their shoot formation response. In support of the observations in the current study on the difference in the IAA metabolism between cultivars of gentian, other people have also noticed differences in IAA metabolism between various plant species and genotypes in IAA metabolism (Feung et al., 1975; Nakamura et al., 1985; Kohler et al., 2004). Therefore, another new hypothesis to investigate would be on the possibility of involvement of metabolite X on shoot formation, which can be tested in future by quantification and comparing the metabolite in presence and absence of ethephon in cultivars of gentian which are naturally of different shoot formation habit.

8.3.2. Ethephon on cytokinin level

As illustrated in Figure 8-1, application of cytokinin in the culture medium of the explants in current study, increased shoot formation which was similar to the effect of ethephon on increasing shoot number (refer to Chapter 7, Figure 7-1). As any increase in cytokinin supply would contribute in decreasing the ratio of auxin: cytokinin, these results provided the evidence to support the hypothesis that lowering auxin: cytokinin ratio (Christianson and Warnick, 1983; Coenen and Lomax, 1997) was responsible for the increase in shoot formation (Sudha et al., 1998; Huang et al., 2000; Haver et al., 2002). Ethephon/ethylene therefore was hypothesised to increase the endogenous level of cytokinin and consequently reduce the ratio of auxin: cytokinin at the node of the explants resulting in an increase in shoot formation. This hypothesis was tested and supported by the data enhancing the concentration of cytokinin on petunia (Haver et al., 2002). However, future experiments on the quantification of endogenous cytokinins as a result of ethephon application would be needed to support the result in gentian.

As explored below, in current study the production of more shoots by explants exposed to either cytokinins or ethephon, presumably resulted from extra bud primordia which developed either accessorily and/or adventitiously at the node position. In the absence of PGRs (Control), explants of 'Little Pinkie' produced a maximum of two shoots which, as detailed in Chapter 3, arose from axillary buds. This is compared to explants exposed to ethephon and cytokinins which produced more than 2 shoots. Both microscopic and macroscopic evidence presented in Chapter 3 illustrated that ethephon had its effect on increasing shoot formation by stimulating co-lateral and/or adventitious buds to form shoots and initiate new adventitious buds. While in the current experiment microscopic examination was not undertaken of the node position of explants exposed to cytokinins, given both the number of shoots (more than two) and location of shoots (all around the node; associated and non-associated with the location of axillary buds), it was considered most likely that the extra shoots were derived either accessorily or from adventitious buds. Similarly, effect of cytokinins on stimulation of explants to produce adventitious shoots has been reported by others previously (Kim and Ernst, 1994; Peres and Kerbauy, 1999; Huang et al., 2000). In another study also, encouraging effect of cytokinins on formation of multiple (more than two) shoots has been noted, which was interpreted to be from a combination of adventitious and axillary buds (Sudha et al., 1998). Different cultivars of gentian with different number of bud primordia at their node and different response to ethephon in shoot formation are also

hypothesised to differ in their endogenous content of cytokinins that is strongly suggested to be quantified in future.

In current study, increase in shoot formation from the explants treated with exogenously applied cytokinin, as the distance of their position increased from the tip, provided evidence to support the hypothesis that the auxin: cytokinin ratio was also the mechanism underlying the increase in shoot formation from bud primordia in 'Little Pinkie'. As the distance of the position of the explants increased from the tip, number of bud primordia at the node increased (refer to Chapter 3, Figure 3-13). Therefore explants were potentially capable of producing propagules with more number of shoots (refer to Chapter 2, Figure 2-6), being hypothesised as attributed to a decreasing trend in the ratio of the auxin: cytokinin. In the natural situation most of the bud primordia (axillary, co-lateral and/or adventitious) at the node, remain dormant and, only one to two buds develop to shoots probably due to apical dominance. Exogenous application of cytokinin on explants increased shoot formation in explants as the distance of the explant increased from the tip, which supported the hypothesised effect of increasing cytokinin level on increasing shoot formation (refer to Chapter 7). Also, shoot formation was increased even more as the concentration of exogenous applied cytokinin increased. Cytokinins would be expected to affect shoot formation by contributing to the outgrowth of dormant buds, under apical dominance, by triggering buds to export auxin (Tanaka et al., 2006; Kalousek et al., 2010a). As noted within Section 8.1, vascular traces are needed to connect bud primordia to the main stem, and stimulating the development of vascular traces is under control of the ratio of auxin: cytokinin. Increasing shoot formation, as the distance of the position that explants originated from increased from the tip, following the exogenous application of cytokinin, therefore was suggested to be due to initiation and/or releasing more number of bud primordia due to the reduction in the ratio of auxin: cytokinin. In gentian however, quantitative measurements of the endogenous concentration of phytohormones in various positions along the shoots remains to be done in future.

8.4. Ethephon and *in vitro* flowering

Within this study, apart from direct effect on shoot formation increase in 'Little Pinkie', ethephon as reported in Appendices 15 & 16, indirectly increased number of shoots through reducing frequency of flowering explants *in vitro*. Given the determinate shoot growth habit of gentian, initiation of flowering *in vitro* might be expected to affect the vegetative growth of the apical bud, imposes an end to vegetative growth of the explant and, therefore, is

unwanted. Reduction in frequency of flowering was also affected by the position of the explant, which again was hypothesized to be through modifications in endogenous hormonal balance within the explant (Figure 8-1). Propensity of explants to flower which decreased as the distance of the position of the explant increased from the shoot tip (refer to Chapter 2, Section B) has been suggested to be related to the difference in the type of buds, juvenility and capacity of their cells to respond to a gradient of a floral promoter, inhibitor or their combination, existing in each position (Wardell and Skoog, 1969; Fu et al., 1995). Meristems forming at the tip probably are of young tissue but physiologically 'less juvenile' (Fortanier and Jonkers, 1975) due to experiencing more number of cycles of cell division to make various positions, and therefore hypothesised to differ in their sensitivity to, and/or concentration of, floral promoters and/or inhibitors (Firn, 1986) and more committed to produce flowers, compare to meristems forming on the lower part of the stem. Effect of ethephon on reducing flowering frequency in 'Little Pinkie' (cultivar of gentian more susceptible to early flowering) (refer to Appendices 15 & 16), as well as in 'Showtime Diva' (less susceptible to early flowering; data not published), which changed the fate of cells already committed to differentiate and develop flowers to grow into vegetative shoots, provided another evidence of hypothesized ethephon's effect on manipulation of endogenous balance of phytohormones toward changing the morphology of the explants. While current research was not focused on providing further data to support this hypothesis, ethephon probably lowered the ratio of auxin: cytokinins which triggered vegetative growth.

Within this study, exposing explants to short photoperiod (8h) and cold temperature (2ºC) completely inhibited *in vitro* flowering (refer to Chapter 2, Section B), probably provided an opportunity for inducing cessation of active vegetative growth and development, perhaps resetting the endogenous phytohormones levels and developmental programs controlling biological activities for the next growth season (Horvath et al., 2004). As far as manipulation of endogenous level of phytohormones by exogenous application of PGRs on shoot formation and *in vitro* flowering is concerned, application of gibberellins (GA_3) in the medium increased shoot development but even at high concentration (up to 0.2 $mg.L^{-1}$) did not induce *in vitro* flowering in gentian (Zhang and Leung, 2002). In contrast, application of BA (cytokinin) resulted in a high level of *in vitro* flowering while it increased shoot formation. Therefore, gibberellins are also another class of phytohormones that are likely to play a role in shoot formation and/or *in vitro* flowering due to their effect on axillary bud outgrowth. In some plant species such as citrus, snapdragon and sweet cherry, gibberellin stimulated

axillary bud development resulted in enhancement in shoot formation (Marth et al., 1956; Elfving et al., 2011). Also, reports were found on the interactions between gibberellin and cytokinins in the positive control of axillary bud outgrowth while strigolactones inhibited shoot formation (Ni et al., 2015). In gentian, application of GA_3 increased emergence of crown buds to shoots *in vivo* (Samarakoon et al., 2016) and development of shoots from the leaf axil of nodal explants cultured *in vitro* (Zhang and Leung, 2002). Shoot formation can be thought of as four steps – initiation of the meristem, development of the bud, release of the bud and finally subsequent growth of the bud. Gibberellins are normally involved in subsequent growth, and also release from dormancy in specific cases involving temperature and day length. Therefore, although not the focus in current study, the effect of gibberellins on the growth and development of adventitious shoots is suggested to be investigated in future for similarity of their effect to ethephon on shoot formation.

Although as a comparatively recently discovered plant growth regulator, data on the interaction between ethylene and strigolactones has been understandably limited, a crosstalk between auxin, cytokinin and strigolactones in root formation has been suggested (Vanstraelen and Benkova, 2012). Analysis of the effect of hormonal treatments on root hair elongation in PGR-signalling mutants of *Arabidopsis* has led to the hypothesis that strigolactones and ethylene regulate root hair elongation via a common regulatory pathway, in which the effect of strigolactones on root hair elongation requires ethylene synthesis. Auxin signalling was not necessary, but enhanced root hair response to strigolactones, implied that the strigolactone and auxin hormonal pathways are connected for regulation of root hair elongation. The ethylene pathway requirement for the root hair response to strigolactones was used to hypothesise that ethylene forms a cross-talk junction between the strigolactone and auxin pathways (Kapulnik et al., 2011; Koltai, 2011). Based on previous reports indicating the regulatory effect of ethylene on auxin biosynthesis and transport in roots (Růžička et al., 2007), it was then suggested that ethylene is part of the mechanism by which strigolactones regulate the auxin transport capacity in their control over root architecture (Koltai, 2011).

It seems possible that the crosstalk between auxin, cytokinin and strigolactones is also involved in other functions of strigolactones involving auxin, such as bud outgrowth and shoot formation. Initiation of bud outgrowth has been believed to be correlated with exporting auxin from buds (Muller and Leyser, 2011) and therefore, there would be a competition among all buds for releasing their auxin into the main PAT stream in the stem.

Active buds export auxin and therefore inhibit the auxin export from other buds into the PAT stream (Sachs et al., 1976; Domagalska and Leyser, 2011). As a result, active buds will grow but the rest of the buds remain suppressed. In highly branched mutants of Arabidopsis, auxin transport was higher than the wild type but decreased following the application of an auxin transport inhibitor and resulted in a significant inhibition of bud outgrowth (Ishikawa et al., 2005; Bennett et al., 2006; Arite et al., 2007). Basal application of synthetic strigolactone analogue, GR24, also reduced basipetal auxin transport in wild type and some mutants (Crawford et al., 2010). In another study, application of GR24 directly to the axillary buds of pea plants that were strigolactone deficient, inhibited shoot branching that for many years was thought to be related to only auxin, cytokinin and abscisic acid (Gomez-Roldan et al., 2008). Therefore, strigolactones was suggested to inhibit shoot branching via its regulation on auxin transport. From the results of the current thesis, ethylene released from ethephon increased shoot formation in 'Little Pinkie' which was opposite to what was reported by Gomez-Roldan et al. (2008) on shoot formation affected by strigolactones. Therefore, it is also possible that rather than the direct inhibition on PAT, ethylene may act through reducing strigolactones. To support this possibility, in a study using Strigolactone-deficient plants, concentration of cytokinins in the xylem reduced while apically derived auxin was present (Beveridge, et al., 1997). Also, number of shoots developed from axillary buds, which had been stimulated by cytokinin, were reduced by application of strigolactones in Zantedeschia (*in vitro*) and pea stems (Manandhar, 2016). Therefore, it has been suggested that there might be an antagonistic interaction between cytokinins and strigolactones on bud release. In doing so, the reduction in endogenous concentration of strigolactones by ethephon, could be responsible for increasing cytokinins which would probably lead to the hypothesised reduction in the ratio of auxin: cytokinin and increased shoot formation. Bud release may also cause by down-regulation of the BRC1 gene (a gene regulator that inhibits bud outgrowth) (Braun et al., 2011) and therefore, reduction in the expression of BRC1 may increase the sensitivity of the buds toward cytokinin present within the buds. Strigolactones increase the expression of BRC1 but cytokinin decrease it (Braun et al., 2011). Other hormones such as abscisic acid which has an important role in endo-dormancy of bud (Hocking & Hillman, 1975) may also have a role in shoot branching regulation in perennial plants. Therefore, in the current research on 'Little Pinkie' as a perennial plant, it is likely that this crosstalk between auxin, cytokinin, abscisic acid and strigolactones is involved in shoot formation treated with ethephon. Hence, the possibility of increasing shoot number in 'Little Pinkie' treated with ethephon through direct or indirect (through other hormones) effect on reducing

strigolactones remains to be investigated in future. Also, previously it has shown that, the concentration of strigolactones in the guttation fluid differed between high and low branched cultivars of Zantedeschia grown *in vivo* (Manandhar, 2016). Although investigations on the concentration of strigolactones in the guttation fluid of gentian were carried out during research for this thesis, results were not obtained due to the difficulties in collection of guttation fluid. However, further attempt in future are suggested in order to quantify differences in concentration of strigolactones between cultivars of gentian such as 'Little Pinkie' with high branching growth habit compare to 'Showtime Diva' with low branching growth habit.

8.5. Summarized future direction

Future investigations using the results of current research has been mentioned in earlier sections of this thesis in detail. However, a brief summary is presented as below:

- Repeating the experiment to test the hypothesized inhibitory effect of ethephon/ethylene on PAT by including ethylene/ethephon in the system (e.g. gaseous ethylene or ethephon in donor agar) during the auxin transport and increasing number of replicates and keep some plant material until the end of *in vitro* growth cycle to quantify and confirm the explant's response to ethephon.

- Investigation on identification and quantification of various IAA metabolites in particular, metabolite X, in cultivars of gentian in presence and absence of ethephon.
- Quantification of endogenous concentration of phytohormones (auxin and cytokinins) in explants of various positions along the shoots in different cultivars of gentian in presence and absence of ethephon
- Investigation over the complete mechanism of various pathways of shoot formation from buds of different origin
- Macroscopic and microscopic investigation on the effect of gibberellins and cytokinins on shoot formation compare to the effect of ethephon
- Investigation over the hypothesised involvement of specific metabolites or chemical component of ethephon on shoot formation, utilizing ethylene inhibitors such as AVG, 1-MCP, etc.

- Quantification of endogenous concertation of ethylene in the explants, as well as the amount of ethephon remaining in the medium
- Microscopic investigations over the effect of various concentrations of ethephon on bud initiation and alteration in shoot formation in cultivars with low and high frequency of shoot formation.
- Investigation over the positive but indirect effect of ethephon on regeneration of xylem and tracheid in gentian and study various aspects of the complete mechanism that may differ between various pathways of shoot formation from buds of different origin in gentian 'Little Pinkie'.

8.6. Protocol improvement, research and commercial implications

Result of current research as listed below, can be applied to improve protocols to benefit future research as well as commercial production of pot plants of gentian 'Little Pinkie' with high number of shoots.

- Ethephon is suggested to be included in the *in vitro* culture medium to increase the number of shoots in explants and produce propagules with high number of shoots (more than two), which can be used to produce pot plants with high number of shoots.

- Cytokinins also produced similar encouraging effect to ethephon's effect on shoot formation *in vitro* and could be utilised to produce propagules and pot plants with high number of shoots if for any reason utilising ethephon would not be possible. However, application of cytokinins may not be cost efficient because instead of concentration of 0.3 $mg.L^{-1}$ which has been used in common protocols in the tissue culture lab on gentians so far, high concentration of 9 $mg.L^{-1}$ was shown to be needed to increase shoot formation (refer to chapter 7).

- Prior to this research, there was no reported method to measure the concentration of ethylene in vessels capable of gas exchange *in vitro*. The protocol that was developed could be suggested to be applied in other tissue culture labs in the future (refer to Chapter 4).

- As a result of current research, a detailed methodology was developed that could be applied in future investigations on polar transport of auxin in explants cultured *in vitro*.

- Microscopic observation on the unique meristematic activity at the node of the explants of 'Little Pinkie' can be of future application in researching the mechanism of adventitious bud initiation and development *in vitro*.

Prompted by problematic situations arising, additional investigations carried out throughout this thesis resulted in outcomes which can be applied to improve the current protocols for generating propagules, and are of the value for commercial application:

- The knowledge quantifying the growth response of plantlets originating from various positions along the donor shoot, is of commercial value (refer to Chapter 2). Explants derived from upper nodes produced plantlets of the greatest uniformity, but not with high number of shoots, whereas explants from the lower nodes produced plantlets which produced high number of shoots but not uniform in their growth. Given that for commercial propagation it is important to get uniform plantlets that produce a uniform number of usable explants, explants from the lower nodes might not be ideal as a source of uniform plant material. There was a significant non-uniformity in growth response of explants from the lower nodes, but they would give explants with high number of shoots appropriate for producing high-quality pot plants. Therefore, modifying the protocol of the propagation system from mass propagation to selective propagation is suggested; upper nodes which would give more uniform plantlets can be used for continuous culture; but from the same original plantlet, lower nodes can be used to generate material with high number of shoots to be used for production of potted plants. Explants from the Base position which have not previously been included as plant material for sub culturing cycles in the tissue culture lab, as a result of current study, are suggested to be used as propagules for production of potted plants because they were naturally capable of producing propagules with the most number of shoots compared to other positions (refer to Chapter 2). Basal explants can be treated with either cytokinin or ethephon in order to produce propagules with even more number of shoots (refer to Chapter 7) within a commercial situation.

- Some of underlying reasons for observation of unwanted early flowering in pot plants of gentian, as well as in the propagules of gentian cultured *in vitro*, were identified and modifications to the protocol are suggested to reduce it (refer to Chapter 2). Exposing explants to a short photoperiod (8h) and cold temperature (2ºC) for a duration of more than 7 weeks is suggested to inhibit early flowering *in vitro*

completely. Also, other strategies detailed below can be used to inhibit early flowering to some extent. Removing explants taken from the tip with the highest propensity to flower, and using nodes from subtending positions is suggested to increase the multiplication rates. Also, a maximum of 5-week sub-culturing interval, in the absence of ethephon, is suggested for *in vitro* production of propagules of gentian 'Little Pinkie' (refer to Chapter 2). Ethephon application is suggested to decrease flowering and increase the number of shoots However, for producing potted plants with high number of shoots, future research might benefit from transplanting explants into a PGR-free medium after a duration (between two to four weeks) exposure to ethephon (refer to Chapter 5) in order to release those shoots into rapid growth. Rejuvenation of explants by taking them from the base position, as well as application of the culture medium with half MS and free of PGRs are also other strategies suggested to decrease the frequency of early flowering (refer to Chapter 2).

- Although early flowering might have disadvantages within the propagation system but 'Little Pinkie' was introduced as a cultivar susceptible to flowering *in vitro* which could be beneficial to research and of commercial applications. Flowering *in vitro* is suggested to provide a controlled system to study molecular and genetic mechanisms of flower induction and morphogenesis, as well as the commercial production of flowers and specific compounds from floral organs. In breeding programmes, *in vitro* flowering is suggested to be used to speed up the process of multiple generational cycles, enabling pre-selections of progenies based on likely breeding objectives including flower colour. For gentian as a perennial plant which typically has a juvenile phase of three years from seed, *in vitro* flowering or *in vitro* induction of flowering is suggested to be of potential value in commercial applications to reduce the time to flower as it has been applied in gentian breeding programs (Ed Morgan, pers. comm.).

8.7. Conclusion

As a result of ethephon application, shoots (axillary, co-lateral and/or adventitious) were developed, and also additional adventitious buds were initiated, at the node of the explants of 'Little Pinkie', and resulted in production of propagules with high number of shoots. Although there might have been a chronological sequence of bud/shoot formation from various origins (axillary, co-lateral and/or adventitious buds), presumably result in various

shoot growth, ethephon seem to stimulate all of them. Therefore, morphologically the mechanism by which ethephon stimulated development of shoots was suggested to be similar for all types of buds. The unique characteristic of meristematic activity all around the node (not only associated with axillary buds) of the explants of 'Little Pinkie' (cultivar with high number of shoots) but not in 'Showtime Diva' (cultivar with low number of shoots), was hypothesised to result from the presence of a number of cells differing in their sensitivity to endogenous balance of phytohormones. Ethephon's effect on increasing number of shoots was hypothesized to be through lowering the ratio of auxin: cytokinin by reducing auxin concentration due to inhibition in polar auxin transport and/or increasing cytokinin level by stimulating cytokinin synthesis. Ethephon and NPA both increased shoot formation in 'Little Pinkie', which was an evidence to support the hypothesised effect of ethephon on reducing auxin concentration through the inhibition of PAT. However, once basipetal transport of auxin was quantified, NPA as a PAT inhibitor inhibited the basipetal transport of auxin whereas ethephon unexpectedly did not. Therefore, effect of ethephon on reduction of endogenous auxin concentration might have also resulted from reduction in auxin synthesis. Explants of 'Little Pinkie', as a cultivar with high number of shoots, metabolised more auxin, contained more metabolite X compared to 'Showtime Diva' as a cultivar with low number of shoots. As a result of application of NPA, metabolites including metabolite X reduced in 'Little Pinkie' whereas it did not change in 'Showtime Diva'. Application of ethephon did not seem to be effective on the IAA metabolism in basipetal direction but resulted in a slight increase in the metabolites in acropetal direction as well as reduction in metabolite X only in 'Little Pinkie'. Possible increase in the metabolism together with the reduction of auxin synthesis as a result of ethylene application, reduced endogenous concentration of auxin (although not quantified here) which in turn resulted in the reduction in auxin: cytokinin ratio and increased shoot formation in 'little Pinkie. Also, the increase in number of shoots produced in 'Little Pinkie' following the application of ethephon or cytokinin provided the evidence to support the hypothesis that lowering auxin: cytokinin ratio was the underlying mechanism responsible for the increase in shoot formation. Other influential factors on shoot formation such as increasing the distance of the position of explants from the tip along the donor shoot, was also suggested to be through lowering the ratio of auxin: cytokinin which might also affect early flowering in explants of gentian cultured *in vitro*.

Bibliography

Aharoni, N. and M. Lieberman. 1979. Ethylene as a regulator of senescence in tobacco leaf-disks. Plant Physiol. 64:801-804.

Allan, H.H. 1961. Flora of New Zealand. Government Printer, Wellington, New Zealand.

Aloni, R., P. Feigenbaum, N. Kalev, and S. Rozovsky. 2000. Hormonal control of vascular differentiation in plants: the physiological basis of cambium ontogeny and xylem evolution. Bios Scientific Publishers Ltd, Oxford.

Aloni, R., K.S. Pradel, and C.I. Ullrich. 1995. The 3-dimensional structure of vascular tissues in agrobacterium tumefaciens-induced crown galls and in the host stems of *Ricinus communis* l. Planta 196:597-605.

Aremu, A.O., L. Plackova, M.W. Bairu, O. Novak, L. Plihalova, K. Dolezal, J.F. Finnie, and J. Van Staden. 2014. How does exogenously applied cytokinin type affect growth and endogenous cytokinins in micropropagated *Merwilla plumbea*? Plant Cell Tiss. Org. Cult. 118:245-256.

Arigita, L., B. Fernandez, A. Gonzalez, and R.S. Tames. 2005. Effect of the application of benzyladenine pulse on organogenesis, acclimatisation and endogenous phytohormone content in kiwi explants cultured under autotrophic conditions. Plant Physiol. Biochem. 43:161-167.

Arigita, L., R.S. Tamés, and A. González. 2003. 1-Methylcyclopropene and ethylene as regulators of *in vitro* organogenesis in kiwi explants. Plant Growth Regu. 40:59-64.

Arite T., Iwata H., Ohshima K., Maekawa M., Nakajima M., Kojima M., Sakakibara H. & Kyozuka J. 2007. *DWARF10*, an *RMS1/MAX4/DAD1* ortholog, controls lateral bud outgrowth in rice. The Plant journal 51:1019–1029.

Audley, B.G. 1979. Structure and properties of a 2-chloroethylphosphonic acid (ethephon) metabolite from *Hevea brasiliensis* bark. Phytochem. 18:53-60.

Audley, B.G., B.L. Archer, and I.B. Carruthers. 1976. Metabolism of ethephon (2-chloroethylphosphonic acid) and related compounds in *Hevea brasiliensis*. Arch. Environ. Contam. Toxicol. 4:183-200.

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

Barkawi, L.S., Y.Y. Tam, J.A. Tillman, B. Pederson, J. Calio, H. Al-Amier, M. Emerick, J. Normanly, and J.D. Cohen. 2008. A high-throughput method for the quantitative analysis of indole-3-acetic acid and other auxins from plant tissue. Anal. Biochem. 372:177-188.

Barrick, W.E. and K.C. Sanderson. 1973. Influence of photoperiod, temperature, and node position on vegetative shoot growth of greenhouse azaleas, *Rhododendron* cv. J. Am. Soc. Hort. Sci. 98:331-334.

Bartlett, M. 1975. Gentians. Somerset, Hillman printers (Frome) Ltd.

Beaudry, R.M. and S.J. Kays. 1987. Effects of physical and environmental factors on the release kinetics of ethylene from (2-chloroethyl) phosphonic acid and (2-chloroethyl)-methylbis (phenylmethoxy) silane. J. Am. Soc. Hort. Sci. 112:352-359.

Becker, M., Y. Becker, K. Green, and B. Scott. 2016. The endophytic symbiont *Epichloe festucae* establishes an epiphyllous net on the surface of *Lolium perenne* leaves by development of an expressorium, an appressorium-like leaf exit structure. New Phytol. 211:240-254.

Bennett, T., Sieberer, T., Willett, B., Booker, J., Luschnig, C., & Leyser, O. 2006. The Arabidopsis MAX pathway controls shoot branching by regulating auxin transport. Current Biology, 16(6), 553-563.

Bernier, G. 1977. Absence of rhythmic response of shoot apex of *Sinapis alba* to applied cytokinin. Ann. Bot. 41:1089-1090.

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

Beyer, E.M. and P.W. Morgan. 1969. Ethylene modification of an auxin pulse in cotton stem sections. Plant Physiol. 44:1690-1694.

Beyer, E.M.J. and P.W. Morgan. 1970. Effect of ethylene on the uptake, distribution, and metabolism of indoleacetic acid-1-14C and-2-14C and naphthaleneacetic acid-1-14C. Plant Physiol. 46:157-162.

Bicknell, R. 1984. Seed propagation of *Gentiana scabra*. Combined proceedings-International Plant Propagators' Society (USA).

Biddington, N.L. 1992. The influence of ethylene in plant tissue culture. Plant Growth Regu. 11:173-187.

Biddle, E., D.G. Kerfoot, Y.H. Kho, and K.E. Russell. 1976. Kinetic studies of the thermal decomposition of 2-chloroethylphosphonic acid in aqueous solution. Plant Physiol. 58:700-702.

Biondi, S., S. Scaramagli, F. Capitani, G. Marino, M.M. Altamura, and P. Torrigiani. 1998. Ethylene involvement in vegetative bud formation in tobacco thin layers. Protoplasma 202:134-144.

Blankenship, S.M. and J.M. Dole. 2003. 1-Methylcyclopropene: a review. Postharvest Biol. Technol. 28:1-26.

Bodhipadma, K. and D.W.M. Leung. 2003. *In vitro* fruiting and seed set of *Capsicum annuum* L. cv. Sweet banana. In Vitro Cell. & Develop. Biol. Plant 39:536-539.

Bowes, B.G. 1996. A colour atlas of plant structure. Manson Publishing Ltd. London.

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

Brewer, P.B., E.A. Dun, B.J. Ferguson, C. Rameau, and C.A. Beveridge. 2009. Strigolactone acts downstream of auxin to regulate bud outgrowth in pea and Arabidopsis. Plant Physiol. 150:482-493.

Burg, S.P. and E.A. Burg. 1965. Ethylene action and ripening of fruits - ethylene influences growth and development of plants and is hormone which initiates fruit ripening. Science 148:1190-1196.

Burg, S.P. and E.A. Burg. 1966a. Auxin-induced ethylene formation: its relation to flowering in the pineapple. Science (New York, N.Y.) 152:1269.

Burg, S.P. and E.A. Burg. 1966b. The interaction between auxin and ethylene and its role in plant growth. Proceedings of the National Academy of Sciences 55:262-269.

Burg, S.P. and E.A. Burg. 1967. Inhibition of polar auxin transport by ethylene. Plant Physiol. 42:1224-1228.

Burtin, D., J. Martin-Tanguy, M. Paynot, M. Carré, and N. Rossin. 1990. Polyamines, hydroxycinnamoylputrescines, and root formation in leaf explants of tobacco cultivated *in vitro* effects of the suicide inhibitors of putrescine synthesis. Plant Physiol. 93:1398-1404.

Cacho, M., M. Morán, M.T. Herrera, and J. Fernández-Tárrago. 1991. Morphogenesis in leaf, hypocotyl and root explants of *Digitalis thapsi* L. cultured *in vitro*. Plant Cell Tiss. Org. Cult. 25:117-123.

Campos, K.O. and G.B. Kerbauy. 2004. Thermoperiodic effect on flowering and endogenous hormonal status in *Dendrobium* (Orchidaceae). J. Plant Physiol. 161:1385-1387.

Chang, W.c. and Y.i. Hsing. 1980. *In vitro* flowering of embryoids derived from mature root callus of ginseng (*Panax ginseng*).

Chatfield, S.P., P. Stirnberg, B.G. Forde, and O. Leyser. 2001. The hormonal regulation of axillary bud growth in Arabidopsis. The Plant Journal 24:159-169.

Chen, C.L., Y.J. Tsai, and J.M. Sung. 2010. Photoperiod effects on flowering and seed setting of *Hypericum perforatum*. Exp. Agric. 46:393-400.

Chen, C.M., J.R. Ertl, S.M. Leisner, and C.C. Chang. 1985. Localization of cytokinin biosynthetic sites in pea plants and carrot roots. Plant Physiol. 78:510-513.

Chi, G.L. and E.C. Pua. 1989. Ethylene inhibitors enhanced denovo shoot regeneration from cotyledons of *Brassica campestris* ssp chinensis (chinese cabbage) *in vitro*. Plant Sci. 64:243-250.

Chiatante, D., G.S. Scippa, and A. Di Iorio. 2008. Anatomical investigations of resprouting in *Cardopatum corymbosum* L.(Asteraceae): A hemicryptophyta living on erosion-prone slopes in a Mediterranean climate. Plant Biosyst 142:366-374.

Christianson, M.L. and D.A. Warnick. 1983. Competence and determination in the process of *in vitro* shoot organogenesis. Dev. Biol. 95:288-293.

Clarke, S.M., S.M. Cristescu, O. Miersch, F.J. Harren, C. Wasternack, and L.A. Mur. 2009. Jasmonates act with salicylic acid to confer basal thermotolerance in *Arabidopsis thaliana*. New Phytol. 182:175-187.

Coenen, C. and T.L. Lomax. 1997. Auxin-cytokinin interactions in higher plants: old problems and new tools. Trends Plant Sci. 2:351-356.

Coenen, C. and T.L. Lomax. 1998. The diageotropica gene differentially affects auxin and cytokinin responses throughout development in tomato. Plant Physiol. 117:63-72.

Corbesier, L., G. Bernier, and C. Perilleux. 2002. C : N ratio increases in the phloem sap during floral transition of the long-day plants *Sinapis alba* and *Arabidopsis thaliana*. Plant Cell Physiol. 43:684-688.

Cosic, T., V. Motyka, M. Raspor, J. Savic, A. Cingel, B. Vinterhalter, D. Vinterhalter, A. Travnickova, P.I. Dobrev, B. Bohanec, and S. Ninkovic. 2015. *In vitro* shoot

organogenesis and comparative analysis of endogenous phytohormones in kohlrabi (*Brassica oleracea* var. gongylodes): effects of genotype, explant type and applied cytokinins. Plant Cell Tiss. Org. Cult. 121:741-760.

Crawford, S., N. Shinohara, T. Sieberer, L. Williamson, G. George, J. Hepworth, D. Muller, M.A. Domagalska, and O. Leyser. 2010. Strigolactones enhance competition between shoot branches by dampening auxin transport. Science Signalling 137:2905-2913.

Cristescu, S., S. Persijn, S. te Lintel Hekkert, and F. Harren. 2008. Laser-based systems for trace gas detection in life sciences. Appl. Phys. B 92:343-349.

D'Onofrio, C. and S. Morini. 2006. Somatic embryo, adventitious root and shoot regeneration in *in vitro* grown quince leaves as influenced by treatments of different length with growth regulators. Sci. Hortic. 107:194-199.

Da Silva, J.A.T. 2013. The effect of ethylene inhibitors (AgNO3, AVG), an ethylene-liberating compound (CEPA) and aeration on the formation of protocorm-like bodies of hybrid Cymbidium (Orchidaceae). Frontiers in Biology 8:606-610.

Da Silva, J.A.T., G.B. Kerbauy, S.J. Zeng, Z.L. Chen, and J. Duan. 20014. *In vitro* flowering of orchids. Crit. Rev. Biotechnol. 34:56-76.

Da Silva, J.A.T., S.J. Zeng, J.C. Cardoso, J. Dobranszki, and G.B. Kerbauy. 2014. *In vitro* flowering of Dendrobium. Plant Cell Tiss. Org. Cult. 119:447-456.

Daniel, S.G., D.L. Rayle, and R.E. Cleland. 1989. Auxin physiology of the tomato mutant diageotropica. Plant Physiol. 91:804-807.

De Melo Ferreira, W., G.B. Kerbauy, J.E. Kraus, R. Pescador, and R.M. Suzuki. 2006. Thidiazuron influences the endogenous levels of cytokinins and IAA during the flowering of isolated shoots of *Dendrobium*. J. Plant Physiol. 163:1126-1134.

De Paepe, A. and D. Van Der Straeten. 2005. Ethylene biosynthesis and signaling: An overview, p. 399-430. In: G. Litwack (ed.), Plant Hormones. Elsevier Academic Press Inc, San Diego.

Del Tredici, P. 2001. Sprouting in temperate trees: A morphological and ecological review. Bot. Rev. 67:121-140.

DeMason, D.A. and R. Chawla. 2004. Roles for auxin during morphogenesis of the compound leaves of pea (*Pisum sativum*). Planta 218:435-448.

Dias, L.L., C. Santa-Catarina, D.M. Ribeiro, R.S. Barros, E.I. Floh, and W.C. Otoni. 2009. Ethylene and polyamine production patterns during *in vitro* shoot organogenesis of two passion fruit species as affected by polyamines and their inhibitor. Plant Cell Tiss. Org. Cult. 99:199-208.

Dickens, C. and J. van Staden. 1988. The *in vitro* Flowering of *Kalanchoe blossfeldiana* Poellniz I. Role of culture condition and nutrients. J. Exp. Bot. 39:461-471.

Dimasi-Theriou, K. and A. Economou. 1995. Ethylene enhances shoot formation in cultures of the peach rootstock GF-677 (*Prunus persica x P. amygdalus*). Plant Cell Rep. 15:87-90.

Dimasi-Theriou, K., A. Economou, and E. Sfakiotakis. 1993. Promotion of petunia (*Petunia hybrida* L.) regeneration *in vitro* by ethylene. Plant Cell Tiss. Org. Cult. 32:219-225.

Doi, H., R. Takahashi, T. Hikage, and Y. Takahata. 2010. Embryogenesis and doubled haploid production from anther culture in gentian (*Gentiana triflora*). Plant Cell Tiss. Org. Cult. 102:27-33.

Doi, H., S. Yokoi, T. Hikage, M. Nishihara, K. Tsutsumi, and Y. Takahata. 2011. Gynogenesis in gentians (*Gentiana triflora, G. scabra*): production of haploids and doubled haploids. Plant Cell Rep. 30:1099-1106.

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

Domir, S.C. and C.L. Foy. 1978. A study of ethylene and CO_2 evolution from ethephon in tobacco. Pestic. Biochem. Physiol. 9:1-8.

Eason, J.R., E.R. Morgan, A.C. Mullan, and G.K. Burge. 2004. Display life of *Gentiana* flowers is cultivar specific and influenced by sucrose, gibberellin, fluoride, and postharvest storage. N. Z. J. Crop Hortic. Sci. 32:217-226.

Eaton, C.J., M.P. Cox, B. Ambrose, M. Becker, U. Hesse, C.L. Schardl, and B. Scott. 2010. Disruption of signaling in a fungal-grass symbiosis leads to pathogenesis. Plant Physiol. 153:1780-1794.

Eklund, L. and C.A. Little. 2000. Transport of [1-14C]-indole-3-acetic acid in *Abies balsamea* shoots ringed with Ethrel. Trees 15:58-62.

Elfving, D.C., D.B. Visser, and J.L. Henry. 2011. Gibberellins stimulate lateral branch development in young sweet cherry trees in the orchard. International J. Fruit Sci. 11:41-54.

Emery, R., N.E. Longnecker, and C.A. Atkins. 1998. Branch development in *Lupinus angustifolius* L. II. Relationship with endogenous ABA, IAA and cytokinins in axillary and main stem buds. J. Exp. Bot. 49:555-562.

Ernest, L.C. and J.G. Valdovinos. 1971. Regulation of auxin levels in *Coleus blumei* by ethylene. Plant Physiol. 48:402-406.

Evert, R.F. 2006. Esau's Plant anatomy meristems, cells, and tissues of the plant body-third structure, function, and development. Wiley-interscience.

Faria, J.L.C. and J. Segura. 1997a. *In vitro* control of adventitious bud differentiation by inorganic medium components and silver thiosulfate in explants of *Passiflora edulis* F. flavicarpa. In Vitro Cell. & Develop. Biol. Plant 33:209-212.

Faria, J.L.C. and J. Segura. 1997b. Micropropagation of yellow passionfruit by axillary bud proliferation. Hort. Sci. 32:1276-1277.

Fekih, R., N. Yamagishi, and N. Yoshikawa. 2016. Apple latent spherical virus vector-induced flowering for shortening the juvenile phase in Japanese gentian and lisianthus plants. Planta 244:203-214.

Feng, J., K. Maguire, and B.R. MacKay. 2003. Factors affecting ethylene production of Hayward kiwifruit, p. 203-209. In: H. W. Huang (ed.), Proceedings of the Fifth International Symposium on Kiwifruit. Int. Soc. Horti. Sci., Leuven 1.

Feng, X.Q., A. Apelbaum, E.C. Sisler, and R. Goren. 2004. Control of ethylene activity in various plant systems by structural analogues of 1-methylcyclopropene. Plant Growth Regu. 42:29-38.

Ferguson, B.J. and C.A. Beveridge. 2009. Roles for auxin, cytokinin, and strigolactone in regulating shoot branching. Plant Physiol. 149:1929-1944.

Feung, C.S., R.H. Hamilton, and R.O. Mumma. 1975. Metabolism of 2, 4-dichlorophenoxyacetic acid. VII. Comparison of metabolites from five species of plant callus tissue cultures. J. Agric. Food Chem. 23:373-376.

Firn, R.D. 1986. Growth substance sensitivity: The need for clearer ideas, precise terms and purposeful experiments. Physiol. Plant. 67:267-272.

Fischer, C., V. Speth, S. FleigEberenz, and G. Neuhaus. 1997. Induction of zygotic polyembryos in wheat: Influence of auxin polar transport. Plant Cell 9:1767-1780.

Fiuk, A. and J.J. Rybczynski. 2008. Genotype and plant growth regulator-dependent response of somatic embryogenesis from *Gentiana* spp. leaf explants. In Vitro Cell. & Develop. Biol. Plant 44:90-99.

Foo, E., E. Bullier, M. Goussot, F. Foucher, C. Rameau, and C.A. Beveridge. 2005. The branching gene RAMOSUS1 mediates interactions among two novel signals and auxin in pea. The Plant Cell Online 17:464-474.

Forney, C.F. and D.G. Brandl. 1992. Control of humidity in small controlled-environment chambers using glycerol-water solutions. Horttech. 2:52-54.

Forni, C., R. Braglia, F. Harren, and S. Cristescu. 2012. Stress responses of duckweed (*Lemna minor* L.) and water velvet (*Azolla filiculoides* Lam.) to anionic surfactant sodium-dodecyl-sulphate (SDS). Aquat. Toxicol. 110:107-113.

Fortanier, E. and H. Jonkers. 1975. Juvenility and maturity of plants as influenced by their ontogenetical and physiological ageing, p. 37-44. In: Symposium on Juvenility in Woody Perennials 56.

Foster, K.R., D.M. Reid, and R.P. Pharis. 1992. Ethylene biosynthesis and ethephon metabolism and transport in barley. Crop Sci. 32:1345-1352.

Friml, J. 2003. Auxin transport - shaping the plant. Curr. Opin. Plant Biol. 6:7-12.

Fu, Y., H. Li, and F. Meng. 1995. The possible role of zearalenone in the floral gradient in *Nicotiana tabacum* L. J. Plant Physiol. 147:197-202.

Funnell, K., E.R. Morgan, G.E. Clark, and J. Chuah, B. 2013. Geophyte research and production in New Zealand, p. 553. In: R. Kamenetsky and H. Okubo (eds.), Ornamental Geophytes From Basic Science to Sustainable Production. CRC Press Taylor & Francis Group, USA.

Funnell, K.A. 2008. Growing degree-day requirements for scheduling flowering of *Scadoxus multiflorus* subsp. katharinae (Baker) Friis & Nordal. Hort. Sci. 43:166-169.

Funnell, K.A., M. Bendall, W.F. Fountain, and E.R. Morgan. 2003. Maturity and type of cutting influences flower yield, flowering time, and quality in *Limonium* 'Chorus Magenta'. N. Z. J. Crop Hortic. Sci. 31:139-146.

Funnell, K.A., E.W. Hewett, I.J. Warrington, and J.A. Plummer. 1998. Leaf mass partitioning as a determinant of dry matter accumulation in *Zantedeschia*. J. Am. Soc. Hort. Sci. 123:973-979.

Funnell, K.A., A.N. Seleznyova, R. Kaji, J. Chen, S. Manandhar, and D.J. Wooley. 2015. Diverse growth and sylleptic branching patterns in Japanese maple cultivars, p. 143-

148. In: R. A. Criley (ed.), Xxix International Horticultural Congress on Horticulture: Sustaining Lives, Livelihoods and Landscapes.

Gagliardi, R.F., G.P. Pacheco, J.F.M. Valls, and E. Mansur. 2002. Germplasm preservation of wild Arachis species through culture of shoot apices and axillary buds from *in vitro* plants. Biol. Plant. 45:353-357.

Galston, A.W. and R. Kaur-Sawhney. 1987. Polyamines as endogenous growth regulators, p. 280-295, Plant hormones and their role in plant growth and development. Springer.

Galweiler, L., C.H. Guan, A. Muller, E. Wisman, K. Mendgen, A. Yephremov, and K. Palme. 1998. Regulation of polar auxin transport by AtPIN1 in Arabidopsis vascular tissue. Science 282:2226-2230.

Garrido, G., J.R. Guerrero, E.A. Cano, M. Acosta, and J. Sanchez-Bravo. 2002. Origin and basipetal transport of the IAA responsible for rooting of carnation cuttings. Physiol. Plant. 114:303-312.

Gaspar, T., C. Kevers, C. Penel, H. Greppin, D.M. Reid, and T.A. Thorpe. 1996. Plant hormones and plant growth regulators in plant tissue culture. In Vitro Cell. & Develop. Biol. Plant 32:272-289.

Geldner, N., J. Friml, Y.D. Stierhof, G. Jurgens, and K. Palme. 2001. Auxin transport inhibitors block PIN1 cycling and vesicle traficking. Nature. 413:425-428.

Glenny, D. 2003. A revision of the genus *Gentianella* in New Zealand.

Goebel, K. 1900. Organography of Plants. Clarendon Press, Oxford.

Goldsmith, M. 1977. The polar transport of auxin. Annual Review of Plant Physiol. 28:439-478.

Goldsmith, M.H.M. 1966. Maintenance of polarity of auxin movement by basipetal transport. Plant Physiol. 41:749-754.

Gomez-Roldan, V., S. Fermas, P.B. Brewer, V. Puech-Pagès, E.A. Dun, J.-P. Pillot, F. Letisse, R. Matusova, S. Danoun, and J.-C. Portais. 2008. Strigolactone inhibition of shoot branching. Nature. 455:189-194.

Goren R, JM. Bukovac and JA. Flore, 1974. Mechanism of indol-3-acetic acid conjugation. No induction of ethylene. Plant physiol. 53:164-166.

Goudey, J.S., Saini, H. S. and Spencer, M. S. 1987a. Uptake and fate of ethephon (2-chloroethyljphosphonic acid) in dormant weed seeds. Plant Physiol. 85:155-157.

Goudey, J.S., Saini, H. S., Spencer, M. S. 1987b. Factors affecting the decomposition of 2-chloroethylphosphonic acid in soils. Plant Cell Environ. 10:347-349.

Hackett, W.P. 1985. Juvenility, maturation, and rejuvenation in woody plants. Horticultural Reviews, Volume 7:109-155.

Hall, S.M. and J.R. Hillman. 1975. Correlative inhibition of lateral bud growth in *Phaseolus vulgaris* L. Timing of bud growth following decapitation. Planta 123:137-143.

Hansen, H. and K. Grossmann. 2000. Auxin-induced ethylene triggers abscisic acid biosynthesis and growth inhibition. Plant Physiol. 124:1437-1448.

Harbage, J.F. and D.P. Stimart. 1996. Ethylene does not promote adventitious root initiation on apple microcuttings. J. Am. Soc. Hort. Sci. 121:880-885.

Hasenstein, K.H. and M.L. Evans. 1988. Effects of cations on hormone transport in primary roots of *Zea mays*. Plant Physiol. 86:890-894.

Haver, D. and U. Schuch. 2001. Influence of root restriction and ethylene exposure on apical dominance of petunia (*Petunia x hybrida* Hort. Vilm. Andr.). Plant Growth Regu. 35:187-196.

Haver, D.L., U.K. Schuch, and C.J. Lovatt. 2002. Exposure of petunia seedlings to ethylene decreased apical dominance by reducing the ratio of auxin to cytokinin. J. Plant Growth Regul. 21:459-468.

Heide, O.M. 2001. Photoperiodic control of dormancy in *Sedum telephium* and some other herbaceous perennial plants. Physiol. Plant. 113:332-337.

Hermans, C., S. Porco, F. Vandenbussche, S. Gille, J. De Pessemier, D. Van Der Straeten, N. Verbruggen, and D.R. Bush. 2011. Dissecting the role of CHITINASE-LIKE1 in nitrate-dependent changes in root architecture. Plant Physiol. 157:1313-1326.

Hermans, C., M. Vuylsteke, F. Coppens, S.M. Cristescu, F.J. Harren, D. Inzé, and N. Verbruggen. 2010. Systems analysis of the responses to long-term magnesium deficiency and restoration in *Arabidopsis thaliana*. New Phytol. 187:132-144.

Ho, T., Liu, S. 2001. A Worldwide Monograph of *Gentiana*. Science Press Beijing New York.

Hocking, T. J., & Hillman, J. R. 1975. Studies on the role of abscisic acid in the initiation of bud dormancy in *Alnus glutinosa* and *Betula pubescens*. Planta, 125(3), 235-242.

Hoffman, N.E. and S.F. Yang. 1982. Enhancement of wound-induced ethylene synthesis by ethylene in preclimacteric cantaloupe. Plant Physiol. 69:317-322.

Horvath, D.R., J.V. Anderson, W.S. Chao, and M.E. Foley. 2004. Knowing when to grow: signals regulating bud dormancy. Trends Plant Sci. 9:534-540.

Hosokawa, K., M. Nakano, Y. Oikawa, and S. Yamamura. 1996. Adventitious shoot regeneration from leaf, stem and root explants of commercial cultivars of *Gentiana*. Plant Cell Rep. 15:578-581.

Hosokawa, K., Y. Oikawa, and S. Yamamura. 1998. Mass propagation of ornamental gentian in liquid medium. Plant Cell Rep. 17:747-751.

Huang, C., M. Hsieh, W. Hsieh, A. Sagare, and H. Tsay. 2000. *In vitro* propagation of *Limonium wrightii* (Hance) Ktze.(Plumbaginaceae), an ethnomedicinal plant, from shoot-tip, leaf-and inflorescence-node explants. In Vitro Cell. & Develop. Biol. Plant 36:220-224.

Huang, C.W. and C.C. Chen. 2005. Physical properties of culture vessels for plant tissue culture. Biosys. Eng. 91:501-511.

Huberman, M., J. Riov, E.E. Goldschmidt, A. Apelbaum, and R. Goren. 2014. The novel ethylene antagonist, 3-cyclopropyl-1-enyl-propanoic acid sodium salt (CPAS), increases grain yield in wheat by delaying leaf senescence. Plant Growth Regu. 73:249-255.

Huxter, T.J., T.A. Thorpe, and D.M. Reid. 1981. Shoot initiation in light- and dark-grown tobacco callus: the role of ethylene. Physiol. Plant. 53:319-326.

Ishimori, T., Y. Niimi, and D.S. Han. 2009. *In vitro* flowering of *Lilium rubellum* Baker. Sci. Hortic. 120:246-249.

Ivana, M.c.c., D. G.c, and M. N. 1997. Micropropagation of four *Gentiana* species (*G. lutea, G. cruciata, G. purpurea and G. acaulis*). Plant Cell Tiss. Org. Cult. 49:141-144.

James, A.T. and A.J.P. Martin. 1952. Gas-liquid partition chromatography - the separation and micro-estimaton of volatile fatty acids from formic acid to dodecanoic acid. Biochem. J. 50:679-690.

Jana, S. and G.S. Shekhawat. 2011. Plant growth regulators, adenine sulfate and carbohydrates regulate organogenesis and *in vitro* flowering of *Anethum graveolens*. Acta Physiol. Plant. 33:305-311.

Jin Goh, C., S. Keng Ng, P. Lakshmanan, and C. Shiong Loh. 1997. The role of ethylene on direct shoot bud regeneration from mangosteen (*Garcinia mangostana* L.) leaves cultured *in vitro*. Plant Sci. 124:193-202.

Johnson, X., T. Brcich, E.A. Dun, M. Goussot, K. Haurogné, C.A. Beveridge, and C. Rameau. 2006. Branching genes are conserved across species. Genes controlling a novel signal in pea are coregulated by other long-distance signals. Plant Physiol. 142:1014-1026.

Jomori, H., Y. Takahata, and N. Kaizuma. 1995. Plant regeneration from leaf-derived calli of gentians and their protoplast culture, p. 81-86. In: T. Nishio and C. Dore (eds.), Genetic Improv. of Hort. Crops by Biotech.

Jumin, H. and M. Ahmad. 1999. High-frequency *in vitro* flowering of *Murraya paniculata* (L.) Jack. Plant Cell Rep. 18:764-768.

Kalousek, P., D. Buchtová, J. Balla, V. Reinöhl, and S. Procházka. 2010a. Cytokinins and polar transport of auxin in axillary pea buds. Acta Univ. Agric. Silvic. Mendel. Brun. 58:79-88.

Kalousek, P., D. Buchtová, J. Balla, V. Reinöhl, and S. Procházka. 2010b. Cytokinins and polar transport of auxin in axillary pea buds. Acta Universitatis Agriculturae et Silviculturae Mendelianae Brunensis 58.

Kapulnik, Y., N. Resnick, E. Mayzlish-Gati, Y. Kaplan, S. Wininger, J. Hershenhorn, and H. Koltai. 2011. Strigolactones interact with ethylene and auxin in regulating root-hair elongation in *Arabidopsis*. J. Exp. Bot. 62:2915-2924.

Katz, E., J. Riov, D. Weiss, and E.E. Goldschmidt. 2005. The climacteric-like behaviour of young, mature and wounded citrus leaves. J. Exp. Bot. 56:1359-1367.

Kende, H. 1993. Ethylene biosynthesis. Annu. Rev. Plant physiol. Plant mol. Biol. 44:283-307.

Kerbauy, G.B. and S. Colli. 1997. Increased conversion of root tip meristems of *Catasetum fimbriatum* into protocorm-like bodies mediated by ethylene. Lindleyana 12:59-63.

Kevers, C., N. Boyer, J.C. Courduroux, and T. Gaspar. 1992. The influence of ethylene on proliferation and growth of rose shoot cultures. Plant Cell Tiss. Org. Cult. 28:175-181.

Khalafalla, M.M. and K. Hattori. 2000. Ethylene inhibitors enhance *in vitro* root formation on faba bean shoots regenerated on medium containing thidiazuron. Plant Growth Regu. 32:59-63.

Kieber, J.J. 2002. Tribute to folke skoog: recent advances in our understanding of cytokinin biology. J. Plant Growth Regul. 21:1-2.

Kieber, J.J., M. Rothenberg, G. Roman, K.A. Feldmann, and J.R. Ecker. 1993. CTR1, a negative regulator of the ethylene response pathway in arabidopsis, encodes a member of the Raf family of protein kinases. Cell 72:427-441.

Kim, K.-N. and S.G. Ernst. 1994. Effects of inhibitors on phenocritical events of *in vitro* shoot organogenesis in tobacco thin cell layers. Plant Sci. 103:59-66.

Kim, M.S., C.M. Schumann, and N.B. Klopfenstein. 1997. Effects of thidiazuron and benzyladenine on axillary shoot proliferation of three green ash (*Fraxinus pennsylvanica* Marsh) clones. Plant Cell Tiss. Org. Cult. 48:45-52.

Klein, I., S. Lavee, and Y. Ben-Tal. 1979. Effect of water vapor pressure on the thermal decomposition of 2-chloroethylphosphonic acid. Plant Physiol. 63:474-477.

Kohlein, F. 1991. Gentians. Portland, United States, Timber Press, Inc.

Kohler, E.A., C.S. Throssell, and Z.J. Reicher. 2004. 2,4-D rate response, absorption, and translocation of two ground ivy (*Glechoma hederacea*) populations. Weed Technol. 18:917-923.

Koltai, H. 2011. Strigolactones are regulators of root development. New Phytol. 190:545-549.

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

Kong, L.S. and E.C. Yeung. 1994. Effects of ethylene and ethylene inhibitors on white spruce somatic embryo maturation. Plant Sci. 104:71-80.

Koornneef, M., C. Alonso-Blanco, A.J.M. Peeters, and W. Soppe. 1998. Genetic control of flowering time in arabidopsis. Annu. Rev. Plant physiol. Plant mol. Biol. 49:345-370.

Kostenyuk, I., B.J. Oh, and I.S. So. 1999. Induction of early flowering in *Cymbidium niveo-marginatum* Mak *in vitro*. Plant Cell Rep. 19:1-5.

Kristiansen, K., H. Ornstrup, and K. Brandt. 1999. *In vitro* PPFD and media composition affect both in and ex vitro performance of *Alstroemeria* Butterfly-hybrids. Plant Cell Tiss. Org. Cult. 56:145-153.

Kumar, A.A., P. Mishra, K. Kumari, and K.C.S. Panigrahi. 2012. Environmental stress influencing plant development and flowering. Frontiers in bioscience (Scholar edition) 4:1315-1324.

Kumar, P.P., P. Lakshmanan, and T.A. Thorpe. 1998. Regulation of morphogenesis in plant tissue culture by ethylene. In Vitro Cell. & Develop. Biol. Plant 34:94-103.

Kumar, P.P., D.M. Reid, and T.A. Thorpe. 1987. The role of ethylene and carbon dioxide in differentiation of shoot buds in excised cotyledons of *Pinus radiata in vitro*. Physiol. Plant. 69:244-252.

Kumar, P.P. and T.A. Thorpe. 1991. A setup for incubating plant cultures under continuous flow of gases. In Vitro Cell. & Develop. Biol. Plant 27:43-44.

Lai, C.C., T.A. Yu, S.D. Yeh, and J.S. Yang. 1998. Enhancement of *in vitro* growth of *Papaya multishoots* by aeration. Plant Cell Tiss. Org. Cult. 53:221-225.

Lakshmanan, P., S.K. Ng, C.S. Loh, and C.J. Goh. 1997. Auxin, cytokinin and ethylene differentially regulate specific developmental states associated with shoot bud morphogenesis in leaf tissues of mangosteen (*Garcinia mangostana* L) cultured *in vitro*. Plant Cell Physiol. 38:59-64.

Lammi, J. 2005. Online photoperiodic calculatore, Sonera Finland. Retrived 29-07-20016 from http://www.sci.fi/~benefon/sol.html.

Lang, A. 1965. Physiology of flower initiation, p. 1380-1536, Differenzierung und Entwicklung/Differentiation and Development. Springer.

Le Bris, M., A. Champeroux, P. Bearez, and M. Le Page-Degivry. 1998. Basipetal gradient of axillary bud inhibition along a rose (*Rosa hybrida* L.) stem: growth potential of primary buds and their two most basal secondary buds as affected by position and age. Ann. Bot. 81:301-309.

Leopold, A. 1967. The polarity of auxin transport. Ann. N. Y. Acad. Sci. 144:94-101.

Leopold, A.C. 1964. Plant growth and development. McGraw-Hill Book Co.

Levyadun, S. and R. Aloni. 1995. Differentiation of the ray system in woody-plants. Bot. Rev. 61:45-84.

Leyser, O. 2005. The fall and rise of apical dominance. Curr. Opin. Genet. Dev. 15:468-471.

Leyser, O. 2009. The control of shoot branching: an example of plant information processing. Plant Cell Environ. 32:694-703.

Lieberman, M. 1979. Biosynthesis and action of ethylene. Annu. Rev. Plant Physiol 30:533-591.

Linsmaier, E.M. and F. Skoog. 1965. Organic growth factor requirement of tobacco tissue cultures. Physiol. Plant. 18:100-127.

Ljung, K., A.K. Hull, M. Kowalczyk, A. Marchant, J. Celenza, J.D. Cohen, and G. Sandberg. 2002. Biosynthesis, conjugation, catabolism and homeostasis of indole-3-acetic acid in *Arabidopsis thaliana*. Plant Mol. Biol. 49:249-272.

Lloyd, A.J., J. William Allwood, C.L. Winder, W.B. Dunn, J.K. Heald, S.M. Cristescu, A. Sivakumaran, F.J. Harren, J. Mulema, and K. Denby. 2011. Metabolomic approaches reveal that cell wall modifications play a major role in ethylene-mediated resistance against *Botrytis cinerea*. The Plant Journal 67:852-868.

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

Luthen, H. 2015. What We Can Learn from Old Auxinology. J. Plant Growth Regul. 34:702-707.

Magdalita, P.M., I.D. Godwin, R.A. Drew, and S.W. Adkins. 1997. Effect of ethylene and culture environment on development of papaya nodal cultures. Plant Cell Tiss. Org. Cult. 49:93-100.

Manandhar, S. 2016. Strigolactones and hormonal interaction in control of branching in Zantedeschia and other horticultural species : a thesis presented in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Plant Science at Massey University, Palmerston North, New Zealand

Maneesh & Pathak, M., Pathak, R. K. 2001. Effect of nodal position and season on *in vitro* shoot proliferation in aonla (*Emblica officinalis* Gaertn.). J. Applied Hort. 3:103-104.

Marcelis-Van Acker, C.A.M. 1994. Development and growth-potential of axillary buds in roses as affected by bud age. Ann. Bot. 74:437-443.

Marin, J.M., O.J. Pozo, J. Beltran, and F. Hernandez. 2006. An ion-pairing liquid chromatography/tandem mass spectrometric method for the determination of ethephon residues in vegetables. Rapid Commun. Mass Spectrom. 20:419-426.

Marks, T.R., Y.Y. Ford, R.W.F. Cameron, C. Goodwin, P.E. Myers, and H.L. Judd. 2002. A role for polar auxin transport in rhizogenesis. Plant Cell Tiss. Org. Cult. 70:189-198.

Marth, P.C., W.V. Audia, and J.W. Mitchell. 1956. Effects of gibberellic acid on growth and development of plants of various genera and species. Botanical Gazette 118:106-111.

Martin, G.C. 1987. Apical Dominance. Hort. Sci. 22:824-833.

Mason, M.G., J.J. Ross, B.A. Babst, B.N. Wienclaw, and C.A. Beveridge. 2014. Sugar demand, not auxin, is the initial regulator of apical dominance. Proceedings of the National Academy of Sciences 111:6092-6097.

Matsoukas, I.G. 2014. Attainment of reproductive competence, phase transition, and quantification of juvenility in mutant genetic screens. Frontiers in Plant Science 5:1-5.

McCullough, P.E. and S.S. Sidhu. 2014. Ethephon Absorption and Transport Associated with Annual Bluegrass Inflorescence Suppression, p. 845-850, Crop Sci.

McDaniel, B.K. and B.M. Binder. 2012. Ethylene receptor 1 (ETR1) is sufficient and has the predominant role in mediating inhibition of ethylene responses by silver in *Arabidopsis thaliana*. J. Biol. Chem. 287:26094-26103.

McDaniel, C.N., K.A. Sangrey, and S.R. Singer. 1989. Node counting in axillary buds of *Nicotiana tabacum* Cv Wisconsin-38, a day-neutral plant. Am. J. Bot. 76:403-408.

McManus, M.T. 2012. The plant hormone ethylene/edited by Michael T. McManus. Oxford : Wiley-Blackwell, 392 p.

McSteen, P. and O. Leyser. 2005. Shoot branching. Annu. Rev. Plant biol. 56:353-374.

Meijer, E.G. and J. Simmonds. 1988. Polyamine levels in relation to growth and somatic embryogenesis in tissue *Medicago sativa*. J. Exp. Bot. 39:787-794.

Mensualisodi, A., M. Panizza, and F. Tognoni. 1995. Endogenous ethylene requirement for adventitious root induction and growth in tomato cotyledons and Lavandin microcuttings in vitro. Plant Growth Regu. 17:205-212.

Métraux, J.-P. and H. Kende. 1983. The role of ethylene in the growth response of submerged deep water rice. Plant Physiol. 72:441-446.

Millenaar, F.F., M. Van Zanten, M.C. Cox, R. Pierik, L.A. Voesenek, and A.J. Peeters. 2009. Differential petiole growth in *Arabidopsis thaliana*: photocontrol and hormonal regulation. New Phytol. 184:141-152.

Miller, A.R., W.L. Pengelly, and L.W. Roberts. 1984. Introduction of xylem differentiation in Lactuca by ethylene. Plant Physiol. 75:1165-1166.

Mishiba, K.i., M. Nishihara, Y. Abe, T. Nakatsuka, H. Kawamura, K. Kodama, T. Takesawa, J. Abe, and S. Yamamura. 2006. Production of dwarf potted gentian using wild-type *Agrobacterium rhizogenes*. Plant Biotechnol. 23:33-38.

Mohiuddin, A.K.M., M.K.U. Chowdhury, Z.C. Abdullah, and S. Napis. 1997. Influence of silver nitrate (ethylene inhibitor) on cucumber *in vitro* shoot regeneration. Plant Cell Tiss. Org. Cult. 51:75-78.

Mok, D.W.S. and M.C. Mok. 2001. Cytokinin metabolism and action. Annu. Rev. Plant physiol. Plant mol. Biol. 52:89-118.

Morgan, E.R. 2004. Use of in ovulo embryo culture to produce interspecific hybrids between *Gentiana triflora* and *Gentiana lutea*. N. Z. J. Crop Hortic. Sci. 32:343-347.

Morgan, E.R. 2015. Gentiana plant named 'Little Pinkie'. The New Zealand Institute For Plant And Food Research Ltd., Google Patents.

Morgan, E.R., B.L. Hofmann, and J.E. Grant. 2003. Production of tetraploid *Gentiana triflora* var. japonica 'Royal Blue' plants. N. Z. J. Crop Hortic. Sci. 31:65-68.

Morgan, E.R.B., R. M.. Bicknell, R. A. 1997. *In vitro* propagation of *Gentiana cerina* and *Gentiana corymbifera*. N. Z. J. Crop Hortic. Sci. 25:1-8.

Morris, D.A. 2000. Transmembrane auxin carrier systems - dynamic regulators of polar auxin transport. Plant Growth Regu. 32:161-172.

Muday, G.K. 2000. Maintenance of asymmetric cellular localization of an auxin transport protein through interaction with the actin cytoskeleton. J. Plant Growth Regul. 19:385-396.

Muday, G.K. and A.S. Murphy. 2002. An emerging model of auxin transport regulation. Plant Cell 14:293-299.

Müller, D., & Leyser, O. 2011. Auxin, cytokinin and the control of shoot branching. Annals of Botany, 107(7), 1203-1212.

Mur, L.A., A. Sivakumaran, J. Mandon, S.M. Cristescu, F.J. Harren, and K.H. Hebelstrup. 2012. Haemoglobin modulates salicylate and jasmonate/ethylene-mediated resistance mechanisms against pathogens. J. Exp. Bot. 63:4375-4387.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-497.

Nakajima, E., K. Hasegawa, K. Yamada, S. Kosemura, and S. Yamamura. 2001. Effects of the auxin-inhibiting substances raphanusanin and benzoxazolinone on apical dominance of pea seedlings. Plant Growth Regu. 35:11-15.

Nakamura, C., M. Nakata, M. Shioji, and H. Ono. 1985. 2, 4-D resistance in a tobacco cell culture variant: cross-resistance to auxins and uptake, efflux and metabolism of 2, 4-D. Plant Cell Physiol. 26:271-280.

Nakano, M., K. Hosokawa, T. Oomiya, and S. Yamamura. 1995. Plant regeneration from protoplasts of *Gentiana* by embedding protoplasts in gellan gum. Plant Cell Tiss. Org. Cult. 41:221-227.

Nakatsuka, T., Y. Abe, Y. Kakizaki, A. Kubota, N. Shimada, and M. Nishihara. 2009. Over-expression of Arabidopsis FT gene reduces juvenile phase and induces early flowering in ornamental gentian plants. Euphytica 168:113-119.

Nakatsuka, T., E. Yamada, M. Saito, T. Hikage, Y. Ushiku, and M. Nishihara. 2012. Construction of the first genetic linkage map of Japanese gentian (Gentianaceae). BMC Genomics 13:1-15.

Nakurte, I., A. Keisa, and N. Rostoks. 2012. Development and Validation of a Reversed-Phase Liquid Chromatography Method for the Simultaneous Determination of Indole-3-Acetic Acid, Indole-3-Pyruvic Acid, and Abscisic Acid in Barley (*Hordeum vulgare* L.). J. Analyt. Methods in Chemist.:6-10.

National Gardening Association, I. 2001. http://garden.org/onlinecourse/Intro.htm.

Nguyen Thi, Q., Kozai, T. 2007. Effect of temperature and nodal cutting position on the growth of *in vitro* cultured coffee plants under photoautotrophic conditions. Jpn J. Trop. Agric 51:5-11.

Ni, J., C. Gao, M.-S. Chen, B.-Z. Pan, K. Ye, and Z.-F. Xu. 2015. Gibberellin promotes shoot branching in the perennial woody plant *Jatropha curcas*. Plant Cell Physiol.:1655-1666.

Nir, G. and S. Lavee. 1981. Persistence, uptake and translocation of ethephon-c-14 (2-chloroethyl phosphonic acid) in perlette and cardinal grapevines. Aust. J. Plant Physiol. 8:57-63.

Nitsch, L., W. Kohen, C. Oplaat, T. Charnikhova, S. Cristescu, P. Michieli, M. Wolters-Arts, H. Bouwmeester, C. Mariani, W.H. Vriezen, and I. Rieu. 2012. ABA-deficiency results in reduced plant and fruit size in tomato. J. Plant Physiol. 169:878-883.

Nordström, A., P. Tarkowski, D. Tarkowska, R. Norbaek, C. Åstot, K. Dolezal, and G. Sandberg. 2004. Auxin regulation of cytokinin biosynthesis in *Arabidopsis thaliana*: A factor of potential importance for auxin–cytokinin-regulated development. Proc. Natl. Acad. Sci. U S A 101:8039-8044.

Normanly, J. and B. Bartelt. 1999. Redundancy as a way of life-IAA metabolism. Curr. Opin. Plant Biol. 2:207-213.

Nour, K.A. and T.A. Thorpe. 1994. The effect of the gaseous state on bud induction and shoot multiplication *in vitro* in eastern white cedar. Physiol. Plant. 90:163-172.

Ohkawa, K. 1983. Gentiana. CRC Hand Book of Flowering. CRC Press, Boca Raton, Florida:351-355.

Osborne, D.J. and M.G. Mullins. 1969. Auxin, ethylene and kinetin in a carrier protein model system for the polar transport of auxins in petiole segments of *Phaseolus vulgaris*. New Phytol. 68:977-991.

Panizza, M., A. Mensuali-Sodi, and F. Tognoni. 1993. Role of ethylene in axillary shoot proliferation of lavandin interaction with benzyladenine and polyamines. J. Exp. Bot. 44:387-394.

Pellegrineschi, A. and D. Tepfer. 1993. Micropropagation and plant regeneration in *Sesbania rostrata*. Plant Sci. 88:113-119.

Pennazio, S. 2003. " Florigen": an intriguing concept of plant biology. Riv. Biol. 97:33-51.

Peres, L.E.P. and G.B. Kerbauy. 1999. High cytokinin accumulation following root tip excision changes the endogenous auxin-to-cytokinin ratio during root-to-shoot conversion in *Catasetum fimbriatum* Lindl (Orchidaceae). Plant Cell Rep. 18:1002-1006.

Perez-Bermudez, P., M. Cornejo, and J. Segura. 1985. A morphogenetic role for ethylene in hypocotyl cultures of *Digitalis obscura* L. Plant Cell Rep. 4:188-190.

Petrasek, J., M. Elckner, D.A. Morris, and E. Zazimalova. 2002. Auxin efflux carrier activity and auxin accumulation regulate cell division and polarity in tobacco cells. Planta 216:302-308.

Petrasek, J., J. Mravec, R. Bouchard, J.J. Blakeslee, M. Abas, D. Seifertova, J. Wisniewska, Z. Tadele, M. Kubes, M. Covanova, P. Dhonukshe, P. Skupa, E. Benkova, L. Perry,

P. Krecek, O.R. Lee, G.R. Fink, M. Geisler, A.S. Murphy, C. Luschnig, E. Zazimalova, and J. Friml. 2006. PIN proteins perform a rate-limiting function in cellular auxin efflux. Science 312:914-918.

Pierik, R., G.C. Whitelam, L.A.C.J. Voesenek, H. De Kroon, and E.J.W. Visser. 2004. Canopy studies on ethylene-insensitive tobacco identify ethylene as a novel element in blue light and plant–plant signalling. The Plant Journal 38:310-319.

Powell, G.R. 2008. On buds man. Fr. Chron 84:590-594.

Pramod, S., P.B. Patel, and K.S. Rao. 2013. Influence of exogenous ethylene on cambial activity, xylogenesis and ray initiation in young shoots of *Leucaena leucocephala* (lam.) de Wit. Flora 208:549-555.

Pranamornkith, T., A. East, and J. Heyes. 2012. Influence of exogenous ethylene during refrigerated storage on storability and quality of *Actinidia chinensis* (cv. Hort16A). Postharvest Biol. Technol. 64:1-8.

Prusinkiewicz, P., S. Crawford, R.S. Smith, K. Ljung, T. Bennett, V. Ongaro, and O. Leyser. 2009. Control of bud activation by an auxin transport switch. Proceedings of the National Academy of Sciences 106:17431-17436.

Pua, E.C. and G.L. Chi. 1993. De-novo shoot morphogenesis and plant-growth of mustard (*Brassica juncea*) *in vitro* in relation to ethylene. Physiol. Plant. 88:467-474.

Pua, E.C., X. Deng, and A. Tiong-Chew Koh. 1999. Genotypic variability of de novo shoot morphogenesis of *Brassica oleracea in vitro* in response to ethylene inhibitors and putrescine. J. Plant Physiol. 155:598-605.

Pumisutapon, P., R.G.F. Visser, and G.J. De Klerk. 2011. Hormonal control of the outgrowth of axillary buds in *Alstroemeria* cultured *in vitro*. Biol. Plant. 55:664-668.

Purnhauser, L., P. Medgyesy, M. Czakó, P.J. Dix, and L. Márton. 1987. Stimulation of shoot regeneration in *Triticum aestivum* and *Nicotiana plumbaginifolia* Viv. tissue cultures using the ethylene inhibitor $AgNO_3$. Plant Cell Rep. 6:1-4.

Rademacher, W. 2015. Plant Growth Regulators: Backgrounds and Uses in Plant Production. J. Plant Growth Regul. 34:845-872.

Rahman, A., A. Bannigan, W. Sulaman, P. Pechter, E.B. Blancaflor, and T.I. Baskin. 2007. Auxin, actin and growth of the *Arabidopsis thaliana* primary root. Plant J. 50:514-528.

Reis, L.B., V.B. Paiva Neto, E.A.T. Picoli, M.G.C. Costa, M.M. Rego, C.R. Carvalho, F.L. Finger, and W.C. Otoni. 2003. Axillary bud development of passionfruit as affected by ethylene precursor and inhibitors. In Vitro Cell. & Develop. Biol. Plant 39:618-622.

Rhodes, M.J.C. 1980. The maturation and ripening of fruits. Senescence in plants.:157-205.

Rijnders, J.G., Yang, YY., Kamiya, Y., Takahashi, N. . 1997. Ethylene enhances gibberellin levels and petiole sensitivity in flooding-tolerant *Rumex palustris* but not in flooding-intolerant *R. acetosa*. Planta Med. 203:20-25.

Riov, J., E. Dagan, R. Goren, and S.F. Yang. 1990. Characterization of abscisic acid-induced ethylene production in citrus leaf and tomato fruit tissues. Plant Physiol. 92:48-53.

Riov, J. and S.F. Yang. 1982. Effects of exogenous ethylene on ethylene production in citrus leaf tissue. Plant Physiol. 70:136-141.

Robbins, J.A., M.S. Reid, J.L. Paul, and T.L. Rost. 1985. The effect of ethylene on adventitious root-formation in mung bean (*Vigna radiata*) cuttings. J. Plant Growth Regul. 4:147-157.

Rodrigues-Pousada, R.A., R. De Rycke, A. Dedonder, W. Van Caeneghem, G. Engler, M. Van Montagu, and D. Van Der Straeten. 1993. The Arabidopsis 1-aminocyclopropane-1-carboxylate synthase gene 1 is expressed during early development. The Plant Cell Online 5:897-911.

Roeder, S., K. Dreschler, M. Wirtz, S.M. Cristescu, F.J. van Harren, R. Hell, and B. Piechulla. 2009. SAM levels, gene expression of SAM synthetase, methionine synthase and ACC oxidase, and ethylene emission from *N. suaveolens* flowers. Plant Mol. Biol. 70:535-546.

Roldan, M., C. Gomez-Mena, L. Ruiz-Garcia, J. Salinas, and J.M. Martinez-Zapater. 1999. Sucrose availability on the aerial part of the plant promotes morphogenesis and flowering of *Arabidopsis* in the dark. Plant J. 20:581-590.

Rose, R.J., R.T. Clarke, and S.B. Chapman. 1998. Individual variation and the effects of weather, age and flowering history on survival and flowering of the long-lived perennial *Gentiana pneumonanthe*. Ecography 21:317-326.

Rubery, P.H. and A.R. Sheldrake. 1974. Carrier-mediated auxin transport. Planta 118:101-121.

Rubio, V., R. Bustos, M.L. Irigoyen, X. Cardona-Lopez, M. Rojas-Triana, and J. Paz-Ares. 2009. Plant hormones and nutrient signaling. Plant Mol. Biol. 69:361-373.

Růžička, K., K. Ljung, S. Vanneste, R. Podhorská, T. Beeckman, J. Friml, and E. Benková. 2007. Ethylene regulates root growth through effects on auxin biosynthesis and transport-dependent auxin distribution. The Plant Cell Online 19:2197-2212.

Sachs, R., A. Kofranek, and W. Hackett. 1976. Evaluating new pot plant species. Florists' Review (USA):35-84.

Sagee, O.R., J. and Goren, R. 1990. Ethylene-enhanced catabolism of [14C]lndole-3-acetic acid to lndole-3-carboxylic acid in citrus leaf tissues. 91: 54-60.

Saha, S., T. Dey, S. Adhikari, S. Mukhopadhyay, C. Sengupta, and P. Ghosh. 2016. Effects of plant growth regulators on efficient plant regeneration efficiency and genetic stability analysis from two *Ocimum tenuiflorum* L. morphotypes. Rendiconti Lincei 27:609-628.

Sahoo, Y., S.K. Pattnaik, and P.K. Chand. 1997. *In vitro* clonal propagation of an aromatic medicinal herb *Ocimum basilicum* L. (sweet basil) by axillary shoot proliferation. In Vitro Cell. & Develop. Biol. Plant 33:293-296.

Salman, A., H. Filgueiras, S. Cristescu, F. Lopez-Lauri, F. Harren, and H. Sallanon. 2009. Inhibition of wound-induced ethylene does not prevent red discoloration in fresh-cut endive (*Cichorium intybus* L.). Eur. Food Res. Technol. 228:651-657.

Samarakoon, U.C. 2012. The physiology and control of crown bud formation and development in gentians: a thesis presented in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Plant Physiology at Massey University, Palmerston North, New Zealand.

Samarakoon, U.C., K.A. Funnell, D.J. Woolley, B.A. Ambrose, and E.R. Morgan. 2013. Anatomical investigations determining the origin of crown buds on the transition zone of gentians. N. Z. J. Bot. 51:264-274.

Samarakoon, U.C., K.A. Funnell, D.J. Woolley, B.A. Ambrose, and E.R. Morgan. 2014. The Architectural Complexity of Crown Bud Clusters in Gentian: Anatomy, Ontogeny, and Origin. J. Am. Soc. Hort. Sci. 139:13-21.

Samarakoon, U.C., K.A. Funnell, D.J. Woolley, and E.R. Morgan. 2012. Temperature impacts changes in crown buds and flowering of gentian 'Spotlight'. Sci. Hortic. 143:49-55.

Samarakoon, U.C., K.A. Funnell, D.J. Woolley, and E.R. Morgan. 2015. Influence of photoperiod regime and exogenous plant growth regulators on crown bud formation in gentian. Sci. Hortic. 182:56-64.

Samarakoon, U.C., D.J. Woolley, E.R. Morgan, and K.A. Funnell. 2016. Ontogeny of Crown Bud Clusters and Dormancy-breaking Treatments Influence Productivity and Spread of Harvest Maturity of *Gentian* 'Showtime Diva'. HortScience 51:829-837.

Santana-Buzzy, N.C.-F., A. Barahona-Perez, F. Montalvo-Peniche, M. D. Zapata-Castillo, P. Y. Solis-Ruiz, A. Zaldivar-Colli, A. Gutierrez-Alonso, O. Miranda-Ham, M. L. 2005. Regeneration of habanero pepper (*Capsicum chinense* Jacq.) via organogenesis. Hort. Sci. 40:1829-1831.

Saritha, K.V. and C.V. Naidu. 2007. *In vitro* flowering of *Withania somnifera* Dunal. An important antitumor medicinal plant. Plant Sci. 172:847-851.

Sasaki, K., K. Shimomura, H. Kamada, and H. Harada. 1994. IAA metabolism in embryogenic and non-embryogenic carrot cells. Plant Cell Physiol. 35:1159-1164.

Scorza, R. and J. Janick. 1980. *In vitro* flowering of *Passiflora suberosa* L.[6-benzylamino purine]. J. Am. Soc. Hort. Sci.

Seabrook, J.E.A. 2005. Light effects on the growth and morphogenesis of potato (*Solanum tuberosum*) *in vitro*: A review. A. J. Potato Research 82:353-367.

Seelye, J.F., D.J. Maddocks, G.K. Burge, and E.R. Morgan. 1994. Shoot regeneration from leaf-disks of *Limonium pergrinum* using thidiazuron. N. Z. J. Crop Hortic. Sci. 22:23-29.

Sensor Sense B.V. Sensor sense user guide. www.sensor-sense.nl.

Sethi, U., A. Basu, and S. Guhamukherjee. 1990. Control of cell-proliferation and differentiation by modulators of ethylene biosynthesis and action in *Brassica* hypocotyl explants. Plant Sci. 69:225-229.

Sgamma, T., A. Jackson, R. Muleo, B. Thomas, and A. Massiah. 2014. TEMPRANILLO is a regulator of juvenility in plants. Scientific Reports 4:1-11.

Shah, J. and K. Unnikrishnan. 1969. Ontogeny of Axillary and Accessory Buds in *Clerodendrum phlomidis* L. Ann. Bot. 33:389-398.

Shah, J.J. and K. Unnikrishnan. 1971. Ontogeny of axillary and accessory buds in *duranta-repens* l. Botanical Gazette 132:81-91.

Sharma, N., K.P.S. Chandel, and A. Paul. 1993. *In vitro* propagation of *Gentiana kurroo*. An indigenous threatend plant of medicinal importance. Plant Cell Tiss. Org. Cult. 34:307-309.

Shekafandeh, A.a.Khosh- khui, M. 2008. Effect of bud position and culture medium on shoot proliferation from nodal culture of two mature *Guava* cultivars. Asian. J. Plant Sci. 7:177-182.

Shimizu-Sato, S. and H. Mori. 2001. Control of outgrowth and dormancy in axillary buds. Plant Physiol. 127:1405-1413.

Shimizu-Sato, S., M. Tanaka, and H. Mori. 2009. Auxin–cytokinin interactions in the control of shoot branching. Plant Mol. Biol. 69:429-435.

Shou, S.Y., L.X. Miao, W.S. Zai, X.Z. Huang, and D.P. Guo. 2008. Factors influencing shoot multiplication of lotus (*Nelumbo nucifera*). Biol. Plant. 52:529-532.

Simon, S. and J. Petrášek. 2011. Why plants need more than one type of auxin. Plant Sci. 180:454-460.

Singh, B., S. Sharma, G. Rani, G.S. Virk, A.A. Zaidi, and A. Nagpal. 2006. *In vitro* flowering in embryogenic cultures of *Kinnow mandarin* (*Citrus nobilis* Lour x *C. deliciosa Tenora*). African J. Biotech. 5:1470-1474.

Sińska, I. 1989. Interaction of ethephon with cytokinin and gibberellin during the removal of apple seed dormancy and germination of embryos. Plant Sci. 64:39-44.

Skoog, F. and C.O. Miller. 1957. Chemical regulation of growth and organ formation in plant tissues cultured in vitro. Symp. Soc. Exp. Biol. 11:118-130.

Snow, R. 1937. On the nature of correlative inhibition. New Phytol. 36:283-300.

Statistics New Zealand Plant and Food Research. 2013. <http://www.stats.govt.nz/>

Struwe, L., and V.A Albert. 2002. Gentianaceae Systematic and Natural History. Cambridge University Press, United Kingdom.

Sudha, C., P. Krishnan, and P. Pushpangadan. 1998. *In vitro* propagation of *Holostemma annulare* (Roxb.) K. Schum., a rare medicinal plant. In Vitro Cell. & Develop. Biol. Plant 34:57-63.

Suge, H., T. Nishizawa, H. Takahashi, and K. Takeda. 1997. Phenotypic plasticity of internode elongation stimulated by deep-seeding and ethylene in wheat seedlings. Plant Cell Environ. 20:961-964.

Suttle, J.C. 1988. Effect of ethylene treatment on polar IAA transport, net IAA uptake and specific binding of N-1-naphthylphthalamic acid in tissues and microsomes isolated from etiolated pea epicotyls. Plant Physiol. 88:795-799.

Taiz, L. and E. Zeiger. 2002. Plant Physiology (2002).

Takahashi, H., T. Imamura, A. Miyagi, and H. Uchimiya. 2012. Comparative metabolomics of developmental alterations caused by mineral deficiency during *in vitro* culture of *Gentiana triflora*. Metabolomics 8:154-163.

Tanaka, M., K. Takei, M. Kojima, H. Sakakibara, and H. Mori. 2006. Auxin controls local cytokinin biosynthesis in the nodal stem in apical dominance. The Plant Journal 45:1028-1036.

Tanimoto, S. and H. Harada. 1981. Chemical factors controlling floral bud formation of *Torenia* stem segments cultured *in vitro* I. Effects of mineral nutrients and sugars. Plant Cell Physiol. 22:533-541.

Tanimoto, S., A. Miyazaki, and H. Harada. 1985. Regulation by abscisic acid of *in vitro* flower formation in *Torenia* stem segments. Plant Cell Physiol. 26:675-682.

Taylor, N.J., M.E. Light, and J. Van Staden. 2005. *In vitro* flowering of *Kniphofia leucocephala*: influence of cytokinins. Plant Cell Tiss. Org. Cult. 83:327-333.

Te-chato, S., P. Nujeen, and S. Muangsorn. 2009. Paclobutrazol enhance budbreak and flowering of Friederick's Dendrobium orchid *in vitro*. J. Ag. Tech. 5:157-165.

Tee, C.S., M. Maziah, and C.S. Tan. 2008. Induction of *in vitro* flowering in the orchid *Dendrobium* Sonia 17. Biol. Plant. 52:723-726.

Teixeira da Silva, J.A., G.B. Kerbauy, S. Zeng, Z. Chen, and J. Duan. 2014. *In vitro* flowering of orchids. Crit. Rev. Biotechnol. 34:56-76.

Thakur, R., P.S. Rao, and V.A. Bapat. 1998. *In vitro* plant regeneration in *Melia azedarach* L. Plant Cell Rep. 18:127-131.

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

Thomann, A., E. Lechner, M. Hansen, E. Dumbliauskas, Y. Parmentier, J. Kieber, B. Scheres, and P. Genschik. 2009. *Arabidopsis* CULLIN3 genes regulate primary root growth and patterning by ethylene-dependent and -independent mechanisms. PLoS Genet. 5:1-14.

Thomson, K.S., R. Hertel, S. Muller, and J.E. Tavares. 1973. 1-N-naphthylphthalamic acid and 2,3,5-triiodobenzoic acid. *In vitro* binding to particulate cell fractions and action on auxin transport in corn coleoptiles. Planta 109:337-352.

Thomson, K.S. and A.C. Leopold. 1974. *In vitro* binding of morphactins and 1-n-naphthylphthalamic acid in corn coleoptiles and their effects on auxin transport. Planta 115:259-270.

Tisserat, B. and T. Murashige. 1977. Effects of ethephon, ethylene, and 2,4-dichlorophenoxyacetic acid on asexual embryogenesis *in vitro*. Plant Physiol. 60:437-439.

Trevisan, F. and B.M.J. Mendes. 2005. Optimization of *in vitro* organogenesis in passion fruit (*Passiflora edulis* F. flavicarpa). Scientia Agricola 62:346-350.

Trewavas, A.J. 1981a. How do plant growth substances work? plant cell & environment 4:203-228.

Trewavas, A.J. 1981b. What is the function of growth substances in the intact growing plant? Joint DPGRG and BPGRG Symposium.

Trewavas, A.J. 1982. Growth substance sensitivity: the limiting factor in plant development. Physiol. Plant. 55:60-72.

Trewavas, A.J. 1983. Is plant development regulated by changes in the concentration of growth substances or by changes in the sensitivity to growth substances. Trends Biochemical Science 7:354-357.

Trujillo-Moya, C.a.G., Carmina 2012. The influence of ethylene and ethylene modulators on shoot organogenesis in tomato. Plant Cell Tiss. Org. Cult. 111:41-48.

Tuominen, H., A. Ostin, G. Sandberg, and B. Sundberg. 1994. A novel metabolic pathway for indole-3-acetic acid in apical shoots of *Populus tremula* (L.) x *Populus tremuloides* (Michx.). Plant Physiol. 106:1511-1520.

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

Vain, P., H. Yean, and P. Flament. 1989. Enhancement of production and regeneration of embryogenic type II callus in *Zea mays* L. by $AgNO_3$. Plant Cell Tiss. Org. Cult. 18:143-151.

van Aartrijk, J. and G.J. Blom-Barnhoorn. 1983. Adventitious bud formation from bulb scale explants of *Lilium speciosum* Thunb. Effects of wounding, TIBA and temperature. J. Plant Physiol. 110:335-363.

van Aartrijk, J. and G.J. Blom-Barnhoorn. 1984. Adventitious bud formation from bulb scale explants of *Lilium speciosum* Thunb. *In vitro*, interacting effects of NAA, TIBA, wounding and temperature. J. Plant Physiol. 116:409-416.

van Aartrijk, J., G.J. Blom-Barnhoorn, and J. Bruinsma. 1985a. Adventitious bud formation from bulb-scale explants of *Lilium speciosum* thunb. *In vitro* effects of aminoethoxyvinyl-glycine, 1-aminocyclopropane-1-carboxylic acid, and ethylene. J. Plant Physiol. 117:401-410.

van Aartrijk, J., G.J. Blom-Barnhoorn, and J. Bruinsma. 1985b. Adventitious bud formation from bulb-scale explants of *Lilium speciosum* Thunb. *in vitro*. Production of ethane and ethylene. J. Plant Physiol. 117:411-422.

van den Dungen, R., S.T. Hekkert, S.M. Cristescu, and F.J.M. Harren. 2011. Highly sensitive ethylene detector for on line measurements on Kiwifruits, p. 651-656. In: G. Costa and A. R. Ferguson (eds.), VII International Symposium on Kiwifruit. Int. Soc. Hort. Sci., Leuven 1.

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

van Hooijdonk, B.M., D.J. Woolley, I.J. Warrington, and D.S. Tustin. 2011. Initial alteration of shoot architecture by dwarfing apple rootstocks involves shoot/root/shoot signalling between auxins, gibberellins and cytokinins, p. 857-863. In: T. L. Robinson (ed.), Acta Hortic. Int. Soc. Hort. Sci., Leuven, Belgium.

Vanstraelen, M. and E. Benkova. 2012. Hormonal interactions in the regulation of plant development, p. 463-487. In: R. Schekman, L. Goldstein, and R. Lehmann (eds.), Annual Review of Cell and Developmental Biology, Vol 28. Annual Reviews, Palo Alto.

Vaz, A.P.A., R.D.L. Figueiredo-Ribeiro, and G.B. Kerbauy. 2004. Photoperiod and temperature effects on *in vitro* growth and flowering of *P. pusilla*, an epiphytic orchid. Plant Physiol. Biochem. 42:411-415.

Vidal, N., G. Arellano, M.C. San-Jose, A.M. Vieitez, and A. Ballester. 2003. Developmental stages during the rooting of *in vitro* cultured *Quercus robur* shoots from material of juvenile and mature origin. Tree Phys. 23:1247-1254.

Vijayaraghavan, S.J. and W.L. Pengelly. 1986. Bound auxin metabolism in cultured crown-gall tissues of tobacco. Plant Physiol. 80:315-321.

Volkeart, H., J. Schoofs, A. Pieters, and E. Delanghe. 1990. Influence of explant source on *in vitro* axillary shoot formation in oak seedlings. Tree Phys. 6:87-93.

Vu, N.H., P.H. Anh, and D.T. Nhut. 2006. The role of sucrose and different cytokinins in the *in vitro* floral morphogenesis of rose (hybrid tea) cv. "First Prize". Plant Cell Tiss. Org. Cult. 87:315-320.

Wang, G.Y., M.F. Yuan, and Y. Hong. 2002. *In vitro* flower induction in roses. In Vitro Cell. & Develop. Biol. Plant 38:513-518.

Wang, Y. 2014. Seasonal changes of non-structural carbohydrates related to the growth and development of gentians: a thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Plant Science at Massey University, Palmerston North, New Zealand. The author.

Wang, Z., L. Wang, and Q. Ye. 2009a. High frequency early flowering from *in vitro* seedlings of *Dendrobium nobile*. Sci. Hortic. 122:328-331.

Wang, Z.H., L. Wang, and Q.S. Ye. 2009b. High frequency early flowering from *in vitro* seedlings of Dendrobium nobile. Sci. Hortic. 122:328-331.

Wardell, W.L. and F. Skoog. 1969. Flower formation in excised tobacco stem segments; I. Methodology and effects of plant hormones. Plant Physiol. 44:1402-1406.

Warner, H.L. and A.C. Leopold. 1969. Ethylene evolution from 2-chloroethylphosphonic acid. Plant Physiol. 44:156-158.

Watkins, C.B. 2006. The use of 1-methylcyclopropene (1-MCP) on fruits and vegetables. Biotechnol. Adv. 24:389-409.

Went, F. 1941. Polarity of auxin transport in inverted Tagetes cuttings. Botanical Gazette:386-390.

White, J. 1979. The plant as a metapopulation. Annu. Rev. Ecol. Syst. 10:109-145.

Williams, L.J. and H. Abdi. 2010. Fisher's least significant difference (LSD) test. Encyclopedia of research design:1-6.

Winer, L., R. Goren, and J. Riov. 2000. Stimulation of the oxidative decarboxylation of indole-3-acetic acid in citrus tissues by ethylene. Plant Growth Regu. 32:231-237.

Wingler, A. 2011. Interactions between flowering and senescence regulation and the influence of low temperature in *Arabidopsis* and crop plants. Ann. Appl. Biol. 159:320-338.

Woltering, E.J., F. Harren, and H.A. Boerrigter. 1988. Use of a laser-driven photoacoustic detection system for measurement of ethylene production in *Cymbidium* flowers. Plant Physiol. 88:506-510.

Wood, B. 1985. Effect of ethephon on IAA transport, IAA conjugation, and antidotal action of NAA in relation to leaf abscission of pecan. J. Am. Soc. Hort. Sci.

Xu, C., K.L. Liberatore, C.A. MacAlister, Z.J. Huang, Y.H. Chu, K. Jiang, C. Brooks, M. Ogawa-Ohnishi, G.Y. Xiong, M. Pauly, J. Van Eck, Y. Matsubayashi, E. van der Knaap, and Z.B. Lippman. 2015. A cascade of arabinosyltransferases controls shoot meristem size in tomato. Nat. Genet. 47:784-+.

Yamada, Y., Shoyama, Y., Nishioka, T., Kohda, H., Namera, A., and Okamot, T. 1991. Clonal micropropagation of *Gentiana scabra* Bunge var. buergeri Maxim, and examination of the homogenity concerning the gentiopicroside content. Chem. Eng. News 39:204-206.

Yamaguchi, M., C.W. Chu, and S. Yang. 1971. fate of 14C (2-chloroethyl) phosphonic acid in summer squash, cucumber, and tomato. Amer Soc Hort Sci J.

Yang, S.F. 1969. Ethylene evolution From 2-chloroethylphosphonic acid. Plant Physiol. 44:1203-1204.

Yang, S.F. and N.E. Hoffman. 1984. Ethylene biosynthesis and its regulation in higher plants. Annu. Rev. Plant. Physiol. 35:155-189.

Yang, S.J., W. Amaki, and H. Higuchi. 1999. Effects of cultivars and ambient environments on *in vitro* flowering in *Kalanchoe blossfeldiana* Poellniz. J. Jpn. Soc. Hort. Sci. 68:1170-1177.

Yen, C.Y.T., T.W. Starman, Y.T. Wang, and G.H. Niu. 2008. Effects of cooling temperature and duration on flowering of the nobile dendrobium orchid. Hort. Sci. 43:1765-1769.

Yordanova, Z.P., E.T. Iakimova, S.M. Cristescu, F.J. Harren, V.M. Kapchina-Toteva, and E.J. Woltering. 2010. Involvement of ethylene and nitric oxide in cell death in mastoparan-treated unicellular alga *Chlamydomonas reinhardtii*. Cell Biol. Int. 34:301-308.

Yu, Y.B. and S.F. Yang. 1979. Auxin-induced ethylene production and its inhibition by aminoethyoxyvinylglycine and cobalt ion. Plant Physiol. 64:1074-1077.

Zhang, F.L., Y. Takahata, and J.-B. Xu. 1998. Medium and genotype factors influencing shoot regeneration from cotyledonary explants of Chinese cabbage (*Brassica campestris* L. ssp. pekinensis). Plant Cell Rep. 17:780-786.

Zhang, S. 2008. Investigations into senescence and oxidative metabolism in gentian and petunia flowers: a thesis presented in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Plant Biotechnology at University of Canterbury, New Zealand.

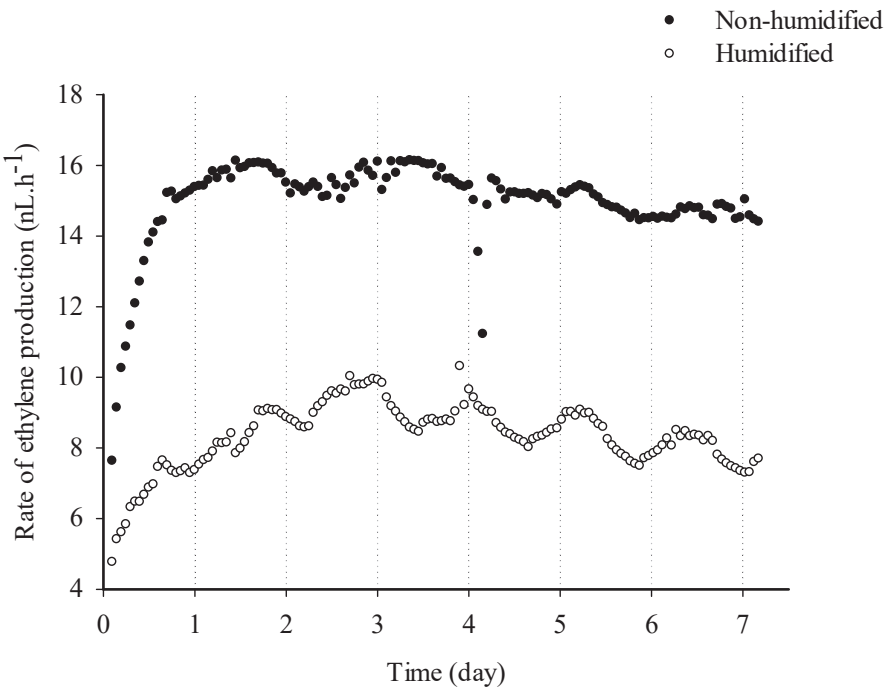
Zhang, Z.M. and D.W.M. Leung. 2002. Factors influencing the growth of micropropagated shoots and *in vitro* flowering of gentian. Plant Growth Regu. 36:245-251.

Zobayed, S.M., C. Kubota, and T. Kozai. 1999. Development of a forced ventilation micropropagation system for large-scale photoautotrophic culture and its utilization in sweet potato. In Vitro Cell. & Develop. Biol. Plant 35:350-355.

Appendices

Appendix 1

Despite endeavouring to keep vessels sterilised, and filter sterilising the incoming air flow, some replications of each treatment (i.e. vessels) had to be removed from the experiment due to contamination. For example, out of three vessels used for each treatment, only one remained non-contaminated for the entire period of observation. Although figure below suggests maximal production reached within ca. 12 h but number of vessels per treatment was not enough to produce statistically reliable results. Production of ethylene continued over the 6 days of measurement.



Rate of ethylene production from medium containing 10 mg.L^{-1} ethephon in sealed culture vessels using 'continuous flow' as the measurement method over the actual time frame of ethylene release (days). Non-humidified air flow (black) and humidified air flow (white), as recorded by ETD at 1 L.h^{-1} flow rate. Initially n = 3 for both treatments but only one of each remained uncontaminated during the entire measurement time.

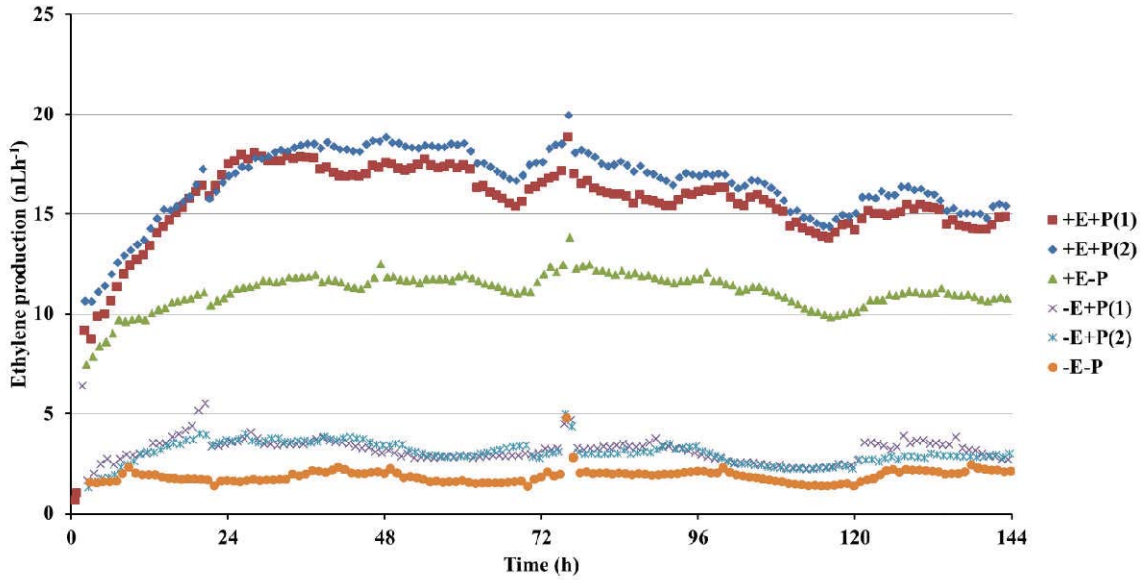
Appendix 2

In order to investigate the possibility of the interaction of ethephon applied in the medium on the biological activities of explants of gentian, concentration of ethylene in the headspace of vessels was evaluated. In doing so, only explants of 'Little Pinkie' were used and 6 vessels, each containing 50 mL of agar medium and a combination of different treatments, were sealed and connected to ETD using a continuous flow of 1 $L.h^{-1}$ air for a time period of 6 days as below:

- 2 vessels each containing 10 $mg.L^{-1}$ ethephon in the medium and 8 explants
- 2 vessels each containing 0 $mg.L^{-1}$ ethephon in the medium and 8 explants
- 1 vessel only containing 10 $mg.L^{-1}$ ethephon in the medium and no explant
- 1 vessel only containing 0 $mg.L^{-1}$ in medium and no explant

As illustrated in figure below the trends of each graph, showing concentration of ethylene measured in the headspace of vessels over the time, were different in different treatments. Culture vessels with ethephon added to their medium, resulted in increased production of ethylene compare to those without ethephon in the medium. Regardless of the presence or absence of ethephon in the medium, ethylene production was greater in the presence of explants, which presumably could be attributed to ethylene biosynthesis by the explants.

It is acknowledged however, that in the current experiment results were based on a single repeated-measurement experiment with not enough replication per treatments. Therefore, further repetition of the experiment was planned, so as to create more confidence about the effect of the explants on ethylene production within the experimental system. In addition, 'sampling' method was planned to be used in next experiments, in order to measure the concentration of ethylene in the headspace of the non-sealed culture vessel.



Rate of ethylene production in sealed culture vessels containing medium with 10 mg.L^{-1} ethephon including explants (blue +E+P) and excluding explants (red +E-P), and medium with 0 mg.L^{-1} ethephon including explants (green -E+P) and excluding explants (-E-P), recorded by ETD using continuous flow as the measurement method at 1L.h^{-1} flow rate. n = 1-2 .

Appendix 3

Concentration of ethylene (nL.L^{-1}) in the headspace of vessels including only ethephon (+E), explants of 'Little Pinkie' in medium amended with ethephon (+E LP), explants of 'Showtime Diva' in medium amended with ethephon (+E SD), no ethephon (-E), explants of 'Little Pinkie' in medium without ethephon (-E LP) and explants of 'Showtime Diva' in medium without ethephon (-E SD) over time, measured by ETD at flow rate of 5 L.h^{-1} using repeated sampling method. n=3 vessels each containing 8 explants.

Average of ethylene concentration (nL.L^{-1}) over time				
	Day 1	Day 2	Day 4	Day 6
Time	6 h	48 h	96 h	144 h
+E	65.02 ± 12.7 e[Z]	69.52 ± 3.1 e	53.26 ± 0.8 e	44.68 ± 2.5 e
+E LP	42.15 ± 9.5 e	55.84 ± 16.6 e	53.05 ± 3.6 e	54.24 ± 2.4 e
+E SD	65.93 ± 3.1 e	85.54 ± 5.6 e	66.31 ± 5.5 e	50.30 ± 4.2 e
-E	0.00 ± 0.0 a	0.32 ± 0.2 cd	0.05 ± 0.0 ab	0.39 ± 0.2 cd
-E LP	2.01 ± 0.2 d	1.06 ± 0.1 d	0.99 ± 0.1 d	1.15 ± 0.2 d
-E SD	1.08 ± 0.2 d	1.12 ± 0.0 d	0.99 ± 0.3 d	0.72 ± 0.1 cd

[Z] Groupings for mean separation are based on using the alternative LSD test where the Studentized Range statistic was used instead of Student's t test at $P \leq 0.05$ on log transformed data. Back transformed mean values followed by different letters within the columns and rows are significantly different at $P < 0.05$.

Appendix 4

As a part of the results and discussion of Section 5.1.2, the influence of positional effects on growth variables presented here. Similar to the results of some other experiments in this thesis which included plant material from various nodal positions, after 8 weeks of growth *in*

vitro, there was an increasing trend in the growth variables such as number of shoots, number of short shoots and total weight of the explant, as the distance of the position increased from the tip toward the base of the original shoot which explants were taken from ($P \leq 0.05$). Explants of basal origin produced more shoots and consequently more total fresh weight (~2 times more) compared to explants taken from other positions (Table 5-2). Compared with other experiments in this thesis however, for the explants taken from various positions, growth variables such as number of shoots and fresh weight slightly decreased (refer to Chapter 2; Tables 2-1 & 2-2). For example, explants of the Base position previously produced 4.8 times the number of shoots; which was reduced to only 2.54 times the controls in current experiment (refer to Chapter 2, Table 2-1). Fresh weight of the explants taken from Node 1 reduced from 220 mg in earlier experiments to 187.9 mg in current experiment and for explants of Node 2 from 290 mg to 191.2 mg. Such a reduction in those growth variables recorded currently as compared with previous experiments could be due to non-uniformity among the stock plants of 'Little Pinkie' investigated earlier which resulted in variability in vegetative vigour of the explants used as plant material over the duration that experiments were conducted in this thesis (refer to Chapter 2, Section A). Hence, it could be worth repeating the experiment in future after rejuvenation of the plant material which is used as the source of explants as detailed in Chapter 8 previously.

Growth variables of gentian 'Little Pinkie' cultured *in vitro* as affected by different positions which explants were originated from (tip (Tip), first node below the tip (Node 1), second node below the tip (Node 2) and rooted base (Base)). The data for the effect of different durations of explant exposure to ethylene gas flow on growth variables were integrated in the position factor.

	Variables		
	No. of shoots (1° and/or 2°)	**Total weight (mg)**	**No. short shoots**
Position			
Tip	1.07 a [Z]	170.9 a	0.22 a
Node 1	1.26 a	187.9 a	0.22 a
Node 2	1.20 a	191.2 a	0.43 ab
Base	2.54 b	360.2 b	0.76 b

[Z] Groupings for mean separation are based on Fisher's unprotected LSD test. Means followed by different letters within rows and columns are significantly different at $P < 0.05$. n=3 vessels each containing 8 explants.

Although the difference between explants taken from different positions was not statistically significant in the number of medium and long shoots, there was an increase in the number of short shoots raised from Basal explants by 70% as compared with the Tip and Node 1 explants, and 43% more compared to the Node 2 explants ($P \leq 0.05$). Such an increase was probably the source of the difference in the total number of shoots per explant as well as total

weight. As reported in Chapter 2, the largest number of additional 2º shoots which originated from adventitious buds following the application of ethephon occurred on explants from the Base position as compared with other positions. Therefore, the data for the explants of the Base position only were analysed, in order to check any possible influence of aerial ethylene application on them. There was still no significant difference between the explants of the Base position with or without aerial exposure to ethylene ($P = 0.19$, data not presented).

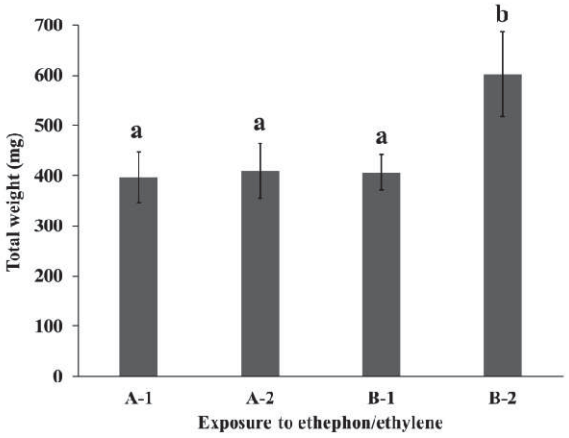
Appendix 5

Number of shoots (1° and/or 2°) developed from explants of gentian 'Little Pinkie' originated from different positions along the shoot as influenced by treatments: **A-1** without any exposure to ethylene either from the medium or the atmosphere in the headspace, by being cultured in ethephon free medium in a petri dish and locating into the ethephon free medium in the vessel, **A-2** without any exposure to ethylene either from the medium or the atmosphere in the headspace, by being cultured into the vessel contained ethephon free medium, **B-1** only aerial exposure to ethylene, by being cultured in a petri dish containing ethephon free medium but locating into the vessel contained medium amended with 10 $mg.L^{-1}$ ethephon, **B-2** aerial exposure to ethylene in the headspace as well as absorption from the medium by being cultured into the culture vessel contained medium amended with 10 $mg.L^{-1}$ ethephon. Data been Square root-transformed, mean comparison was done using Fisher's unprotected least significant difference. Different letters on actual mean values are significantly different at $P \leq 0.05$. Vertical lines represent ± standard error bars, n=8 vessels each containing 8 explants.

Treatment	A-1	A-2	B-1	B-2
Position				
Tip	0.87 a[Z]	1.25 ab	1.00 ab	1.12 ab
Node 1	1.50 abc	1.50 abc	1.62 abc	2.12 abc
Node 2	2.12 abc	3.12 c	2.37 bc	7.12 d
Base	8.12 de	8.87 e	10.12 e	19.12 f

[Z] Groupings for mean separation are based on Fisher's unprotected least significant difference test on square root-transformed data. Back transformed mean values followed by different letters within rows and columns are significantly different at $P < 0.05$.

Appendix 6



A-1: withot exposure to ethylene either from the medium or the atmosphere

A-2: without any exposure to ethylene either from the medium or the atmosphere

B-1: only aerial exposure to ethylene

B-2: aerial exposure to ethelne in the headspace as well as absorbtion from the medium (refer to figure 5-5)

Total fresh weight developed by explants of gentian 'Little Pinkie' as influenced by treatments: **A-1** without any exposure to ethylene either from the medium or the atmosphere in the headspace, by being cultured in ethephon free medium in a petri dish and locating into the ethephon free medium in the vessel, **A-2** without any exposure to ethylene either from the medium or the atmosphere in the headspace, by being cultured into the vessel contained ethephon free medium, **B-1** only aerial exposure to ethylene, by being cultured in a petri dish containing ethephon free medium but locating into the vessel contained medium amended with 10 $mg.L^{-1}$ ethephon, **B-2** aerial exposure to ethylene in the headspace as well as absorption from the medium by being cultured into the culture vessel contained medium amended with 10 $mg.L^{-1}$ ethephon (refer to Figure 5-5). Mean comparison was done using Fisher's unprotected least significant difference. Different letters on mean values are significantly different at $P \leq 0.05$. Vertical lines represent ± standard error bars, n=8 vessels each containing 8 explants.

Appendix 7

Following the experiment presented in Chapter 5, it was hypothesized that, one of the possibilities for inconsistency in the effect of ethephon observed as compared with those experiments reported in earlier chapters (refer to Chapter 1), could be related to the weak vegetative growth condition of donor plantlets at the time that experiment carried out. Hence, experiment was repeated to test the hypothesis. Therefore, donor plantlets of gentian 'Little Pinkie' were stored at low temperature (2ºC) for two months and prepared as detailed before using as the source of plant material (refer to Chapter 2, Section 2.2). Then they were cultured under standard growth condition *in vitro* in a fresh medium for 5 weeks. Explants were under two sub culturing period after taking out of the cold store condition before they were used for taking explants.

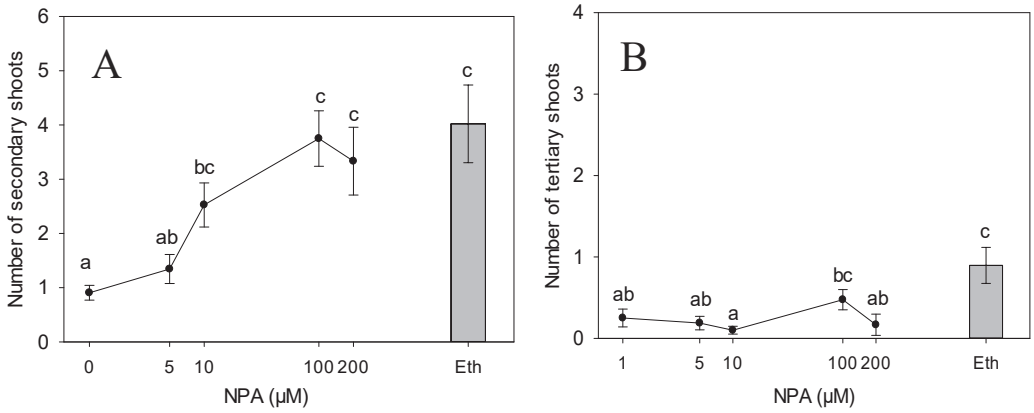
Factors for the experiment comprised chemical treatments (Control, ethephon or a concentration range of NPA) and positions of explants (i.e. Tip, Node 1 Node 2 and Node 3). Concentration range of NPA was 0, 5, 10, 100 and 200 µM. Compare to the first repetition, high concentrations of 1000 and 500 µM of NPA were removed from the concentration range

because their effect was negative on most of the growth variables. Also, low concentration of 2.5 µM of NPA which represented no significant effect on most of the growth variables compare to the control was not included. For treatments which included ethephon, the final concentration of ethephon in the medium was 10 mg.L^{-1}. There were three to six culture vessels as individual replications, each including eight explants from various positions distributed evenly. Growth variables recorded and analysis of the data was done as detailed in Section 6.2.2.

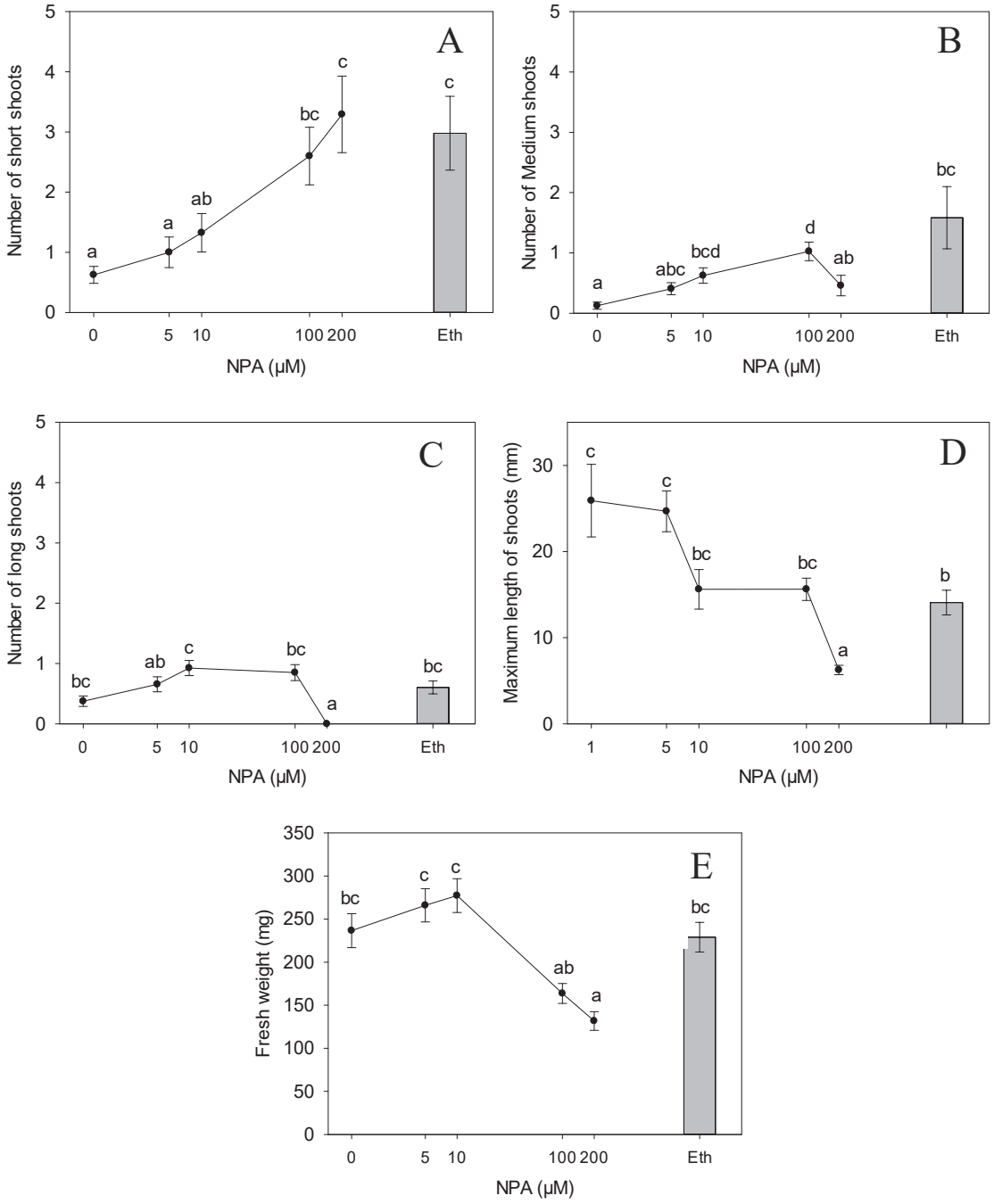
Probability (*P*) values from the analysis of variance for a factorial treatment design comprising chemical treatments (Control, ethephon or a concentration range of NPA) and positions of explants (Tip, Node 1 (N1), Node 2 (N2) or Node 3 (N3)) as factors. The chemical treatment factor was split into two contrasts: control versus NPA versus ethephon, and nested within that NPA concentration. The position factor was also split into two contrasts: Tip versus Nodes, and nested within that Node 1 versus Node 2 versus Node 3. Data were square root-transformed when needed.

Factor	Sqrt No. 2 shoots	Sqrt No. 3 shoots	Sqrt No. short shoots	Sqrt No. medium shoots	No. long shoots	Sqrt Max length	Weight (mg)
Ctrl vs NPA vs Eth	0.008	0.011	0.023	0.006	0.974	0.120	0.863
NPA dose	0.033	0.151	0.014	0.080	0.013	0.005	0.002
Tip vs Node	<.001	<.001	<.001	0.048	<.001	0.066	<.001
N1 vs N2 vs N3	0.025	0.189	0.027	0.070	0.536	0.477	0.027
Tip vs Node.Ctrl vs NPA vs Eth	0.010	0.029	0.067	0.007	0.308	0.007	0.685
Tip vs Node.NPA dose	0.578	0.960	0.398	0.289	0.369	0.319	0.036
Ctrl vs NPA vs Eth.N1 vs N2 vs N3	0.217	0.181	0.087	0.031	0.115	0.031	0.653
NPA Dose. N1 vs N2 vs N3	0.625	0.738	0.393	0.293	0.853	0.768	0.459

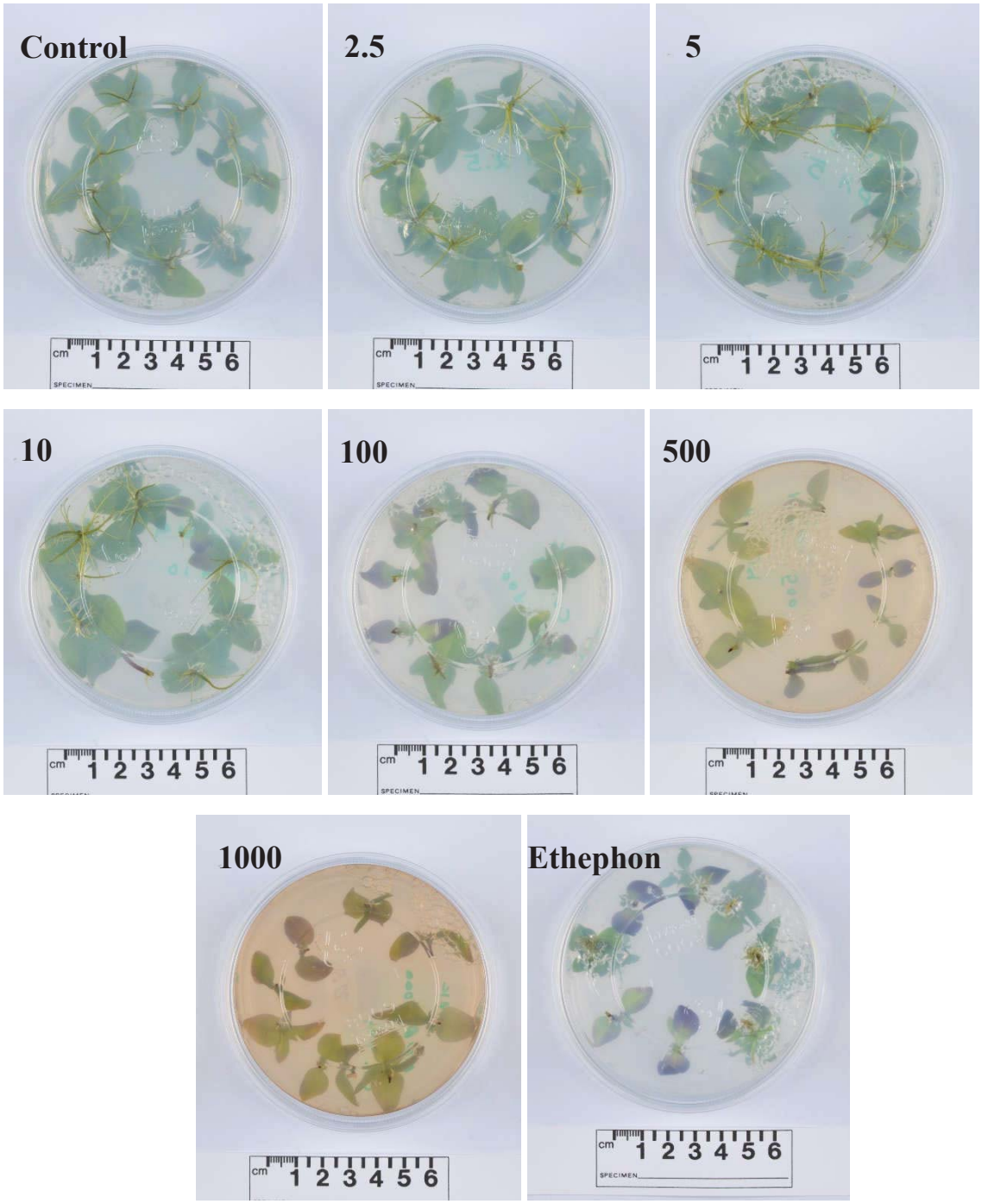
Effect of NPA and ethephon on growth variables:



Mean values for number of **(A)** shoots (1º and/or 2º) and **(B)** tertiary shoots developed from the explants of gentian 'Little Pinkie' as effected by different chemical treatments (NPA at concentrations of 0 (control), 5, 10, 100 and 200 µM compared with ethephon (Eth) at concentration of 10 mg.L^{-1} after 8 weeks growth *in vitro*. Data were square root-transformed. Mean comparison was done using the alternative LSD test where the Studentized Range statistic was used instead of Student's t test at $P \leq 0.05$.. Vertical lines represented ± standard errors. Back-transformed mean values followed by different letters were significantly different at $P \leq 0.05$, n=3 to 6 vessels each containing 8 explants.



Mean values for number of **(A)** short, **(B)** medium and **(C)** long shoots, **(D)** maximum length of shoots and **(D)** total fresh weight the explants of gentian 'Little Pinkie' taken from various positions as effected by different chemical treatments (NPA at concentrations of 0 (control), 5, 10, 100 and 200 µM compared with ethephon (Eth) at concentration of 10 mg.L^{-1} after 8 weeks growth *in vitro*. Data for short and medium shoots were square root-transformed. Mean comparison was done using the alternative LSD test where the Studentized Range statistic was used instead of Student's t test at $P \leq 0.05$. Vertical lines represented ± standard errors. Back-transformed mean values followed by different letters were significantly different at $P \leq 0.05$, n=3 to 6 vessels each containing 8 explants.



Bottom view (root area) of the culture vessels containing explants of gentian 'Little Pinkie' taken from different positions (Tip, Node 1, Node 2 and Node 3), after 8 weeks of growth *in vitro* treated as control, having10 mg.L^{-1} ethephon or different concentrations of NPA (2.5, 5, 10, 100, 500 and 1000 µM) in the medium.

Appendix 8

In a test experiment time duration which was needed for radioactivity transport was investigated prior to the main experiment. Factors were time duration (4 and 12 hours), chemical treatments (control, inclusion of ethephon or NPA in the medium). There were three replications of each treatment combination. Pre-treatments, radioactivity application and counting the amount of radioactivity carried out as detailed in Section 6.4.2.3. Each stem was cut to give three segments (top, middle and bottom) after radioactivity transport. Recorded variables were the radioactivity derived from ^{14}C-IAA that had been transported and counted in the receiver agar, number of ^{14}C-IAA been transported, in the top, middle and bottom segments of the stem and remaining in the donor agar after the transport period.

Number of ^{14}C-IAA transported in various time durations (4 or 12 hours), as affected by pre-treatments (control, ethephon and NPA in their medium) into various parts of transport system (receiver agar, top segment, middle segment, bottom segment and donor agar).

Chemical treatment	Transport parts	Transport duration	
		4 hours	12 hours
Control	**Receiver agar**	905 **cdef** [Z]	77 **ab**
Control	**Top stem**	881 **cdefg**	2324 **cdefg**
Control	**Middle stem**	3593 **efghi**	6826 **ghij**
Control	**Bottom stem**	7277 **hij**	17881 **ijkl**
Control	**Donor agar**	117658 **kl**	90477 **kl**
Ethephon	**Receiver agar**	208 **a**	229 **abc**
Ethephon	**Top stem**	468 **bcde**	898 **cdefg**
Ethephon	**Middle stem**	1978 **defg**	4786 **fghij**
Ethephon	**Bottom stem**	5770 **ghij**	17100 **ijk**
Ethephon	**Donor agar**	139174 **l**	86924 **kl**
NPA	**Receiver agar**	504 **bcde**	336 **abc**
NPA	**Top stem**	208 **abc**	437 **bcd**
NPA	**Middle stem**	2738 **defgh**	5084 **fghij**
NPA	**Bottom stem**	8053 **hij**	27617 **jkl**
NPA	**Donor agar**	130613 **kl**	84081 **kl**

[Z] Mean comparison was done using the alternative LSD test where the Studentized Range statistic was used instead of Student's t test. Actual mean values followed by different letters within rows and columns of each group are significantly different at $P \leq 0.05$ and based on log-transformed data.

Appendix 9

In a small separated experiment two different extraction methods were compared to each other for their efficiency before being applied in the main experiment.

Every four pieces of stem segments taken from either the top, middle or the bottom inserted into separated vials while they were intact or mashed. Three mL Methanol 80% was added to intact tissue in each vial. Vials then were sealed and placed on a shaker at 20°C in darkness for 24 hrs to extract the radioactive compounds. For mashing the tissue, 250µL Methanol 80% was inserted into each vial and tissue was grinded using a pistil. Another 250 µL Methanol 80% was used to wash any residuals of the tissue or radioactivity on the pistil. Vials then stored in darkness overnight before they were measured for their radioactivity content.

Factors for the analysis therefore comprised: 1) stem tissue for the extraction (mashed or intact) and 2) stem segments (tip, middle or bottom). There were three replications of vials for each treatment combination. Result of the current experiment was used to make the decision over using intact tissue for the extraction in the main experiment.

Total radioactivity derived from ^{14}C-IAA retrieved in extracts using different methods (intact or mashed tissue), in different segments of the stem (top or bottom segments) after 4 hours transport period.

	Extraction tissue	
Stem segment	**Mashed**	**Intact**
Top	3963 a[Z]	3936 a
Middle	1103 a	1188 a
Bottom	2303 a	1365 a
***P* value**		
Stem segment	0.9	
Extraction tissue	0.46	
Stem segment × Extracted tissue	0.89	

[Z] Mean comparison was done using the alternative LSD test where the Studentized Range statistic was used instead of Student's t test. Mean values followed by similar letters within rows and columns of each group were not significantly different at $P \leq 0.05$.

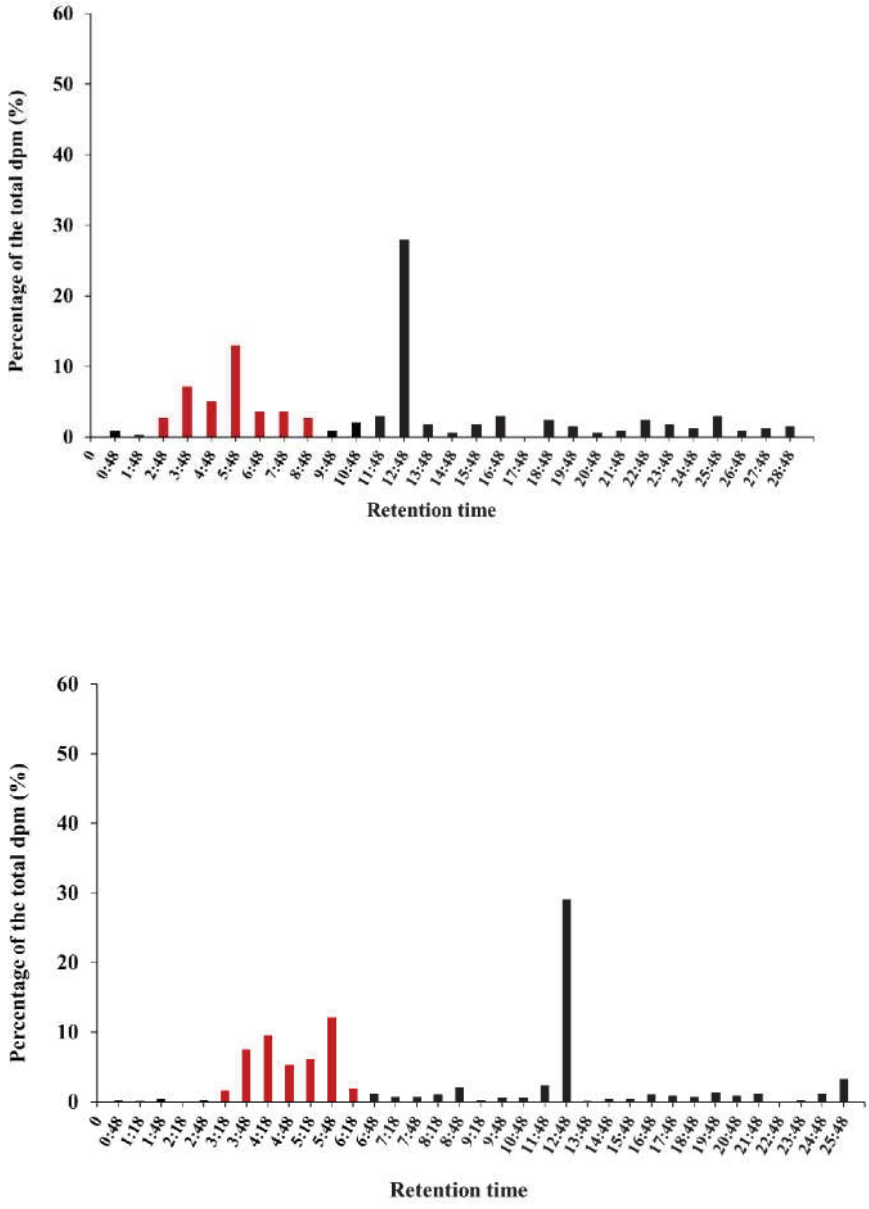
In order to investigate the duration of time intervals between fractions, 50 µL of a test sample was injected in to the HPLC. Collection of fractions started at 48 seconds after the injection based on the information achieved from injecting a standard solution of IAA (Figure 6-10). There was one minute time intervals between collections in order to collect IAA in a single vial at 11:48. Scintillant was added to each vial, shaken, stored in the dark overnight and then counted. Results of the bar chart for the number of dpm counted in each vial over the

retention time represented a major peak at the similar retention time as the standard IAA (Figure 6-10). Therefore, it could be concluded that the majority of the radioactivity which had been transported into the sample was still in the form of IAA.

However, there were some peaks at earlier retention times as IAA which indicated some of the radioactivity had been possibly converted into other IAA metabolites (Figure below red bars). These peaks started at about 1:48 after the injection and continued to about 7:48. Hence, for the second test sample time intervals decreased to every 30 seconds in order to capture early fractions more accurately. Decreasing the duration of time interval between collections from one minute to 30 seconds, indicated that earlier peaks started at 2:48 minutes and continued till 5:48 after the injection.

The results represented in the graphs of the two test injections were utilised to develop a more accurate plan for time intervals between fractions for the rest of the samples. Since there was no peak for the first 1:30, no fraction was collected during that time. Then first fraction was collected 48 seconds later, at 2:18 following by the next one at 3:18. Time interval between collections decreased to 20 seconds for the next 9 fractions from 3:18 to 6:18. Then the rest of the collection continued every one minute. Fractions therefore for the rest of the injections were planned to be collected in scintillation vials at 0:48, 1:48, 2:48, 3:48, 4:08, 4:28, 4:48, 5:08 ,5:28, 5:48, 6:08, 6:28, 6:48, 7:48, 8:48, 9:48, 10:148, 11:48, 12:48, 13:48, 14:48, 15:48, 16:48, 17:48, 18:48, 19:48, 20:48, 21:48, 22:48 and 23:48.

Appendix 10



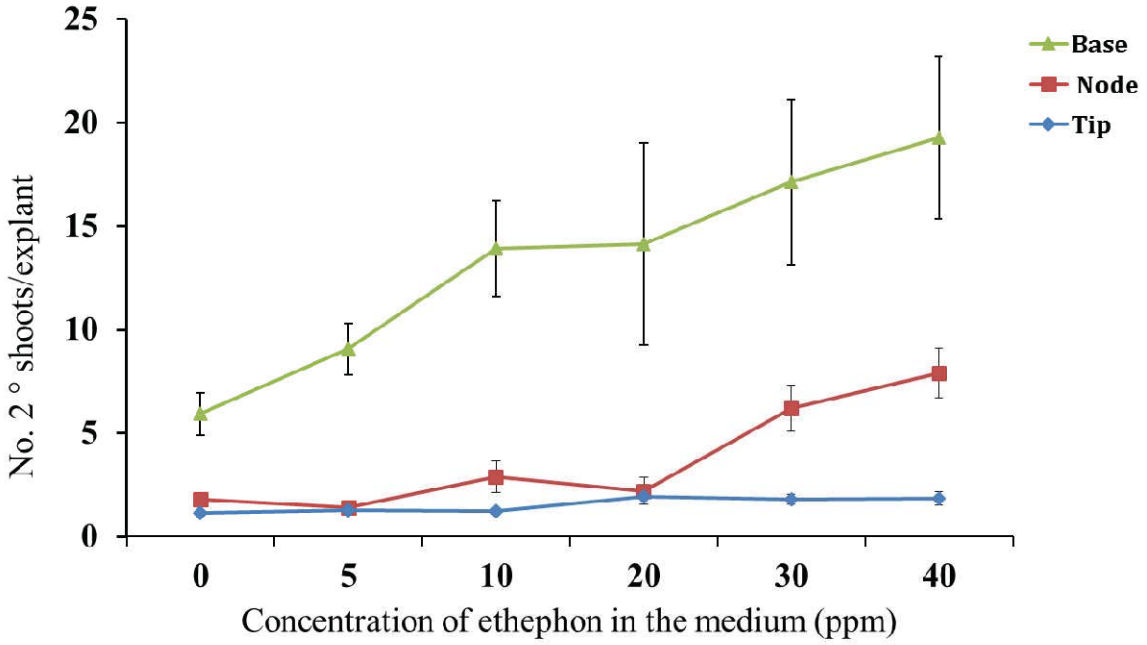
Collected fractions (percentage of each fraction from the total) of a 50 µl solution injected into HPLC, contained radioactivity over the retention time of (A) one minute intervals and (B) 30 seconds intervals. Major peak represented the quantity of the radioactive derived from ^{14}C-IAA. Red peaks represented IAA metabolites.

Appendix 11

Summarised information from the reports of previous researchers on various plant genera, in table below was used to determine the concentration range of 0 to 18 mg.L^{-1} used in Chapter 6, Experiment 2. Table included name of the author, the type of cytokinin applied, concentration range including the optimum concentration highlighted in red, the effect on various plant genera and part of the plant which was used to be cultured *in vitro* as an explants.

Author	Type of cytokinin	Concentration range	effect	Crop	Explant
(Fiuk and Rybczynski, 2008a)	TDZ BAP	(0, 0.25, 0.50, 0.75, 1 mg.L^{-1})	TDZ: direct regeneration BAP: Callus induction	*Gentianella kurroo*	Leaf derived callus
(Kim et al., 1997)	TDZ BAP	TDZ (1, 5, 10, 20, or 40 µM) BAP (1, 5, 10, 20, 40 or 80 µM) 80 µM =17.6 mg.L^{-1}	Maximum axillary shoot proliferation	*Fraxinus pennsylvanica* Marsh	
(Seelye et al., 1994)	Zeatin TDZ	Zeatin (3.0 mg.L^{-1}) TDZ (0, 1.0, 2.0, 3.0, 4.0, or 5.0 mg.L^{-1})	Better shoot regeneration in TDZ 2 days Pulse application of TDZ in liquid media=long application in solid media	*Limonium perigrinum*	leaf discs
(Jomori et al., 1995)	TDZ BAP	TDZ (0,0.5,1,2,4 mg.L^{-1}) BAP (0,0.5,1,2,5 mg.L^{-1})	TDZ more effective than BA on % of shoot regeneration callus	*Gentianella scabra*	Leaf derived callus
(Nakano et al., 1995)	TDZ	1 to 10 mg.L^{-1}	Plants from Protoplast-derived calluses	*Gentianella*	
(Hosokawa et al., 1996)	TDZ 4PU-30 BA Zeatin	5 – 10 mg.L^{-1} and 0.1 mg.L^{-1}	TDZ was the most effective	*Gentianella*	leaf and stem explants
(Hosokawa et al., 1998)	4PU-30 BAP TDZ	0.01-0.5 mg.L^{-1} for all	TDZ was the most effective	*Gentianella triflora* × *G. scabra*	Shoots in Liquid culture
(Fiuk and Rybczynski, 2008b)	Zeatin, Kinetin,CPPU TDZ, BAP	(Zeatin, kinetin, CPPU) (0.25,0.5,1,2) TDZ or BAP (0.5,1,2,3)	BAP the most effective on % of somatic embryogenesis	*Gentianella* (5 cultivars)	leaf explants

Appendix 12



Number of secondary (2°) shoots arised from explants of gentian 'Little Pinkie' taken from various positions (tip (Tip), first and second nodes below the tip (Node) and rooted base (Base)) after 8 weeks growth *in vitro* containing various concentration of ethephon (0, 5, 10, 20, 30 and 40 ppm) in the medium. Mean comparison was done using the alternative LSD test where the Studentized Range statistic was used instead of Student's t test at $P \leq 0.05$. Vertical lines represented ± standard errors, n=7 vessels each containing 8 explants.

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Short communication

Ethephon and secondary shoot induction in Gentian (*Gentiana* spp.) hybrids *in vitro*

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ABSTRACT

The potential effect of ethylene on the production of secondary shoots of *in vitro* explants of two cultivars of gentian (*Gentiana* sp.) with different branching habit, 'Little Pinkie' (naturally highly branched) and 'Showtime Diva' (naturally less branched), was evaluated by supplementing the medium with ethephon ([2-chloroethyl]phosphoric acid). An almost constant concentration of ethylene (60 nL L^{-1}) in the headspace of the culture vessels was measured when the medium was supplemented with ethephon. The number of secondary shoots arising from single node explants of 'Little Pinkie' after 8 weeks increased five times as a result of ethephon application *in vitro*, whereas branching did not increase in explants of 'Showtime Diva'. Microscopic observation supported the hypothesis that secondary shoots additional to those arising from pre-existing axillary buds were adventitious in origin. Future research strategies will be to investigate the mechanism of action of ethephon/ethylene on branching, and determining the basis of genetic differences between cultivars.

1. Introduction

Branched plants, with a high frequency of secondary shoot production (*i.e.* individual shoots arising from nodes on the stem), typically result in high-quality pot plants of gentian (*Gentiana* sp.). While auxin and cytokinin affect plant regeneration of gentians *in vitro* (Hosokawa et al., 1996), the possibility of ethylene's effect on morphogenesis (Biddington, 1992) has not been reported. As explored within the current study, it was hypothesised that ethephon ([2-chloroethyl]phosphoric acid), as a source of ethylene, might influence shoot formation *in vitro*.

Most studies have reported adverse effects of ethylene accumulation following the growth of explants in a sealed system *in vitro*, in several plant species (Chi and Pua, 1989; Kevers et al., 1992; Pua and Chi, 1993; Pua et al., 1999). However, a promoting effect on shoot regeneration has been reported (Kevers et al., 1992). The current study was undertaken as a part of an ongoing research programme to relate the ethylene quantity released in the headspace to the number of secondary shoots of *in vitro* explants of two different cultivars of hybrid gentian: 'Little Pinkie' (naturally highly branched) and 'Showtime Diva' (naturally less branched). We also studied the origin of buds that subsequently grow into secondary shoots *in vitro*.

2. Materials and methods

Plantlets of the *Gentiana* spp. cultivars 'Little Pinkie' and 'Showtime Diva', which had been grown *in vitro* for several propagation cycles, were used as source material. The base medium was as described by Morgan et al. (1997). A final concentration of 10 mg L^{-1} ethephon (Ethrel®; 480 g L^{-1} chlorethephon; Bayer Crop Science, New Zealand), was attained when added *via* filter sterilising (0.2 μm Minisart® filter; Sartorius Stedim Biotech) before the medium cooled. Culture vessels were disposable 290 mL clear plastic (Alto Packaging, Hamilton, New Zealand) tubs with snap-on lids which allowed gas exchange. Each culture vessel contained 50 mL of medium and explants were cultured *in vitro* as described previously (Morgan et al., 1997).

Shoots of plantlets were divided into single-nodal explants, each 10 mm in length, and positioned with the nodes above the surface of the medium. Morphological features of fresh samples of explants were recorded under a binocular microscope (Leica DFC 550, Switzerland), with a Leica M 205 FA digital camera attached, both at day 0 and week 8. At the end of the growth period, samples were dissected to expose the node and viewed under an FEI

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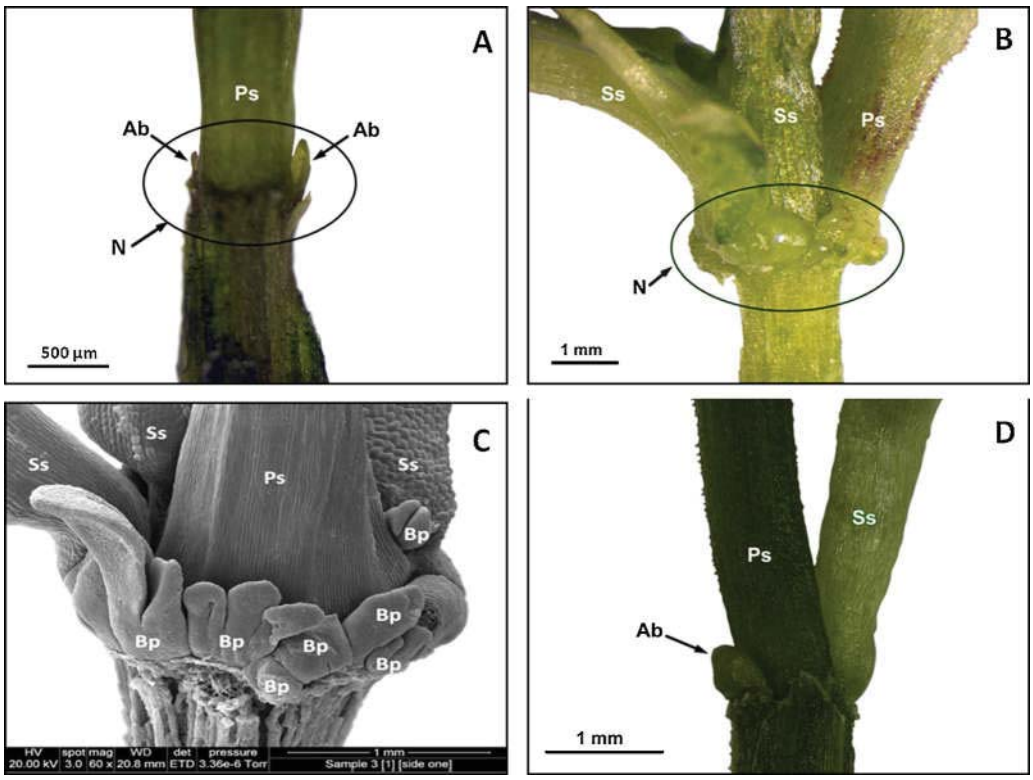

Fig. 1. Morphological features of the node (N) of *in vitro* explants of gentian 'Little Pinkie' at day 0, with axillary buds (Ab) only visible once the leaf sheath was removed from the stem, *i.e.* primary stem (Ps) (A). After 8 weeks growth in medium containing 0 mg L^{-1} ethephon; secondary shoots (Ss) together with an area of meristematic activity at the node position of 'Little Pinkie' under the binocular microscope (B); secondary shoots and bud primordia (Bp) at the meristematic zone of 'Little Pinkie' under the scanning electron microscope (C); and, secondary shoot and bud primordia at the node of 'Showtime Diva' under the binocular microscope (D).

Quanta 200 scanning electron microscope (SEM; FEI Electron Optics of Eindhoven, The Netherlands).

Gas samples were taken from the headspace of culture vessels every 48 h and analysed for ethylene concentration using an ethylene detector (ETD-300, Sensor sense, Nijmegen, The Netherlands); access for sampling was enabled by a rubber septum installed on each lid. The first and last ethylene measurements were done 4 h and 366 h (2 weeks) after adding ethephon to the medium, respectively. Sampling used the 'Sample mode approach' noted by Cristescu et al. (2013). This methodology was extended to calculate the rate of ethylene production.

A completely randomised design was used with a 2×2 factorial arrangement of treatments: media (amended with or without ethephon) and cultivars ('Showtime Diva' and 'Little Pinkie'). Each treatment comprised at least three culture vessels as replicates, with eight explants in each culture vessel. ANOVA was applied to data using Minitab (version 16, 2010, Minitab Inc., USA), with mean comparisons using Tukey's test at $P < 0.001$.

3. Results

Secondary shoots became macroscopically visible 4–8 weeks after transferring the explants *in vitro*, depending on the cultivar. After 8 weeks growth *in vitro*, when treated with ethephon the two cultivars differed in the number of secondary shoots per explant ($P \leq 0.001$). Additionally, the interaction between cultivar and medium was highly significant ($P \leq 0.001$). The number of secondary shoots per explant of 'Little Pinkie' increased from 2 shoots in the absence of ethephon to 10 shoots with ethephon added. In explants of 'Showtime Diva' however, there was no significant difference in the number of secondary shoots between explants grown in media with or without ethephon, with plants in both treatments achieving an average of one secondary shoot per explant ($P = 0.482$). Similarly, in the absence of ethephon the cultivars did not differ in the number of secondary shoots per explant.

At the time nodal explants of both cultivars were transferred to the *in vitro* medium, apart from the two axillary buds, no other meristematic activity was evident at the node (Fig. 1A). After 8 weeks of growth on media without ethephon added, explants of 'Little Pinkie' produced one or two secondary shoots arising from the node, plus several primordia that were microscopically visible at the node, after dissection of the leaf sheath. These additional primordia, arranged almost in a ring at the node, developed after the experiment commenced (Fig. 1B). As they were located randomly around the node, and not necessarily associated with the axillary bud, it supported the hypothesis that the additional bud primordia were adventitious in their origin. This hypothesis was further supported by the SEM images (Fig. 1C). However, in 'Showtime Diva' while one or two secondary shoots were observed arising from the node, no additional primordia were evident (Fig. 1D).

A comparison between microscopic images of 'Little Pinkie' after 8 weeks of growth, in media with or without ethephon added, indicated that ethephon increased the number of bud primordia at the node which developed into secondary shoots (Fig. 2A). In contrast to the results for 'Little Pinkie', multiple bud primordia around the node of explants of 'Showtime Diva' were not evident, irrespective of the presence or absence of ethephon in the medium (Fig. 2B).

The rate of ethylene release from the medium increased during the first 48 h after adding ethephon to the medium, and then decreased over time through to 336 h (Fig. 3). The concentrations of ethylene in the headspace at each measurement time were not significantly different ($P \leq 0.1$) and averaged 60 ± 10 nL L^{-1} over the duration of the experiment (Fig. 3). The average rate of ethylene

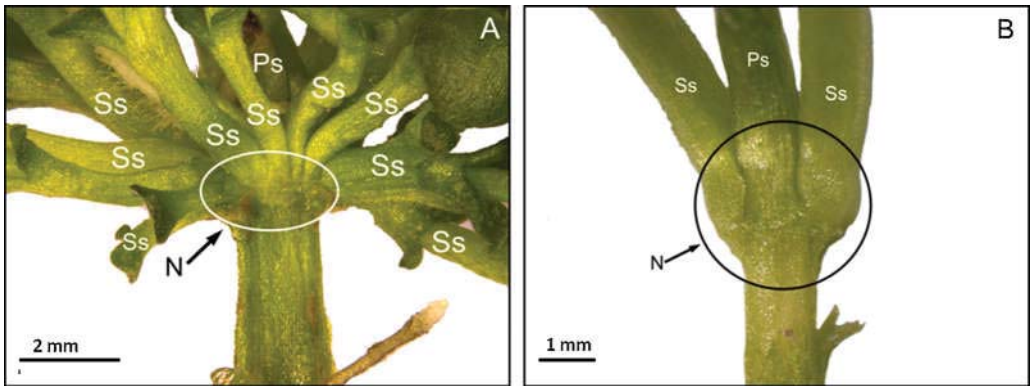

Fig. 2. Morphological features at the node (N) of *in vitro* explants of gentian after 8 weeks growth in medium containing 10 mg L^{-1} ethephon; *i.e.* primary stem (Ps) visible after leaf sheath was removed, secondary shoots (Ss) on 'Little Pinkie (A), and 'Showtime Diva' (B).

release from the medium at the first measurement time (*i.e.* 4 h after adding ethephon to the medium) was calculated to be 5.65 nL h^{-1}.

4. Discussion

Differences between cultivars, stages of culture and/or shoot organogenesis, in their *in vitro* shoot regeneration response to ethephon/ethylene have previously been reported in other plant species (Pua et al., 1999; Huxter et al., 1981; Lakshmanan et al., 1997). Hence, different cultivars at different biological stages within the explant may respond to ethephon differently as a result of differences in sensitivity, affinity and/or endogenous hormonal content (Trewavas, 1982). In the current study the appearance of additional buds at the nodes of 'Little Pinkie' treated with ethephon supports the hypothesis of their adventitious origin. However, due to the limitation of the number of samples that could be examined under SEM, further microscopy is required to confirm whether ethephon was effective only in stimulating the development of existing primordia or was stimulating initiation of new bud primordia as well. In addition, while not explored within the current study, differences in the effective concentrations of ethephon for each genotype could be another factor leading to such differences in response between cultivars.

To calculate the rate of ethylene production, the contents of the culture vessel were flushed through the ethylene detector (ETD), resulting in a peak, which would give rise to a measured headspace concentration of 62.17 nL L^{-1} in just 2.64 h. That the headspace concentration did not increase after this time, despite changes in rate of ethylene release, would in part be due to the continuous gas exchange and ethylene escape *via* the edges of the lids of the non-sealed culture vessels, presumably through mass flow (*i.e.* leakage). The concentration of accumulated ethylene in the headspace at the time of measurement, therefore, would be the net result of an equilibrium between the rate of ethylene released from the agar medium and the rate of leakage over time.

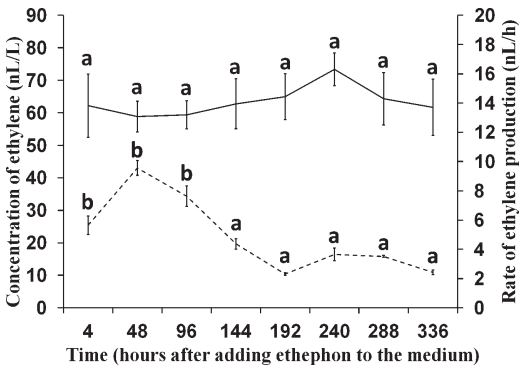

Fig. 3. Daily mean concentration of ethylene in the headspace of non-sealed culture vessels (solid line) and the rate of ethylene production from medium containing 10 mg L^{-1} ethephon (dashed line). $N = 3$. Vertical lines represent standard error. Within each line, mean values with different letters were significantly different at $P < 0.01$.

An increase in the number of secondary shoots arising from explants of 'Little Pinkie' when exposed to approximately 60 nL L^{-1} ethylene, is consistent with a promotional effect of 100 nL L^{-1} on shoot formation of peach (Dimasi-Theriou and Economou, 1995), and that of slightly higher concentrations of 1000 and 1280 nL L^{-1} on multiplication rate of rose (Kevers et al., 1992) and eastern white cedar (Nour and Thorpe, 1994) explants *in vitro*. While in contrast, application of a very high concentration of ethylene (10,000 nL L^{-1}) suppressed the number of shoots in peach, a low concentration (100 nL L^{-1}) increased it (Dimasi-Theriou and Economou, 1995). The results of the aforementioned studies, as well as the result of the current study, therefore support the hypothesis that low concentrations of ethylene in ventilated systems, within a range of 60–1280 nL L^{-1}, can encourage the growth and development of branches in explants whereas high concentrations (10,000 nL L^{-1}) may be inhibitory, especially in sealed vessels where altered levels of other gases (*e.g.* CO_2) as a consequence of tissue growth can also have an inhibitory effect. Another possibility for such differences noted in the literature may, in part, also be due to the lack of highly sensitive equipment to detect the very low concentrations of ethylene determined in the current study.

In addition to the concentration of ethylene applied, time and duration of the application may also be influential on branching. In the current study the concentration of ethylene was measured only during the first 2 weeks of growth; however, others have reported concentrations must be maintained for 3 weeks or even 12 weeks with rose (Kevers et al., 1992) and *Thuja occidentalis* (Nour and Thorpe, 1994), respectively. On the other hand, in a wide range of other studies, enhancement of shoot induction *in vitro*, as well as the increase in the number of shoots per explant, was achieved only by inhibiting ethylene production in the system under investigation (Chi and Pua, 1989; Pua et al., 1999). As evident in other plant systems, variability of ethylene's effect to either inhibit or enhance developmental responses can be related to different species, genotype, tissue, culture system and presence of other plant hormones, which probably affect either endogenous ethylene production or sensitivity to this hormone (McManus, 2012). The use of various ethylene inhibitors in future research may provide further insights into the role of ethephon/ethylene in generating the plant response reported in this study.

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References

Biddington, N.L., 1992. The influence of ethylene in plant-tissue culture. Plant Growth Regul. 11, 173–187.

Chi, G.L., Pua, E.C., 1989. Ethylene inhibitors enhanced de novo shoot regeneration from cotyledons of *Brassica campestris* ssp. *chinensis* (Chinese cabbage) in vitro. Plant Sci. 64, 243–250.

Cristescu, S.M., Mandon, J., Arslanov, D., De Pessemier, J., Hermans, C., Harren, F.J., 2013. Current methods for detecting ethylene in plants. Ann. Bot. 111, 347–360.

Dimasi-Theriou, K., Economou, A., 1995. Ethylene enhances shoot formation in cultures of the peach rootstock GF-677 (*Prunus persica* × *P. amygdalus*). Plant Cell Rep. 15, 87–90.

Hosokawa, K., Nakano, M., Oikawa, Y., Yamamura, S., 1996. Adventitious shoot regeneration from leaf, stem and root explants of commercial cultivars of Gentiana. Plant Cell Rep. 15, 578–581.

Huxter, T.J., Thorpe, T.A., Reid, D.M., 1981. Shoot initiation in light- and dark-grown tobacco callus: the role of ethylene. Physiol. Plant. 53, 319–326, http://dx.doi.org/10.1111/j.1399-3054.1981.tb04507.x.

Kevers, C., Boyer, N., Courduroux, J.C., Gaspar, T., 1992. The influence of ethylene on proliferation and growth of rose shoot cultures. Plant Cell Tissue Organ Cult. 28, 175–181.

Lakshmanan, P., Ng, S.K., Loh, C.S., Goh, C.J., 1997. Auxin, cytokinin and ethylene differentially regulate specific developmental states associated with shoot bud morphogenesis in leaf tissues of mangosteen (*Garcinia mangostana* L) cultured in vitro. Plant Cell Physiol. 38, 59–64.

McManus, M.T., 2012. The Plant Hormone Ethylene, Annual Plant Reviews, vol. 44. Wiley-Blackwell, Oxford.

Morgan, E.R., Butler, R.M., Bicknell, R.A., 1997. In vitro propagation of *Gentiana cerina* and *Gentiana corymbifera*. N. Z. J. Crop Hortic. Sci. 25, 1–8.

Nour, K.A., Thorpe, T.A., 1994. The effect of the gaseous state on bud induction and shoot multiplication in vitro in eastern white cedar. Physiol. Plant. 90, 163–172.

Pua, E.C., Chi, G.L., 1993. De-novo shoot morphogenesis and plant-growth of mustard (*Brassica juncea*) in vitro in relation to ethylene. Physiol. Plant. 88, 467–474.

Pua, E.C., Deng, X., Tiong-Chew Koh, A., 1999. Genotypic variability of de novo shoot morphogenesis of *Brassica oleracea* in vitro in response to ethylene inhibitors and putrescine. J. Plant Physiol. 155, 598–605, http://dx.doi.org/10.1016/S0176-1617(99) 80060-1.

Trewavas, A.J., 1982. Growth substance sensitivity: the limiting factor in plant development. Physiol. Plant. 55, 60–72.

Appendix 14

Sources of morphological non-uniformity in gentian propagated in vitro without plant growth regulators

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Abstract

To be economically valuable as a potted plant it is desirable for gentians to produce uniform plants with multiple branches (secondary shoots) at flowering. Not all propagules of the cultivar 'Little Pinkie' grew equally when propagated in vitro from either a single node explant, or just the apical region of the donor plant in vitro. Explants from the tip position produced a single primary shoot derived from the continued morphological development of the apex and no secondary shoots. In contrast, explants from lower positions produced between 0 and 8 secondary shoots. The number of shoots and total weight of the in vitro plantlets increased up to three- and two-fold, respectively, with increased distance of the explant position from the tip. In addition, with increased distance from the tip, morphological non-uniformity increased for all growth variables. Scanning electron microscopy evidenced the formation of additional vegetative buds (adventitious), especially for explants taken toward the base of the original shoot. However, in spite of the increase in number of secondary shoots raised on the explants taken from lower down the stem, the total number of usable explants was the same for plantlets derived from all positions. The physiological mechanisms and underlying hormonal control will be discussed together with strategies to increase the number of usable explants.

Keywords: nodal position, usable explant, propagule, plantlet, secondary shoot, bud primordia, scanning electron microscopy

INTRODUCTION

The quality of a pot plant is assessed as being "high" when it makes a good display with multiple buds or flowers, and is uniform in flowering time both within a pot and across a population of pots. Hence, some gentian cultivars (primarily *Gentiana triflora*, *G. scabra*, and their hybrids) whose size and form predicates their suitability as potted plants, such as 'Little Pinkie', have been developed recently by The New Zealand Institute for Plant & Food Research.

In vitro methods can provide a propagation system which ideally will generate, in as short a time as possible, a high proportion of useable propagules for gentian (Morgan et al., 1997). The number of new shoots per explant (shoots explant^{-1}) is important in contributing towards the development of a high-yielding, rapid and efficient method for in vitro propagation. The number of shoots explant^{-1} influences the uniformity of explants; their predisposition to form a high-quality potted plant, and directly determines the yield of explants which can be used either for routine propagation to build up volumes of the germplasm, or deflasked for growth of a highly branched potted plant. While the inclusion of plant growth regulators (PGR) such as cytokinins within the growing medium can improve the production of secondary shoots with gentian (Morgan et al., 1997), our experience to date has been that with successive subcultures the productivity of secondary shoots cannot be sustained and use of such PGR progressively becomes detrimental. In most instances therefore, in our laboratory propagation in vitro is conducted using PGR-free medium.

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The effect of the explant position and the distance from the apex of the shoot, which it was taken from, on the subsequent formation of shoots were reported to be slight for gentian (Zhang and Leung, 2002). In contrast, other authors have suggested that shoot regeneration in other species was significantly affected by the original explant position (Gagliardi et al., 2002). Preliminary experiences at Plant & Food Research indicated that not all individual explants of the gentian cultivar 'Little Pinkie' grew equally when clonally propagated in vitro (K. Funnell, pers. commun.). In particular plantlets were not morphologically uniform in height nor in the number of secondary shoot (i.e., individual shoots arising from leaf axils on the main stem (primary shoot) of the explant). Determining the source of such non-uniformity was, therefore, considered important in optimising propagation protocols.

For the current experiment the specific objective was to quantify the influence of shoot position of donor plants in vitro, on the resulting in vitro plantlets. For 'Little Pinkie' explants, this was achieved by examining the growth responses of (i) total fresh weight, (ii) number and length of shoots explant^{-1}, and (iii) number of usable explants harvested.

MATERIALS AND METHODS

Plants of 'Little Pinkie', previously established in vitro (Morgan et al., 1997) and sub-cultured on a 5-week cycle, were used as donor material for explants. The hormone-free-base medium for the experiment was a modified MS (Murashige and Skoog, 1962) medium, comprising MS macro-salts at half strength, MS micro-nutrient salts, LS vitamins (Linsmaier and Skoog, 1965), 7.5 g L^{-1} agar (Davis) and 3% (87.6 mM) sucrose. The pH of the medium was adjusted to 5.7 with either 0.1 N NaOH or 0.1 N HCl, prior to autoclaving for 15 min. Culture vessels were disposable 290 mL (80 mm base diameter × 60 mm deep) tubs with snap-on lids, manufactured by blow moulding of a general purpose styrene plastic (Alto Packaging, Hamilton, New Zealand). Each culture vessel contained 50 mL of medium, with explants inserted with the nodes just above the surface of the medium. Explants were cultured at 25±1°C and a 16-h photoperiod. The photosynthetic photon flux density (PPFD) at the top of culture vessels was 30±5 µmol m^{-2} s^{-1}, provided by cool-white fluorescent tubes.

Shoots of the in vitro donor plant, selected for production of explants, were of a similar physiological age, being of uniform length and number of nodes (generally three expanded internodes). Treatments comprised the position along the shoot from which explants, each 10 mm in length, were derived (Figure 1). The locations of the 3 nodes on the stem were either (i) tip, which consisted of the apex and undeveloped leaves and nodes located at the distal end of the shoot, (ii) node 1, which was the node just below the tip, or (iii) node 2, which was the second node of the shoot down from the tip, closer to the proximal end of the shoot. Each treatment comprised 3 culture vessels as individual replicates, with each culture vessel containing 8 explants as sub-samples, in a completely randomised design.

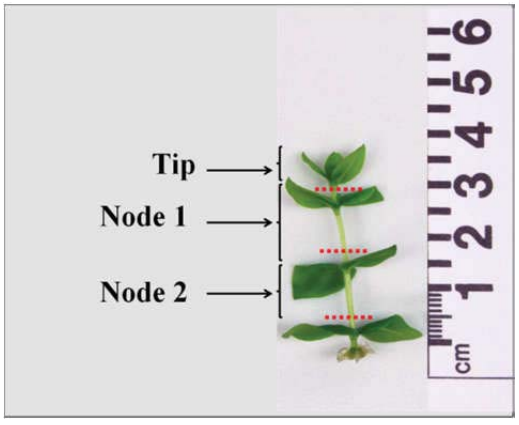

Figure 1. Different positions in gentian 'Little Pinkie' used for taking explants; dashed lines represent where the cuts were made to create the treatments.

After 5 weeks of in vitro growth on basal medium, the number of shoots and the total plant fresh weight per explant were recorded, along with the number of explants 10 mm or longer in length (i.e., useable as a potential propagule). External morphological features of the nodal region of the original explant were recorded under a scanning electron microscope (SEM). For SEM observation, samples were dissected to expose the node, fixed overnight in 3% glutaraldehyde, 2% formaldehyde in 0.1 M phosphate buffer under vacuum, buffer washed, dehydrated in a graded ethanol series, and critical point dried using liquid CO_2 as the critical point fluid. Dried samples were mounted on double-sided tape on SEM specimen stubs, sputter coated with gold, and viewed in an FEI Quanta 200 SEM (FEI Electron Optics of Eindhoven, The Netherlands).

The unit of analysis was the culture vessel; for each culture vessel values were expressed per explant. Following analysis of variance, mean comparison was conducted using Tukey's method (Minitab version 16.1.1, Minitab Inc., State College, Pennsylvania, USA). Box and whisker plots (Sigma Plot version 10, Systat Software Inc., San Jose, CA, USA) were used to quantitatively describe the distribution of the data for growth variables. The 80% spread was calculated based on the number of each variable between the 10th and 90th percentiles. Boundaries of the box indicate 50% of the spread (25th and 75th percentiles).

RESULTS AND DISCUSSION

After 5 weeks of growth, explants developed to form plantlets (miniature plants with developed shoots and roots) which were variable in their overall size. Since explants produced different numbers of shoots with different lengths, total fresh weight could be used as a representative variable for describing the size of plantlets. Explants with more vigorous growth were expected to produce more fresh weight in comparison with those explants which did not grow well.

There was an increase in the total fresh weight of the plantlet as the position along the shoot increased from the tip toward the base (P≤0.05, Table 1). Mean value for the total fresh weight of plantlets derived from node 1 and Node 2 was 27 and 45% greater, respectively, than those derived from the tip (Figure 2A). Furthermore, top 25% of the explants from node 2 produced up to 1.8 times more fresh weight compare to the top 25% of the explants from the tip. The spread of the box plots of each position, demonstrated that the total fresh weight for plantlets derived from node 2 was more (twice) variable than plantlets derived from the other two positions (Figure 2A). Explants from the tip produced plantlets which were most uniform, but with the smallest average weight.

Table 1. Influence of different positions from which explants were sourced on the growth of gentian 'Little Pinkie', after 5 weeks of culture in vitro. All figures are averages per explant.

Treatment	Fresh weight (mg)	No. shoots	No. short Shoots (<5 mm)	No. medium shoots (5-20 mm)	No. long Shoots (>20 mm)	Maximum length of shoots (mm)	No. usable explants
Tip	160 b	1.00 c	0.04 b	0.25 a	0.8 a	27.5 a	4.04 a
Node 1	220 ab	1.75 b	0.16 b	0.58 a	1.00 a	29.6 a	4.83 a
Node 2	293 a	3.36 a	1.90 a	0.72 a	0.8 a	33.0 a	4.98 a

Groupings for mean separation are based on Tukey method. Means followed by different letters within a column are significantly different at *P*<0.05.

The mean number of shoots explant^{-1} arising from different positions were significantly different from each other (P≤0.05; Table 1). A positive trend of an increasing number of shoots explant^{-1} from nodes selected away from the tip towards node 2 was evident, explaining the incremental increase in total fresh weight (Table 1). Explants from node 2 produced three shoots explant^{-1} on average, which was almost 2 and 3 times more than from node 1 and tip positions, respectively. None of the explants from the tip produced more than a single shoot, i.e., they had just the continued growth produced from the original

primary shoot (Figure 2B). In contrast, almost 50% of explants from nodes 1 and 2 produced anywhere up to 2 and 5 shoots explant^{-1} (secondary shoots), respectively (boundaries of boxes, Figure 2B). Furthermore, almost 25% of explants taken from node 2 (upper whisker, Figure 2B) were capable of producing up to 8 shoots explant^{-1}. However, the morphological uniformity in the number of shoots explant^{-1} decreased as the position of the explant along the stem increased from the tip. These results differed from those of Zhang and Leung (2002), who used a different gentian cultivar 'Akita Blue', and reported only a slight effect of explant position on subsequent formation of shoots. Comparing 'Little Pinkie' there was an obvious cultivar difference in shoot proliferation of gentian in vitro. These results contrast to what was described in the literature reporting reduction in the number of shoots explant^{-1}, as the position of the explant was closer to the base (Barrick and Sanderson, 1973). This result was for other plant species and, therefore, could highlight species dependence.

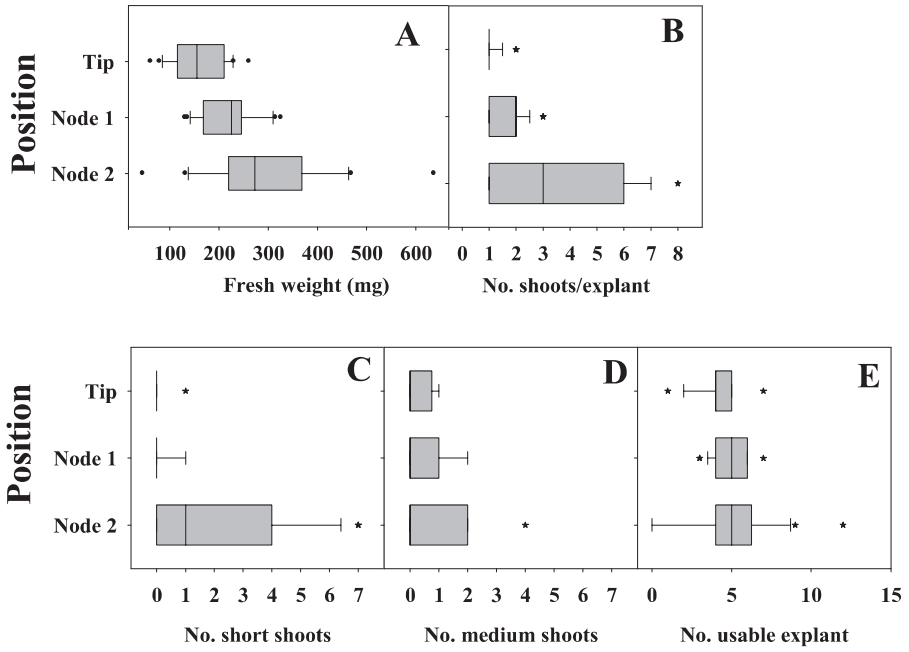

Figure 2. Growth of gentian 'Little Pinkie' explants taken from three positions (Tip, Node 1, and Node 2) after five weeks; A, Total fresh weight (mg), B, Number of shoots per explant, C, Number of shoots with length less than 5 mm (short shoots), D, Number of shoots with length from 5 to 20 mm (medium shoots), and E, Number of usable explants. Vertical line in the centre of the box indicates median value. Boundaries of box indicate 25th and 75th percentiles; whiskers present the range between 10th and 90th percentiles, individuals beyond these boundaries are represented as stars.

There was no significant difference in the maximum length of shoots produced from different positions which explants were derived from (Table 1). Explants from node 1 and 2 typically produced one long shoot (i.e., more than 20 mm, carrying well-developed leaves and internodes) and one shoot of medium length (5-20 mm, Table 1). As supported by the SEM images, the limited number of these long and medium secondary shoots reflected their likely origin as preformed axillary buds (Figure 3A). However, there were some additional medium length shoots arising from 25% of the population of explants from node 1 and node 2 (upper level whiskers, Figure 2C) which, together with the short shoots (i.e., less than 5 mm), were most evident in explants from node 2 (Table 1). These additional shoots were considered to be most likely adventitious in origin. Microscopic observation of additional

primordia at node 2 confirmed the possibility of adventitious buds as the morphological source of non-uniformity.

As illustrated by SEM images, there was an increase in the number of bud primordia as the distance of the explant increased from the tip towards the base (Figure 3). At node 1, only 1 or 2 buds developed to form secondary shoots, which were considered most likely to be axillary (Figure 3A), and in cases where both axillary buds grew to form axillary shoots, there were no additional bud primordia evident. Node 2 in contrast, additionally to the axillary buds, had several bud primordia arranged almost in a ring at the node (Figure 3B). At the time of culture to the in vitro medium, none of the explants selected from all 3 positions showed meristematic activity apart from appearance of 2 axillary buds (data not presented). Hence, additional primordia clearly developed after the experiment commenced.

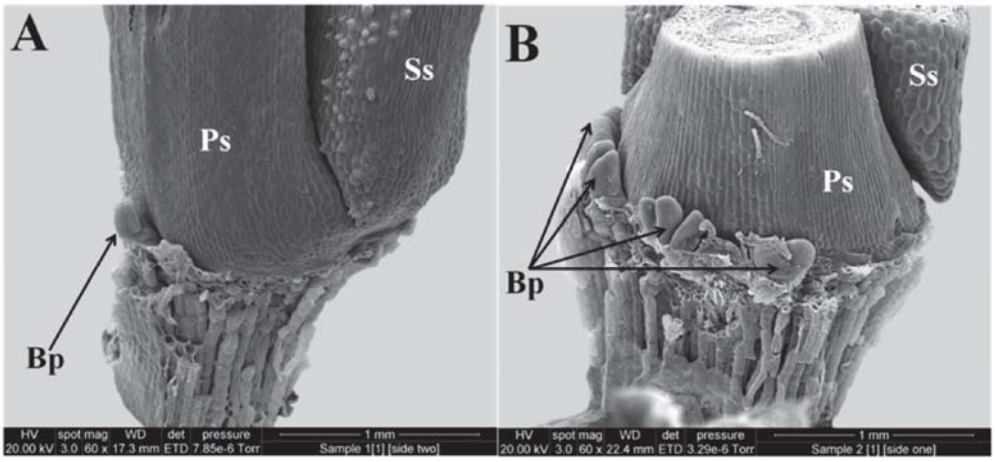

Figure 3. Morphological features of; (A) Node 1 and (B) Node 2, of explants of gentian 'Little Pinkie' after 5 weeks' growth seen using a scanning electron microscope; bud primordia (Bp) were only visible once the leaf sheath was removed from the stem, i.e., primary stem (Ps) and secondary shoot (Ss).

Since secondary shoots raised from the explants of different positions were at different length and developmental stages therefore, as a source of plant material for next subculturing cycle, they might be expected to produce different numbers of new, usable explants for propagation. From our experience, every usable explant for propagation must typically comprise a single node with at least 10 mm length of internode. Only plantlets with shoots of long enough internodes could be used to obtain explants during the multiplication stage. Hence, counting the number of usable explants arising from these shoots was also evaluated. Although more shoots were produced from node 2, the total number of usable explants was the same for plantlets derived from all positions ($P \leq 0.1$; Table 1). This lack of difference was due to the different developmental stage of shoots from node 2 which after 5 weeks were still small. While the number of useable cuttings did not differ between positions, the uniformity in the number of usable explants declined as the distance of the position increased from the tip (Figure 2E). Some explants derived from node 2, at the greatest distance from the tip, produced up to 12 usable explants, whereas others were not developed enough to achieve any.

It is likely that, within the plant system under investigation, correlative inhibition by the apical bud in tip explants likely prevented axillary buds within the leaf axil growing into secondary shoots, resulting in plantlets comprising a single main stem (primary shoot). In explants of node 1 and node 2, the inhibitory effect on shoot development decreased due to the increased distance of the position from apical bud along the shoot. Nodal explants produced up to 3 times more numbers of shoots compared to the tip explants (Table 1). The difference between positions in the number of bud primordia could be due to the differences in tissue age (D'Onofrio and Morini, 2006; Santana-Buzzy et al., 2005) and endogenous

hormonal content (Emery et al., 1998) and, therefore, difference in the sensitivity of their cells to endogenous PGR and hormonal receptor interaction (Firn, 1986; Trewavas and Cleland, 1983). In the current study the age of the original explant was related to the position of nodes on the shoot, with those closer to the apex being younger. Further research is, therefore, still required in order to determine the underlying mechanism of development of secondary shoots in different position of 'Little Pinkie'.

It is important for commercial propagation system to achieve uniform propagules that could be either used for production of uniform in vitro explants or be deflasked and produce uniform pot plants. Since explants derived from different positions produced propagules of variable size, to be used in commercial production, further modification of the protocol in which explants would be graded for their uniformity is suggested.

CONCLUSIONS

The position of the explant on the stem of a donor plant of gentian 'Little Pinkie' in vitro, influenced the growth response and morphological uniformity of in vitro propagated plants. The differences between positions were probably due to diverse physiological conditions in the explant used and different stages of development in different nodes.

Due to the significant non-uniformity in growth response, explants from node 2 might not be ideal as a source of uniform plant material for commercial in vitro propagation, but they would give highly branched explants appropriate for producing high-quality pot plants. Therefore, modifying the protocol of the propagation system from mass propagation to selective propagation is suggested; upper nodes which would give more uniform plantlets could be used for continuous culture; but from the same original plantlet, lower nodes could be used to generate highly branched material to be used for production of potted plants. Further research is suggested to target stimulation and development of non-developed primordia/shoots, arising from node 2, so as to achieve developed shoots.

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Literature cited

Barrick, W.E., and Sanderson, K.C. (1973). Influence of photoperiod, temperature, and node position on vegetative shoot growth of greenhouse azaleas, *Rhododendron* cv. J. Am. Soc. Hortic. Sci. *98*, 331–334.

D'Onofrio, C., and Morini, S. (2006). Somatic embryo, adventitious root and shoot regeneration in in vitro grown quince leaves as influenced by treatments of different length with growth regulators. Sci. Hortic. (Amsterdam) *107* (*2*), 194–199 http://dx.doi.org/10.1016/j.scienta.2005.05.016.

Emery, R., Longnecker, N.E., and Atkins, C.A. (1998). Branch development in *Lupinus angustifolius* L. II. Relationship with endogenous ABA, IAA and cytokinins in axillary and main stem buds. J. Exp. Bot. *49*, 555–562.

Firn, R.D. (1986). Growth substance sensitivity: the need for clearer ideas, precise terms and purposeful experiments. Physiol. Plant. *67* (*2*), 267–272 http://dx.doi.org/10.1111/j.1399-3054.1986.tb02454.x.

Gagliardi, R.F., Pacheco, G.P., Valls, J.F.M., and Mansur, E. (2002). Germplasm preservation of wild Arachis species through culture of shoot apices and axillary buds from in vitro plants. Biol. Plant. *45* (*3*), 353–357 http://dx.doi.org/10.1023/A:1016257315091.

Linsmaier, E.M., and Skoog, F. (1965). Organic growth factor requirement of tobacco tissue cultures. Physiol. Plant. *18* (*1*), 100–127 http://dx.doi.org/10.1111/j.1399-3054.1965.tb06874.x.

Morgan, E., Butler, R.M., and Bicknell, R.A. (1997). In vitro propagation of *Gentiana cerina* and *Gentiana corymbifera.* N. Z. J. Crop Hortic. Sci. *25* (*1*), 1–8 http://dx.doi.org/10.1080/01140671.1997.9513981.

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. *15* (*3*), 473–497 http://dx.doi.org/10.1111/j.1399-3054.1962.tb08052.x.

Santana-Buzzy, N., Canto-Flick, A., Barahona-Pérez, F., del Carmen Montalvo-Peniche, M., Zapata-Castillo, P.Y.,

Solís-Ruiz, A., Zaldívar-Collí, A., Gutiérrez-Alonso, O., and de Lourdes Miranda-Ham, M. (2005). Regeneration of habanero pepper (*Capsicum chinense* Jacq.) via organogenesis. HortScience *40* (*6*), 1829–1831.

Trewavas, A.J., and Cleland, R.E. (1983). Is plant development regulated by changes in the concentration of growth substances or by changes in the sensitivity to growth substances. Trends Biochem. Sci. *8* (*10*), 354–357 http://dx.doi.org/10.1016/0968-0004(83)90359-6.

Zhang, Z.M., and Leung, D.W.M. (2002). Factors influencing the growth of micropropagated shoots and in vitro flowering of gentian. Plant Growth Regul. *36* (*3*), 245–251 http://dx.doi.org/10.1023/A:1016588302549.

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Appendix 15

Explant position influences in vitro flowering of 'Little Pinkie' gentian

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Abstract

When propagules of gentian 'Little Pinkie' were generated in vitro, each progressive subculture resulted in a reduction in the duration to flower production when deflasked plants were subsequently grown in vivo. The initiation of flowering in propagules while growing in vitro reduced the quality of potted plants produced, and reduced the overall efficiency of the micro propagation system by 40%. Hence determining factor(s) influencing the onset of flowering and developing strategies to maximise persistent vegetative growth of explants were considered to be important for commercialisation of this cultivar. The effect of position from which explants originated on flowering in vitro was investigated. A gradient of propensity for explants to flower was found to exist along the shoot from which explants were derived. Explants derived from the tip had the greatest propensity to flower (35%), and the percentage of flowering decreased with distance from the tip. Explants derived from the first node below the tip produced four times fewer flowering plants than those derived from the tip, while only 6% of explants flowered when derived from the second node. To optimise the efficiency of propagation of 'Little Pinkie' therefore, modifying the propagation protocol from mass propagation to selective propagation is suggested, wherein explants derived from the tip of original shoots are not recommended for inclusion. In contrast, lower nodes may be used as propagules for either subculture in vitro or production of potted plants.

Keywords: flower initiation, flowering time, in vitro culture, persistent vegetative, position

INTRODUCTION

Flowering in ornamental plants is a critical developmental stage which involves their transition from a vegetative to reproductive phase. In a commercial production system the timing of flowering and its uniformity are often of particular focus (Funnell, 2008; Chen et al., 2010). As a newly developed cultivar of gentian (*Gentiana*) suited to potted plant production, our research programme initially set out to determine the duration to flowering of in vitro propagated plants of 'Little Pinkie' growing in vivo subsequent to deflasking. As perennial herbs, gentians are reported to require several growth cycles before flowering occurs (Ohkawa, 1983), so time to flower following propagation in vitro was determined in the first experiment reported here.

In order to achieve uniformity in flowering time, factors both in vitro and in vivo might affect the phase change from vegetative to flowering at any stage of the pot plant production system. Given the determinate shoot growth habit of gentian, initiation of flowering in vitro might be expected to affect the vegetative growth of the apical bud and, if so, the efficiency of the micro-propagation system. It was hypothesised that factors affecting in vitro flowering might also affect the time to flower in vivo. Hence, the second experiment was focused on propagation in vitro, and factor/s influencing early flowering in vitro.

The effect of several factors, including the position of the explant, light intensity, pH of the growth medium, as well as the inclusion of gibberellic acid (GA_3) and benzyladenine (BA), on in vitro flowering of *Gentiana triflora* had been previously reported (Zhang and

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Leung, 2002). Using the new cultivar 'Little Pinkie' (derived primarily from *G. triflora* and *G. scabra*), in the current study we also investigated the possible effect of position along the shoot from which explants were derived. With the overall aim of optimising current protocols for propagation of 'Little Pinkie', specific objectives were to quantify: 1) the time needed for rooted plantlets from being deflasked to the date of appearance of the first coloured flower bud when grown in vivo, 2) the effect of explant position on flowering in vitro and subsequent multiplication rate (number of usable explants).

MATERIALS AND METHODS

Experiment 1 – time to flower in vivo

Propagules of the gentian cultivar 'Little Pinkie' were produced in vitro following the protocol described by Keshavarzi et al. (2014). Over a four-week period of establishment, rooted propagules were deflasked into a bark-based growing medium in 60-cell trays (45 mL cell volume) and placed in a fog tent with basal heating (23±2°C for 1 week), before transfer to an open mist bench for a further week in a heated greenhouse (16°C minimum, vented at 24°C). Plants in plug trays were subsequently transferred to an open bench in the greenhouse with capillary irrigation for a further 2 weeks of growth before being potted into 15 cm diameter pots in the same greenhouse environment. The growing medium used comprised A-grade bark fines (50%), bark fibre (30%), and pumice 7 mm (20%) supplemented with 1 kg m^{-3} serpentine super, 1.5 kg m^{-3} dolomite, 2 kg m^{-3} 8-9 month Osmocote® (16N-3.5P-10K, Everris International, Geldermalsen, The Netherlands), and 1 kg m^{-3} 3-4 month Osmocote® (15N-4.8P-10.8K). Throughout the experiment, irrigation was delivered by micro-tubes on a drained capillary matting bench for 10 min three to five times each day, depending on plant requirements. Despite multiple dates at which plants were deflasked, daily air temperature typically ranged between 15 and 24°C, from July until the end of the experiment in November. During the progression of natural photoperiod throughout July to November (southern hemisphere), in order to eliminate any potential effect of photoperiod on flowering, a long day photoperiod regime (2 h night break lighting at 4.6 μmol s^{-1} m^{-2} from 2300 to 0100 HR; Samarakoon et al., 2015) was applied to plants after being deflasked in the greenhouse.

Treatments comprised plantlets which were deflasked on each of five dates at approximate 4-week intervals, i.e., July 8, August 5, September 9, October 7 or November 3. Plants were monitored weekly and, for individual plants, the date of first coloured flower bud, and for each date of deflask the number of saleable pot plants (i.e., three flowers open) was recorded. Within each date of deflask, the experiment was arranged as a completely randomised design, utilising 40 single-plant replicates.

Experiment 2 – explant position in vitro

Plant material originated from plantlets which had been previously grown and sub-cultured in a five-weekly cycle in vitro (Keshavarzi et al., 2014), with plant growth regulators (PGR) to stimulate shoot proliferation or rooting as required (Morgan et al., 1997). For the experiment, plantlets for propagation were selected for uniformity of shoot length and number of nodes on the shoot. Shoots of uniform length with three expanded internodes were selected and cut into nodal positions each 10 mm in length as either: Tip (apex and undeveloped leaves and nodes), Node 1 (first node below the tip) or Node 2 (the second node below the tip). The experiment was conducted as a completely randomised design, utilising six replicates of each treatment (nodal positions), with a culture vessel containing eight explants comprising a replicate.

Data for the number of usable explants (minimum internode length of 10 mm) derived from each explant, as well as the number of explants which flowered in vitro, were recorded after a 5-week period of growth. Following the analysis of the variance, mean separation was conducted using Tukey's method (Minitab version 16.1.1, Minitab Inc., State College, Pennsylvania, USA).

RESULTS AND DISCUSSION

Experiment 1

For the dates of deflask investigated, spanning July to November, there was a significant reduction in the duration from deflask to when the first flower bud showed pink colouration ($P \leq 0.05$). The maximum number of days from deflask to flower was up to 68 days for plants deflasked in July, but was reduced to 47 days for plants deflasked in November. Plants deflasked in November commenced flowering 21 days earlier than those which were deflasked in July (Figure 1). A similar declining trend was observed in the number of plants considered saleable (three flowers open), with the progressive reduction in plant numbers achieving saleable quality over the time. Production efficiency for explants which were deflasked in November was reduced by 40% compared to those in July. Since the use of night break lighting and temperature control should remove seasonal differences between dates of deflask, we interpret this non-uniformity in time to flower as a result of changes occurring prior to deflasking. In vitro plantlets with flowers being either initiated or fully developed, should not be considered for deflasking as their subsequent performance is expected to be poor.

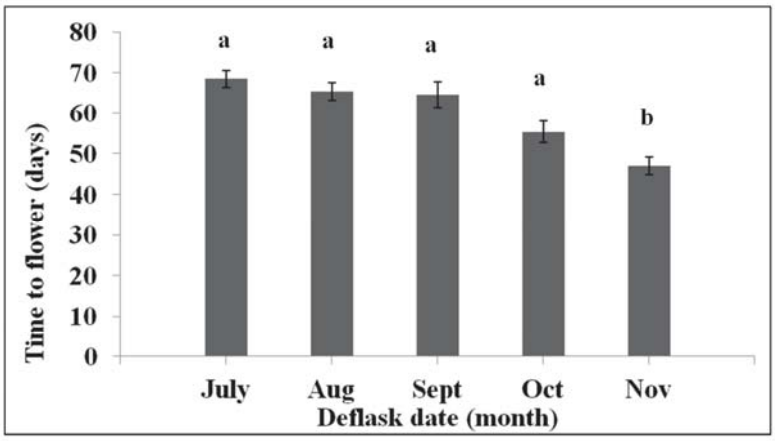

Figure 1. The duration from the date of deflask until when the first apical flower bud on the propagule of 'Little Pinkie' gentian showed colour in the petals for deflask dates from July to November (southern hemisphere). Means with the same letter are not significantly different at $P \leq 0.05$.

Experiment 2

The average number of flowering explants in vitro was influenced by the position from which explants were derived ($P \leq 0.001$; Figure 2). The propensity of explants to flower decreased as the distance of the position of the explant increased from the shoot tip. From a total number of eight explants originating from the Tip position per vessel, on average ~35% produced flowers (Figure 3). In contrast explants derived from the Node 1 and 2 positions achieved about four times less flowering compared to those from the Tip position (Figure 3).

Our present data confirmed the effect of explant position as an influential factor on the propensity for flowering in vitro (Jumin and Ahmad, 1999; Zhang and Leung, 2002), but additionally extended understanding by including the tip of shoots. The difference in flowering propensity between positions has been suggested to be related to the difference in the type of buds, juvenility and capacity of their cells to respond to a gradient of a floral promoter, inhibitor or their combination, existing in each position (Wardell and Skoog, 1969; Fu et al., 1995). As previously illustrated for 'Little Pinkie', shoots arising from Node 1 and 2 positions included those derived from pre-existing axillary shoots, as well as adventitious buds (Keshavarzi et al., 2014). As evident with other cultivars of gentian (Samarakoon et al.,

2015), flowering shoots in the current growth cycle typically arise from crown buds formed in the previous growth cycle. As hypothesised by these authors, in the current research it was considered likely that pre-existing buds (apical and axillary) may have already progressed to flower induction, and would differ in their propensity to flower from buds (adventitious) which develop later during the growth. As compared to apical and axillary buds, adventitious buds are of a younger physiological age.

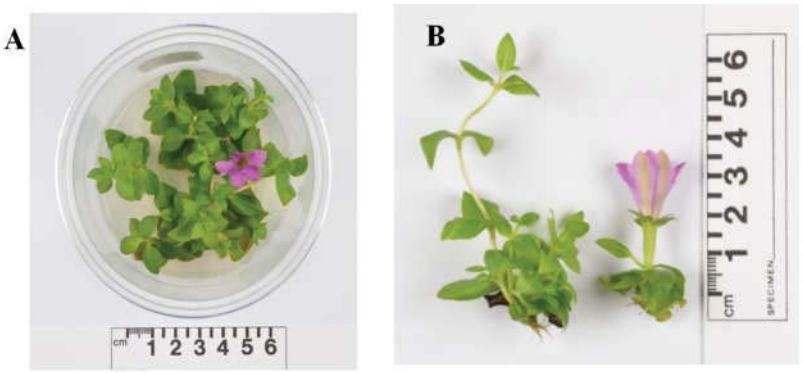

Figure 2. Explants of 'Little Pinkie' gentian after 5 weeks of growth in vitro. (A) Aerial view. (B) Side view of growth response of flowering and vegetative explants.

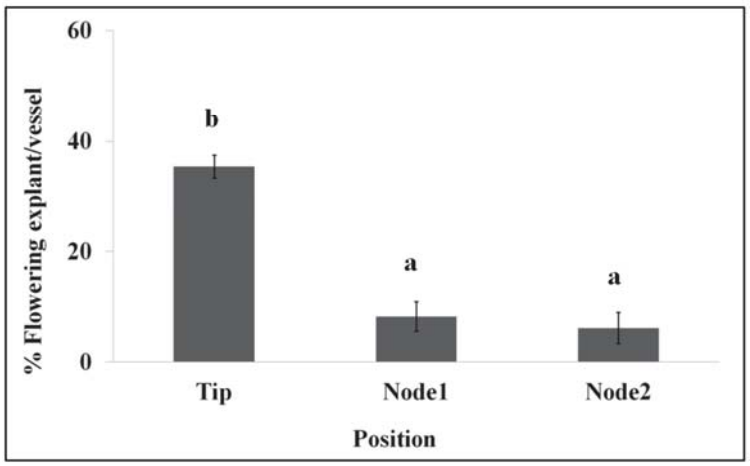

Figure 3. Average percentage of flowering explants of 'Little Pinkie' gentian taken from different nodal positions: Tip, Node 1 (first node below the tip) or Node 2 (second node below the tip) per vessel after 5 weeks' growth in vitro. Means with the same letter are not significantly different at $P \leq 0.05$ (Tukey). Vertical lines represent ± standard error mean, $n=6$.

Similarly, explants taken from different positions along the shoot are different in physiological age and juvenility and therefore vary in their propensity to flower. The concept of juvenility was used by Goebel (1900) for the first time to explain the differences in growth between propagules taken from different parts of the plant. Although meristems forming at the tip are on younger tissue, they may be physiologically 'less juvenile' than meristems forming on the lower part of the stem (Fortanier and Jonkers, 1976). Decrease in juvenility has been defined as onto-genetical aging, which is genetically programmed in terminal meristems and takes into account the number of cycles of cell division from the beginning of the plant (or explant's) life. It is likely therefore those cells from different nodes vary in their sensitivity to, and/or concentration of, floral promoters and/or inhibitors (Firn, 1986). Further research is, however, still required in order to substantiate the application of these

hypotheses to 'Little Pinkie'.

As previously reported, the propensity for 'Little Pinkie' to flower in vitro increased up to 71% when the duration of culture cycle increased from 5 to 8 weeks (Keshavarzi et al., 2016). In the current experiments however, despite keeping time intervals between sub-cultures to no longer than 5 weeks, some explants still continued to flower while in vitro. We interpret this to indicate that initiation of flowering in 'Little Pinkie' will occur in explants of tip origin, and that a growth duration of more than 5 weeks is not the only factor leading explants toward flowering in vitro.

A decline trend in propensity to flower for the explants taken from different positions along the shoot from Tip to Node 2 resulted in a progressive decline in the number of non-vegetative explants discarded from the sub-culturing cycle and, consequently, a significant increase in multiplication rate ($P \leq 0.001$). The multiplication rate of the explants taken from Node 1 and Node 2 positions were, respectively, 2.3 and 3 times more than the multiplication rate of the explants taken from the tip (Figure 4). In a commercial propagation laboratory it would be possible to increase the multiplication efficiency by excluding the tip position from the propagation material in subsequent sub-cultures.

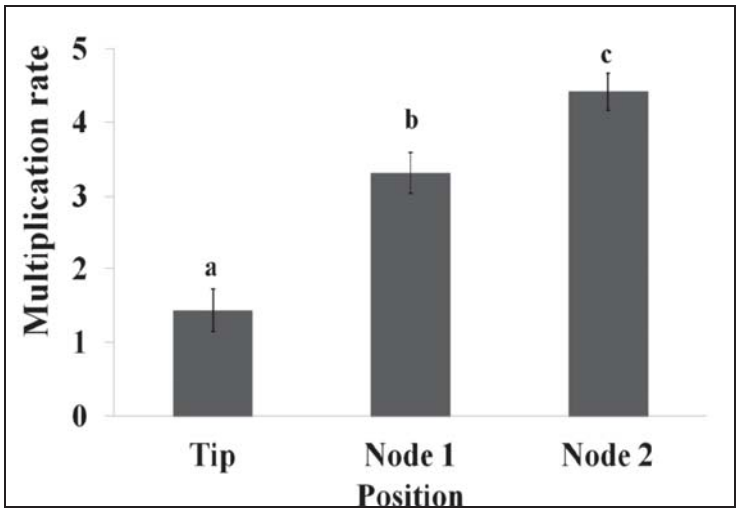

Figure 4. Multiplication rate (number of usable explants taken from mother explant) of 'Little Pinkie' gentian explants taken from different nodal positions: Tip (shoot tip), Node 1 (first node below the tip) or Node 2 (second node below the tip) after 5 weeks' growth in vitro. Means with the same letter are not significantly different at $P \leq 0.05$ (Tukey). Vertical lines represent ± standard error mean, $n=6$.

With a determinant shoot growth habit in gentian, as evident by the decline in the number of vegetative shoots, flowering either stops or at least slows down the vegetative growth of the explant (Figure 2B). This interpretation is consistent with the concept of an irreversible alteration in the endogenous hormonal balance within the explant, which leads the propagule toward changes in the structure of the vegetative bud toward being floral. Initiation of flowering in an in vitro system designed for clonal propagation is typically unwelcome, due to the reduction in efficiency of multiplication in vitro and/or production in vivo. The multiplication rate in flowering explants would decrease because of the reduced number of vegetative shoots per explant. However, identification of flowering while explants are in vitro could save time and resources and, therefore, benefit the overall production efficiency by eliminating flowering explants from the propagation cycle at an early stage before grown in vivo. If flowers only become visibly evident once deflasked, but earlier than expected, not only will the multiplication rate be reduced, but, as evident in our first experiment, when included as plants to be grown in vivo, they do not branch and the production efficiency within the greenhouse phase reduced by up to 40%. From our experience, although early flowering in pot plants of gentian would not permanently stop

the production of further multiple branching, it reduces and/or delays the branching. Therefore early flowering appears to be detrimental both in terms of effective crop scheduling and efficient production.

Early flowering might have disadvantages within the propagation system. However, we believe it was important to introduce 'Little Pinkie' as a cultivar susceptible to flowering in vitro. Flowering in vitro does potentially provide a controlled system necessary to study molecular and genetic mechanisms of flower induction and morphogenesis, as well as the commercial production of flowers and specific compounds from floral organs (Zhang and Leung, 2000; Pennazio, 2004; Teixeira da Silva et al., 2014; da Silva et al., 2014). In breeding programmes, in vitro flowering could be used to speed up the process of multiple generational cycles, enabling pre-selections of progenies based on likely breeding objectives including flower colour (Nakatsuka et al., 2009; da Silva et al., 2014). As noted by Nakatsuka et al. (2009) for gentian as a perennial plant which typically has a juvenile phase of three years from seed, in vitro flowering or in vitro induction of flowering could be of potential value in commercial applications to reduce the time to flower (Nakatsuka et al., 2009) and has been applied in gentian breeding (Ed Morgan, pers. commun.). However, further study is still needed to improve the scheduling of flowering of propagules of gentian in vivo when derived from an in vitro system.

CONCLUSIONS

Early flowering of 'Little Pinkie' in vivo was affected by the nodal position from which each propagule was taken during in vitro propagation. Since explants taken from the tip had the highest propensity to flower, using nodes from subtending positions resulted in significant increases in multiplication rates.

ACKNOWLEDGEMENTS

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Literature cited

Chen, J., Funnell, K.A., and Morgan, E. (2010). A model for scheduling flowering of (*Limonium sinuatum* × *Limonium perezii*) hybrid. HortSci. *45*, 1441–1446.

da Silva, J.A.T., Zeng, S., Cardoso, J.C., Dobránszki, J., and Kerbauy, G.B. (2014). In vitro flowering of *Dendrobium.* Plant Cell Tissue Organ Cult. *119* (*3*), 447–456 http://dx.doi.org/10.1007/s11240-014-0561-x.

Firn, R.D. (1986). Growth substance sensitivity: the need for clearer ideas, precise terms and purposeful experiments. Physiol. Plant. *67* (*2*), 267–272 http://dx.doi.org/10.1111/j.1399-3054.1986.tb02454.x.

Fortanier, E.J., and Jonkers, H. (1976). Juvenility and maturity of plants as influenced by their ontogenetical and physiological ageing. Acta Hortic. *56*, 37–44 https://doi.org/10.17660/ActaHortic.1976.56.2 http://dx.doi.org/10.17660/ActaHortic.1976.56.2.

Fu, Y., Li, H., and Meng, F. (1995). The possible role of zearalenone in the floral gradient in (*Nicotiana tabacum* L). J. Plant Physiol. *147* (*2*), 197–202 http://dx.doi.org/10.1016/S0176-1617(11)81506-3.

Funnell, K.A. (2008). Growing degree-day requirements for scheduling flowering of (*Scadoxus multiflorus* subsp). katharinae (Baker) Friis & Nordal. HortSci. *43*, 166–169.

Goebel, K. (1900). Organography of Plants. I. General Organography, translated by I.B. Balfour (Oxford: Clarendon Press).

Jumin, H., and Ahmad, M. (1999). High-frequency *in vitro* flowering of (*Murraya paniculata* L.) Jack. Plant Cell Rep. *18* (*9*), 764–768 http://dx.doi.org/10.1007/s002990050657.

Keshavarzi, M., Funnell, K.A., Heyes, J.A., and Woolley, D.J. (2014). Ethephon and secondary shoot induction in Gentian (*Gentiana* spp.) hybrids in vitro. Sci. Hortic. (Amsterdam) *179*, 170–173 http://dx.doi.org/10.1016/j.scienta.2014.09.020.

Keshavarzi, M., Woolley, D.J., Heyes, J.A., and Funnell, K.A. (2016). Sources of morphological non-uniformity in gentian propagated in vitro without plant growth regulators. Acta Hortic. *1113*, 157–164 http://dx.doi.org/

10.17660/ActaHortic.2016.1113.23.

Morgan, E.R., Butler, R.M., and Bicknell, R.A. (1997). In vitro propagation of (*Gentiana cerina)* and (*Gentiana corymbifera).* N. Z. J. Crop Hortic. Sci. *25* (*1*), 1–8 http://dx.doi.org/10.1080/01140671.1997.9513981.

Nakatsuka, T., Abe, Y., Kakizaki, Y., Kubota, A., Shimada, N., and Nishihara, M. (2009). Over-expression of Arabidopsis FT gene reduces juvenile phase and induces early flowering in ornamental gentian plants. Euphytica *168* (*1*), 113–119 http://dx.doi.org/10.1007/s10681-009-9899-2.

Ohkawa, K. (1983). *Gentiana*. In CRC Hand Book of Flowering, A.H. Halevy, ed. (Boca Raton, Florida: CRC Press), p.351–355.

Pennazio, S. (2004). "Florigen ": an intriguing concept of plant biology. Riv. Biol. *97* (*1*), 33–51 PubMed.

Samarakoon, U.C., Funnell, K.A., Woolley, D.J., and Morgan, E.R. (2015). Influence of photoperiod regime and exogenous plant growth regulators on crown bud formation in gentian. Sci. Hortic. (Amsterdam) *182*, 56–64 http://dx.doi.org/10.1016/j.scienta.2014.11.023.

Teixeira da Silva, J.A., Kerbauy, G.B., Zeng, S., Chen, Z., and Duan, J. (2014). In vitro flowering of orchids. Crit. Rev. Biotechnol. *34* (*1*), 56–76. PubMed http://dx.doi.org/10.3109/07388551.2013.807219

Wardell, W.L., and Skoog, F. (1969). Flower formation in excised tobacco stem segments; I. Methodology and effects of plant hormones. Plant Physiol. *44* (*10*), 1402–1406. PubMed http://dx.doi.org/10.1104/pp.44.10.1402

Zhang, Z.M., and Leung, D.W.M. (2000). A comparison of in vitro with in vivo flowering in gentian. Plant Cell Tissue Organ Cult. *63*, 223–226 http://dx.doi.org/10.1023/A:1010701332296.

Zhang, Z.M., and Leung, D.W.M. (2002). Factors influencing the growth of micropropagated shoots and in vitro flowering of gentian. Plant Growth Regul. *36* (*3*), 245–251 http://dx.doi.org/10.1023/A:1016588302549.

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Appendix 16

Gentian 'Little Pinkie' and the production of explants as influenced by ethephon and growth duration *in vitro*

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Abstract:

A successful *in vitro* propagation system for gentian (*Gentiana* sp.) 'Little Pinkie' as a potted plant depends on a rapid multiplication rate to produce shoots that, when excised (explants), result in highly branched plants. As a part of an ongoing research programme to improve our understanding of this gentian grown *in vitro*, the effect of growth duration, nodal position from which explants were taken, and inclusion of ethephon in the culture medium were investigated. In the absence of ethephon, increasing the duration of growth from 5 to 8 weeks did not improve branching. Furthermore, the multiplication rate did not change, as after 8 weeks most explants from the tip position became floral, resulting in the total number of usable explants generated being reduced by 30%. Not every explant taken from different nodal positions grew equally, however: fresh weight and number of new shoots per explant taken from the second node below the tip were respectively, 1.5 and 3 times more than the explants taken from the tip. In contrast when ethephon was applied, after 8 weeks growth *in vitro*, explants produced twice as many shoots per explant as the control, and only 10% of the tip-derived explants flowered. The number of shoots produced per explant taken from the second node below the tip was 4.5 times more than tip-derived explants. In spite of the improvement in branching however, ethephon in the growing medium decreased the multiplication rate as a result of a decrease in the length of the longest shoots rendering fewer shoots as useable explants. We conclude that culture for 5 weeks in the absence of ethephon is preferable for *in vitro* propagation of 'Little Pinkie'. Higher multiplication rates may be achievable if ethephon can be used to stimulate the number of branches formed, but also subsequently release those branches into rapid growth.

Keywords: Abiotic stress, Flower initiation, *In vitro* culture, Position effect, Propagation efficiency

INTRODUCTION

For Gentian (*Gentiana* sp.) 'Little Pinkie', bred and developed by The New Zealand Institute of Plant & Food Research Ltd as a potted plant, there is a need to improve protocols for a highly efficient *in vitro* regeneration system. High efficiency of the propagation system is directly dependent on a rapid multiplication rate (number of usable explants for propagation) to produce shoots that, when excised (explants), result in highly branched plants, i.e. a high number of secondary shoots.

We previously reported an increasing trend in the number of secondary shoots produced by explants taken from different positions along the shoot from the tip towards the base (Keshavarzi et al., 2014a). Such difference between nodal positions in branching was attributed to differences in the number of bud primordia, possibly because of the differences

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in tissue age (D'Onofrio et al., 2006; Keshavarzi et al., 2014a). As within these previous studies, age of the original explant was related to the position of nodes on the shoot with those closer to the apex being younger. Based on our earlier findings with 'Little Pinkie', it was hypothesised that by increasing the duration of growth, younger tissue would have more time to develop multiple bud primordia and, consequently, more number of secondary shoots. The first experiment presented in this manuscript was designed to test this hypothesis. The second experiment was based on the findings of another former study in which we reported an increase on the number of secondary shoots raised on the explants taken from the second node below the apex (older tissue) and grown in the agar medium amended with ethephon as an ethylene donor (Keshavarzi et al., 2014b). Within the second experiment the hypothesis that ethephon would increase the multiplication rate by encouraging explants of all positions to produce multiple secondary shoots was tested. In overall Therefore, the current study aimed to improve our understanding of 'Little Pinkie' grown *in vitro*, in which we specifically studied the effect of growth duration and exposure to the plant growth regulator (PGR) ethylene in the culture system on explants of different age.

MATERIALS AND METHODS

Plant material originated from plantlets which had been previously grown and sub-cultured on a five-weekly cycle *in vitro*. The base medium was a PGR-free medium, comprising MS (Murashige and Skoog, 1962) macro-salts at half strength, MS micro-nutrient salts, LS vitamins (Linsmaier and Skoog, 1965), 7.5 g L^{-1} agar and 3% sucrose. For the second experiment, ethephon (Ethrel®; 480 g L^{-1}chlorethephon; Bayer CropScience, New Zealand) at a final concentration of 10 mg L^{-1}, was added to the base medium as the comparative treatment after filter sterilisation (0.2 µm Minisart®filter; Sartorius Stedim Biotech) (Keshavarzi, et al., 2014a). Explants were cultured in disposable plastic culture vessels (Alto Packaging, Hamilton, New Zealand) containing 50 mL of media at 25 ± 1 °C, at a photosynthetic photon flux density of 30 ± 5 µmol m^2 s^{-1} and 16 h photoperiod, provided by cool-white fluorescent tubes.

Shoots of uniform length with three expanded internodes were selected and cut into nodal positions each 10 mm in length as either; Tip (apex and undeveloped leaves and nodes), Node 1 (first node below the tip) or Node 2 (the second node below the tip) (Figure 1).

Treatments for the first experiment comprised two factors; position along the shoot from which explants were derived (Tip, Node 1 or Node 2) and growth duration (5 or 8 weeks). Within the second experiment the treatments comprised a combination of two factors; nodal position (Tip, Node 1 or Node 2) and presence of ethephon (with and without) in the base medium.

Total fresh weight, maximum length, and number of secondary shoots per explant (branching), and multiplication rate (number of explants 10 mm or longer in length, i.e. useable as a potential propagule), were recorded after 5 or 8 weeks for the first experiment, and after 8 weeks in the second experiment. The number of flowering explants per culture vessel was also determined.

Both experiments were planned according to a factorial design, each comprising two factors. Each treatment comprised three culture vessels as individual replicates and each culture vessel contained eight explants. Analysis of variance was conducted using Minitab software (Minitab version 16.1.1, Minitab Inc., State college, Pennsylvania, USA), with mean separation achieved using Tukey's method.

RESULTS AND DISCUSSION

In the absence of ethephon, after 5 weeks of growth the total fresh weight of rooted plantlets derived from explants taken from different positions differed ($P \leq 0.001$, Table 1) by as much as 132 mg. As the distance of the explant from the Tip increased along the shoot, total fresh weight of plantlets increased, with those from Node 2 producing 1.5 times more total fresh weight than the explants from the tip (Table 1; Fig 2). This confirmed our previous findings illustrating the increasing trend in the size of the plantlets raised from the explants taken from different positions along the shoot (Keshavarzi et al., 2014a). Similar to fresh weight, the

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effect of the position which explant derived from, on the number of secondary shoots, was significantly high ($P \leq 0.001$, Table 1). Explants taken from Node 2, which were the most distant from the tip, produced branched plantlets which had 3 times more secondary shoots/explant compared to the explants obtained from the Tip. Explants derived from the Tip typically produced a single shoot, being derived from the continued growth of the original primary shoot. In contrast, explants from Node 1 and Node 2 produced anywhere up to three shoots/explant (secondary shoots) (Table 1). The difference in the number of shoots/explant with respect to positions of explants, was similarly expressed as differences in total fresh weight. In spite of the increase in number of shoots/explant however, there was not a significant difference in the maximum length of their shoots (Table 1). Keshavarzi et al. (2014a) reported that explants taken from Node 1 and 2 typically produced one long shoot (i.e. more than 10 mm, carrying well-developed leaves and internodes) and between one and two shoots of medium length (5-10 mm). It is likely that correlative inhibition by the longest shoot inhibits the growth and elongation of other secondary shoots in the explants of Node 1 and Node 2 positions. Within the current study therefore the additional secondary shoots of medium length arising from nodal positions were not developed enough to be usable explants for propagation, resulting in no differences between positions in multiplication rate (Figure 2).

As evident by no significant improvement in total fresh weight of plantlets, in the absence of ethephon continuation of growth duration from 5 to 8 weeks did not change the overall size of the plantlets (Table 1). However, the position that explants derived from significantly influenced total fresh weight after 8 weeks ($P \leq 0.001$) and followed the same increasing pattern from Tip to Node 2 as evident after 5 weeks. This unchanged total fresh weight was concomitant with no significant change in the number (branching) and length of shoots following the extension of growth duration.

The only variable which was highly affected by both growth duration and position was the number of explants which flowered ($P \leq 0.001$, Table 1). At week 5, in the absence of ethephon the overall percentage of flowering was negligible (only 8% in the whole population) and there was no interaction with the position explants derived from ($P > 0.05$). By increasing the growth duration to 8 weeks, flowering increased to an overall of 83%, and the different positions varied in flowering frequency ($P \leq 0.001$; Table 1). While few explants taken from Node 2 flowered, 71% of the explants taken from the Tip position did; a 6-fold increase in flowering percentage over explants taken from Node 1 was observed (Table 1). As a consequence of changes in flowering frequency, compared to following 5 weeks growth duration, after 8 weeks the multiplication rate of explants derived from the Tip position declined notably (Figure 4). Flowering either imposes an end to vegetative growth of the explant or at least slows it down, possibly as a result of resource consumption for flower development (Reekie and Bazzaz, 1987). Due to their poor subsequent performance, during multiplication *in vitro* flowering explants were normally removed and discarded during the sub-culturing cycle. As a result, the overall multiplication rate of all explants decreased by 33% (Figure 4). Exposure to abiotic stress has been reported to induce flowering in plants, being a recognised survival response to unfavourable conditions (Ahmad and Prasad, 2011) .While specific factor(s) influencing floral initiation has not been identified, when grown *in vitro* gentian appears to be readily induced to flower. While application of a short duration of growth *in vitro* could be used to minimise flowering, growth duration still needs to be long enough for the explant to grow roots and shoots with a number of nodes and adequate length of internodes to achieve new usable explants for propagation. From our experience, for 'Little Pinkie' a 5-weekly propagation cycle *in vitro* provides explants with adequate time for maximum vegetative growth without multiplication rate issues due to floral initiation.

After 8 weeks of growth in the presence of ethephon as an ethylene producer, branching was significantly (two times) greater than without ethephon ($P \leq 0.001$, Table 2). This supported the hypothesis of a promotive effect of ethylene on *in vitro* shoot production (Keshavarzi et al., 2014a; Kevers et al., 1992), and was similar to that observed from application of ethephon on the number of crown buds in gentian cultured *in vivo* (Samarakoon et al., 2015). The effect of position of explants on branching was also significant after 8 weeks

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duration ($P \leq 0.001$, Table 2). Branching in explants derived from Node 2 was about 6 times more than in those from the Tip (Table 2). As noted by Samarakoon et al. (2013), the additional branches/shoots beyond those arising from pre-existing axillary buds were of adventitious origin (Keshavarzi et al., 2014a). In contrast to an increase in the number of shoots, there was a significant decrease in the length of shoots grown in the medium amended with ethephon compared to the shoots grown without ethephon ($P \leq 0.001$, Table 2). With the application of ethephon explants taken from Tip, Node 1 and Node 2 positions produced shoots which were respectively 3, 2.5 and 2 times shorter than the explants from the similar position grown without ethephon. This reduction in length of secondary shoots, previously reported to be due to the reduction of internode length, could partly have resulted from the improvement in branching and competition between shoots (Mele et al., 1982). As a consequence total fresh weight of explants exposed to ethephon did not significantly change compared with the explants without ethephon ($P > 0.05$, Table 2).

Importantly for a propagation system, only 29% of the tip-derived explants treated with ethephon flowered, which was 2.7 times less than occurred for explants from the Tip position in the absence of ethephon (Table 2). Furthermore, there were no flowers produced by the explants taken from other positions. An increase in multiplication rate was expected following the decrease in flowering and improvement in branching, however ethephon in the growing medium decreased the multiplication rate (Figure 4, Table 2). Although multiplication rate of the explants taken from the Tip remained unchanged after the application of ethephon, the multiplication rate of explants derived from Node 1 and Node 2 significantly decreased 2.3 and 2 times than the equivalent positions without ethephon. One of the factors that contributed to such a decrease in multiplication rate could be the decrease in the length of secondary shoots (Figure 4&5). Hence, considering the encouraging effect of ethephon on branching, future work could examine how to release those branches (presumably correlative inhibition) into rapid growth, in order to improve the multiplication rate further.

However, a growth duration more than 5 weeks did not seem to be the only factor leading explants toward flowering, as flowering continued despite intervals between subsequent sub-cultures being less than 5 weeks (unpublished observations). Flowering within a system designed for propagation could be an obstacle to achieving a rapid and highly efficient multiplication system. Thus, future research should aim to determine the effect of other influential factors on flowering of 'Little Pinkie' *in vitro*.

CONCLUSIONS

A gradient for the propensity for branching was evident along the primary shoot that increased with the distance away from the tip that the explant was taken. However, the number of resultant propagules did not differ among positions if they grow *in vitro* for duration shorter than 5 weeks. By extension of the growth duration to 8 weeks significant number of explants specially taken from the Tip position flowered and the multiplication rate decreased. Since the multiplication rate actually declined as an outcome of the extension of growth duration, a 5-week sub-culturing interval, in the absence of ethephon, is preferable for *in vitro* production of propagules of gentian 'Little Pinkie'. However, higher multiplication rates may be achievable if ethephon can be used to stimulate the number of branches formed, but also to release those branches into rapid growth.

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References:

Ahmad, P. and Prasad, M.N.V. (2011). Abiotic stress responses in plants: metabolism, productivity and sustainability. Springer Science & Business Media.

Keshavarzi, M, K.A. Funnell, J.A. Heyes, D.J. Woolley, 2015. Gentian 'Little Pinkie' and the production of explants as influenced by ethephon and growth duration *in vitro*. Presented to: "6th International Symposium on Production and Establishment of Micropropagated Plants", Italy, April 2015. Acta Horticulturae (in Press)

D'Onofrio, C. and Morini, S. (2006). Somatic embryo, adventitious root and shoot regeneration in *in vitro* grown quince leaves as influenced by treatments of different length with growth regulators. Scientia Horticulturae *107*, 194-199.

Keshavarzi, M., Funnell, K.A. Heyes, J.A. and Woolley, D.J. (2014a). Sources of morphological non-uniformity in Gentian. "Ornamental Horticulture in the Global Greenhouse", Symposium 15 in the International Horticultural Congress, Brisbane, August 17-22, 2014. Acta Horticulturae (in Press).

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

Kevers, C., Boyer, N., Courduroux, J.-C., and Gaspar, T. (1992). The influence of ethylene on proliferation and growth of rose shoot cultures. Plant Cell, Tissue and Organ Culture *28*, 175-181.

Linsmaier, E.M. and Skoog, F. (1965). Organic growth factor requirement of tobacco tissue cultures. Physiologia Plantarum *18*, 100-127.

Mele, E., Messeguer, J. and Camprubi, P. (1982). Effect of ethylene on carnation explants grown in sealed vessels. Plant tissue culture: 69-70.

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

Reekie, E.G. and Bazzaz, F.A. (1987). Reproductive effort in plants .3. Effect of reproduction on vegetative activity. American Naturalist *129*, 907-919.

Samarakoon, U.C., Funnell, K.A., Woolley, D.J. and Morgan, E.R. (2015). Influence of photoperiod regime and exogenous plant growth regulators on crown bud formation in gentian. Scientia Horticulturae *182*, 56-64.

Samarakoon U.C., Funnell, K.A., Woolley, D.J., Ambrose, B.A. and Morgan, E.R. (2013). Anatomical investigations determining the origin of crown buds on the transition zone of gentians. New Zealand Journal of Botany *51* (4), 264-274.

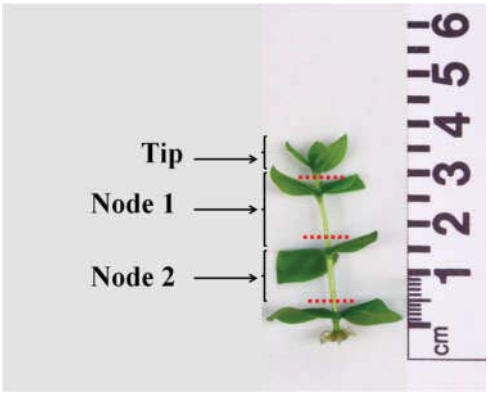

Figure 1. Different positions along the shoot in gentian 'Little Pinkie' from which explants were taken; dashed lines represent where the cuts were made to obtain the explants .

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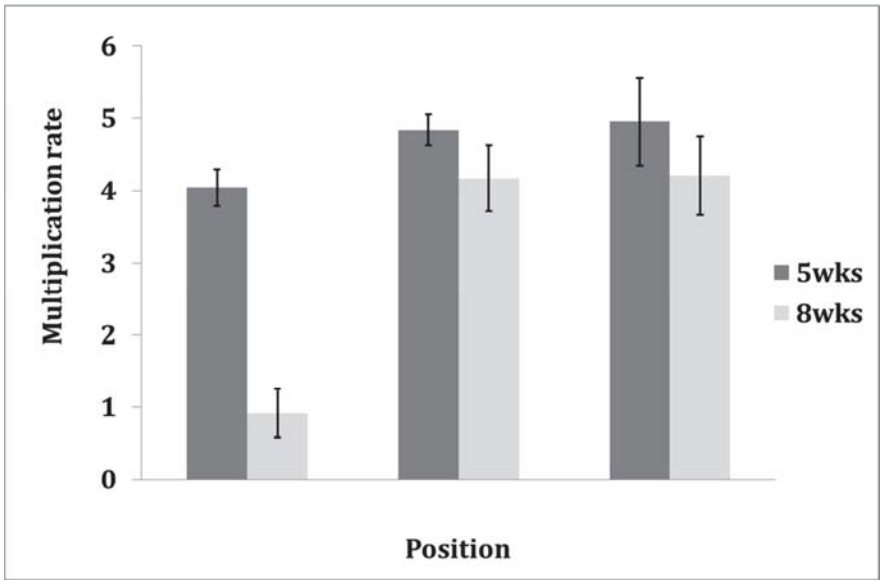

Figure 2. Multiplication rate (number of usable explants per mother explant) of the explants of gentian 'Little Pinkie' derived from different positions (Tip, Node 1 and Node 2) cultured *in vitro* for different growth durations (5 and 8 weeks). *n*=3. Vertical lines represent ± standard error of mean.

Figure 4. Explants of gentian 'Little Pinkie' taken from the Tip position after; 5 and 8 weeks of *in vitro* growth.

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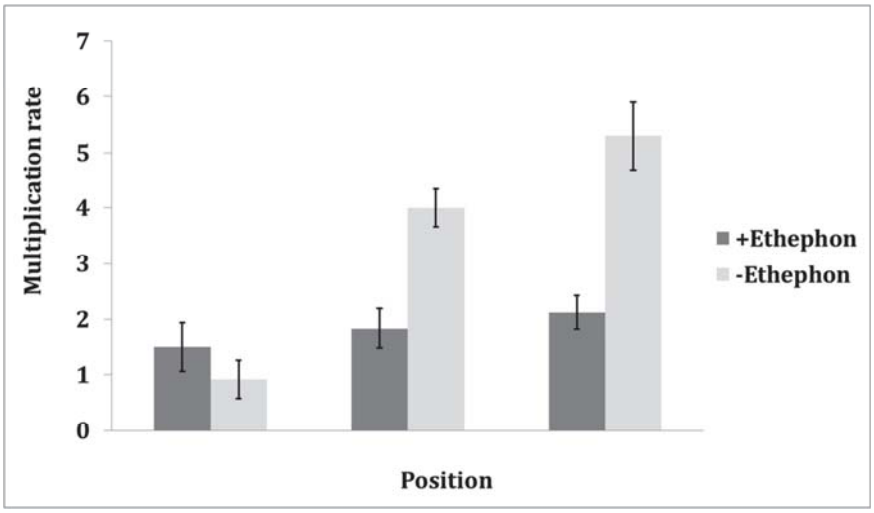

Figure 3. Multiplication rate (number of usable explants per mother explant) in the explants of gentian 'Little Pinkie' derived from different positions (Tip, Node 1 and Node 2) cultured *in vitro* in the presence (+Ethephon) or absence of ethephon (-Ethephon) in the medium. *n*=3. Vertical lines represent ± standard error of mean.

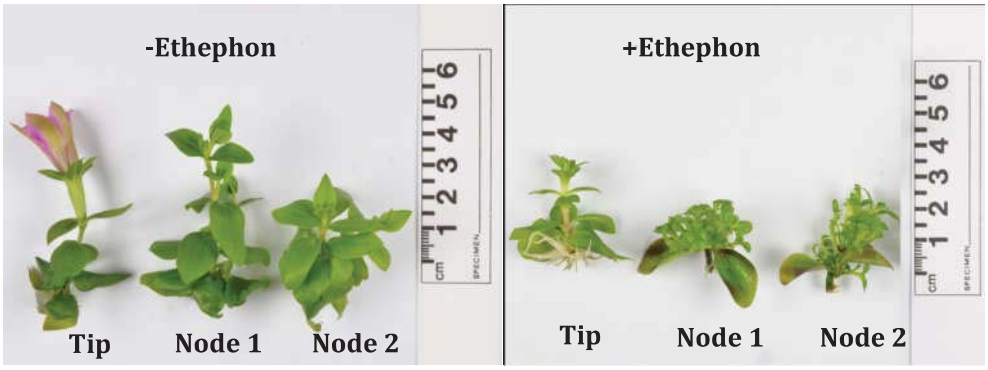

Figure 5. *In vitro* explants of gentian 'Little Pinkie' grown for 8 weeks' in the absence (-Ethephon), or presence (+Ethephon) of ethephon.

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Table 1. Growth of gentian 'Little Pinkie' cultured *in vitro* as affected by different positions and growth durations in the absence of ethephon.

Position		Fresh weight of plantlet (mg) [z]		Maximum shoot length (cm) [z]		Branching (shoot/explant) [z]		Flowering explants (%) [z]	
	Duration	**5 wk**	**8 wk**	**5 wk**	**8 wk**	**5 wk**	**8 wk**	**5 wk**	**8 wk**
Tip		159 b	180 b	27 a	27 a	1.1 c	1.0 c	4 a	71 b
Node 1		219 ab	285 a	24 a	30 a	1.8 bc	2.1 b	4 a	12 a
Node 2		291 a	290 a	24 a	33 a	3.4 a	3.2 a	0 a	0 a
***P* value**									
Duration		NS		NS		NS		≤0.001	
Position		≤0.001		NS		≤0.001		≤0.001	
Duration*Position		NS		NS		NS		≤0.001	

[z] Means with the same letter are not significantly different (NS) at $P \leq 0.05$ (Tukey)

Table 2. Growth of gentian 'Little Pinkie' as influenced by different positions (Tip, Node 1, Node 2) and presence (+E) or absence (-E) of ethephon in the medium after 8 weeks of *in vitro* culture.

Position		Fresh weight of plantlet (mg) [z]		Maximum shoot length (cm) [z]		Branching (shoot/explant) [z]		Flowering explants (%) [z]	
	Treatments	**+E**	**-E**	**+E**	**-E**	**+E**	**-E**	**+E**	**-E**
Tip		206 ab	176 b	9 b	29 a	1.3 bc	1.0 c	29 b	79 a
Node 1		229 ab	284 a	10 b	25 a	3.5 b	2.2 bc	0 c	8 bc
Node 2		258 ab	293 a	12 b	24 a	7.5 a	3.3 b	0 c	4 c
***P* value**									
Treatment		NS		≤0.001		≤0.001		≤0.001	
Position		≤0.001		NS		≤0.001		≤0.001	
Treatment * Position		NS		NS		≤0.001		≤0.001	

[z] Means with the same letter are not significantly different (NS) at $P \leq 0.05$ (according to the Tukey test)

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