Studies on breeding dwarf poinsettias (*Euphorbia pulcherrima* Willd.) and the influence of infective agents

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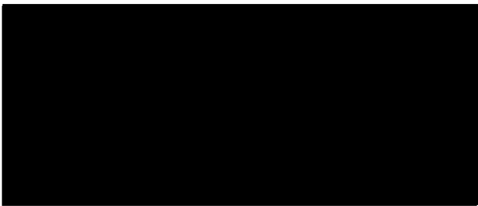
DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any other University, and to the best of my knowledge, is original and contains no material previously published or written by another person, except where due reference is given.



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Summary

Poinsettias are an economically important ornamental potted crop that is grown globally. To satisfy commercial demands, plants must be grown to a height (within certain limits) depending upon factors such as pot size, plant form and market requirements. To achieve the desired height, application of chemical growth regulators is frequently required.

In this thesis, development of genetic dwarf poinsettias is one method proposed as a means to obtain plants that can be produced without application of growth regulators. In addition, studies on infective agents such as viruses and phytoplasmas were initiated to obtain an understanding of the effects these organisms impose on commercially grown poinsettias.

In the first experimental chapter, the effects of poinsettia mosaic virus (PnMV) and poinsettia branch inducing phytoplasma (PoiBI) on plant height were investigated as they frequently infect commercially grown poinsettias. Initially, a survey of the current commercial population was undertaken to determine the extent of PnMV, poinsettia cryptic virus (PnCV) and PoiBI infection. All cultivars were found to be infected with PnMV and PoiBI and two cultivars were infected with PnCV. This is the first report of PoiBI and PnCV presence in Australia. To clarify the methods of transmission of these three infective agents, grafting and seed production experiments were conducted. PnCV was transmissible via both grafting and seed, indicating that the eradication of this virus may be difficult. This is the first conclusive report that demonstrates this virus is both graft and seed transmissible. PnMV and PoiBI were found to be transmissible by grafting, but not through the seed. In addition, a new host for PnMV, PnCV and PoiBI was found.

Following these experiments, techniques to remove the infective agent(s) were implemented to produce 'infection types' of PnMV and PoiBI in two poinsettia cultivars. These infection types enabled the quantification of the individual and combined effects of PnMV and PoiBI on plant height compared to PnMV and PoiBI free plants. There was a significant reduction in height in cultivar (cv.) Freedom red when PoiBI was present. This result was replicated in three consecutive commercial production experiments conducted throughout the year. Plant height was reduced by 11 – 18% compared to non-infected plants and was attributed to a reduction in mean internode length. PoiBI presence also improved post-production performance by maintaining a greater proportion of leaves and bracts on infected plants. PnMV had limited morphological effects, except to synergistically increase stem diameter in

the presence of PoiBI. In another similar experiment conducted with cv. V10 Amy red, all four infection types did not significantly influence plant height. However, a preliminary study indicated a similar effect of PoiBI on post-production performance. The only effect of PnMV in this experiment was to significantly reduce bract size when PoiBI was not present. This is the first report to quantify the effects of PoiBI and PnMV on plant height and morphology in a commercial production environment.

The mechanism by which PoiBI reduced plant height was examined in Experiment 3.5. Phytohormone concentrations were quantified in PoiBI infected and non-infected poinsettias. At the first sampling, the concentration of IAA was significantly reduced in PoiBI-infected plants and the concentration of several cytokinins was significantly increased. At the second sampling, no significant differences were found. Therefore, it was postulated that PoiBI could influence the hormonal balance of poinsettias to induce branching and reduce plant height through reduction in apical dominance at a specific period during crop growth.

Another method of producing shorter poinsettias is to utilise intraspecific hybridisation. Several experiments were performed to determine if poinsettias could be produced that were genetically shorter than current 'short' cultivars. These commenced with studies on the breeding system and efficiency of seed production of selected parents, primarily from the currently available commercial cultivar population. The efficiency of seed and plant production was low. These low efficiencies were attributed to mutational load and inbreeding depression in current commercial cultivars.

Seeds produced from these breeding system investigations were germinated and four vigorous dwarf lines were selected, based (in part) upon mean internode length, time to flower, pedigree, ornamental appeal and rate of leaf unfolding. To determine if these lines were significantly shorter than their progenitors, they were compared to commercial cultivars devoid of PoiBI during a commercial production experiment (Experiment 4.4). At the final scoring date (approximately anthesis) these lines were not significantly shorter than their parents however, two lines achieved the lowest mean heights. This indicated breeding shorter poinsettias via intraspecific hybridisation could be difficult.

This experiment also identified suitable cultivars for use in further studies aimed at breeding shorter poinsettias via intraspecific hybridisation. Using these four lines and two current short commercial cultivars, production of shorter poinsettias was attempted. Initially by intermating

the four lines in complete diallel and secondly by intermating the two short commercial cultivars in a complete diallel. Reciprocal crosses were utilised as a seed derived population (SDP), and self pollination derived seeds as seed derived controls (SDCs). In addition, vegetatively derived controls (VDCs) were used to assist in selection of vigorous, dwarf, ornamentally excellent plants. Two primary selections were made from the SDP with the previously mentioned criteria. The selection intensity was 0.9%. Difficulties arose during this experiment due to limited seed production and therefore, plant production from some lines. Segregation for height was investigated in two populations of seeds derived from self pollination of two commercial cultivars. Analysis indicated height in these two cultivars was determined by several minor genes (quantitative). In addition to the three primary selections made above, 30 other selections were made based entirely on excellent ornamental appeal.

A preliminary study of the total 32 selections after being infected with PoiBI, under commercial production during summer in Australia, showed that one from two primary selections were significantly shorter than both cultivar Freedom red and V10 Amy red. Of the 30 other selections, 14 were significantly shorter than both control cultivars. This study demonstrated that producing shorter plants than commercial cultivars was possible, but further testing of the selected plants is required to determine if a reduction in height occurs under commercial production in all situations.

Introgression of novel genes from related species was also utilised as a means of producing dwarf poinsettias. Cross pollinations between poinsettias and several other *Euphorbia* species resulted in organogenesis and plant production from *in vitro* embryo rescue and ovule culture procedures. The cross combination *E. pulcherrima* x *E. cornastra* yielded many hybrid plants with reduced height and ornamental appeal, thus satisfying the main aim of the project. These plants were generally vigorous, and when grafted to PoiBI containing poinsettias, exhibited free-branching symptoms. This is the first report of interspecific hybrids being generated with *E. pulcherrima*.

Several additional investigations were performed to provide knowledge for future breeding efforts and for the possibility of producing shorter poinsettias. Colchicine was used to double the chromosome number of diploid poinsettias to produce plants with shorter internode lengths, thicker stems and attempt greater seed production than diploids due to genetic buffering of inbreeding depression/mutational load effects. Putative tetraploids were verified by root tip chromosome counts and morphology.

Experiments conducted to produce triploid plants resulted in 42, 35 and 36-39 chromosome progeny. Two progeny from the 56 x 28 chromosome cross were examined in detail. One plant possessed 2n = 42 and the other 2n = 35 chromosomes. Both plants were vigorous and displayed characteristics typical of tetraploids. One plant produced via endosperm culture was investigated. This plant possessed 36 - 39 chromosomes and was sterile with unusual morphology (zigzag stems, swollen nodes, small rounded leaves) compared to the diploid plant produced from the embryo of the same seed.

In conclusion, this thesis demonstrates that several methods can be utilised to develop dwarf poinsettias that do not require growth regulator application to obtain market desired height. These methods include infection with poinsettia branch inducing phytoplasma, intraspecific hybridisation and interspecific hybridisation. In addition to achieving this main aim, several lines were produced that have immediate commercial potential due to dwarfness and excellent ornamental merit.

Abbreviations and notes

Abbreviation	Description
2iP	N^6-[2-isopentyl]adenine
BAP	N^6-benzyladenine
CK	cytokinin
cv.	cultivar
DIF	difference between day and night temperature
DROP	early morning temperature reduction
EC	electrical conductivity (milliSiemens per centimetre, mS cm^{-1})
ELISA	enzyme linked immunosorbent assay
ER	embryo rescue
GR	growth regulator
GA	gibberellic acid
H/N	mean internode length (height per node - mm)
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
IBD	inbreeding depression
ISEM	immunosorbent electron microscopy
M	million
mg/l	milligrams per litre
mm	millimetre
mM	millimole
MS	plant tissue culture media devised by Murashige and Skoog 1962
NAA	1-napthaleneacetic acid
nm	nanometre
p	probability
pmol/gDW	pico moles per gram dry weight
PCR	polymerase chain reaction
PFI	premature floral initiation
PnMV	poinsettia mosaic virus
PoiBI	poinsettia branch inducing phytoplasma
PnCV	poinsettia cryptic virus
PPF	photosynthetic photon flux
RFLP	restriction fragment length polymorphism
SE	somatic embryogenesis
SEs	somatic embryos
T/N	mean node production rate (time per node - days)
WT	wild-type

All literature cited in this thesis using imperial measurements has been converted to metric. Fahrenheit measurements have been converted to Celsius. Foot candles have been converted to PPF ($\mu mol\ m^{-2}\ s^{-1}$) using conversion factors for sunlight as per Apogee (2000). Data measured in weeks has been converted to days or vice versa, in some instances. A number of personal communications are listed in this thesis. These communications were recorded during 1997 - 2000 and the contributors (including area of expertise) are listed in the acknowledgements section.

Chapter 1. General Introduction

The poinsettia (*Euphorbia pulcherrima* Willd.) is among the most popular of ornamental potted plants. Originating in Mexico, it was first commercially grown in California in 1906 (Ecke 1990). Continual development by breeders has resulted in a multitude of cultivars that are available today. Most cultivars were developed in California and Europe and therefore grow optimally in these environments. No attempts have been made to develop cultivars suited to the Australian environment, which is vastly different to the majority of areas within which poinsettias are currently grown. In Sydney for example, during the major poinsettia production period (August – December) the climate is characterised by high day and night temperatures and humidity, especially during the later stages of production. Therefore, to develop cultivars suitable to this climate, heat tolerance and dwarfness/short height need to be improved.

Poinsettias are grown in a wide range of pot sizes and plant forms. A standard product quality and size are necessary for consumer acceptance within each category. In Australia, and throughout the world, the majority of poinsettias sold are branched, red bracted and grown in 150 mm pots (Anonymous 1996b). Grower and consumer surveys have shown that a total product height of 460 mm, including 150 mm pot height, is desirable in U.S.A. markets (Rinehart pers. comm. 1997) and a 380 mm total height is desirable in Australia (White pers. comm. 1997). To meet this need, chemical growth regulators (GRs) are often applied. However, the use of chemical GRs has more recently been deemed environmentally unsound due to the public health issues involved (Berghage and Heins 1991). The purchase and application cost of GRs for each spray treatment amounts to a few cents per plant (Tayama 1992), which reduces the profit margin. In addition, use of certain growth regulating chemicals is illegal in some countries. For example, in Germany all GRs are banned except Cycocel (Ludolph 1992). If this trend continues, other methods of height control will need to be found and implemented.

Alternatives to chemical height control methods include many additive and singular options (such as crop management and production manipulations) of which breeding for compactness and dwarf habit rates highly (Ludolph 1992). Techniques such as utilising differences between day and night temperature and early morning temperature drops can be used to control height to a limited extent. Other methods such as limiting nutrition and increasing plant spacing, could result in an inferior product or inefficient use of space. Many non-

chemical and chemical height control options adversely affect other factors of production such as scheduling, profitability and plant quality. Therefore, genetic methods to reduce plant height would be considered the best option for the production of dwarf poinsettias. At present, shorter growing cultivars are available, but these are generally slow growing (e.g. cv. Lemon Drop and cv. Pepride). The cv. V10 series developed in the 1970's is considered compact and vigorous but is rarely grown now due to undesirable leaf and bract colour and poor post-production performance. Any dwarf poinsettia developed must also possess desirable production traits to be commercially acceptable.

Poinsettia cultivar improvement has been based to date entirely on intraspecific hybridisation and mutation breeding (Rinehart pers. comm. 1997, Sander pers. comm. 1999 and Trees pers. comm. 1999). The effectiveness of intraspecific hybridisation for dwarfness could be limited by a narrow germplasm base (Ling *et al.* 1997, Starman and Abbitt 1997). In addition, low seed set and inbreeding depression (Sink pers. comm. 1997) could limit efficiency in the development of dwarf poinsettias via this method.

Interspecific hybridisation is widely utilised in many crop species to introduce new genes for height and develop new plant forms. This method has not been utilised successfully with poinsettia as shown in the available published literature and could enable development of dwarf plants if a narrow germplasm base is a breeding limitation.

Mutation breeding has been practiced for decades on poinsettias (Love 1966, Rinehart pers. comm. 1997, Sander pers. comm. 1999). The desire is generally to develop variations of already excellent cultivars, while maintaining all other desirable attributes. Dwarf mutations are not reported in the available literature, however a variegated dwarf was recently produced via gamma-ray mutagenesis (Derera pers. comm. 1997).

Other methods that may induce dwarfness include addition of poinsettia branch inducing phytoplasma (PoiBI). This phytoplasma is known to alter morphology and increase branch number in infected plants. There is no report of the effect(s) of PoiBI on plant height during commercial production in the available literature. Addition of poinsettia viruses such as poinsettia mosaic virus (PnMV) and poinsettia cryptic virus (PnCV) as infective agents could affect height and other characteristics and require further investigation.

This study was undertaken to develop genetically dwarf poinsettias. Due to various external factors such as GRs, infective agents, etc. involved in the development of current commercial cultivars, efforts were made to exclude/eliminate the influence of these confounding factors to ascertain expression of the genotype - alone. Therefore, the primary aims of this study were to i. - quantify the effects of PoiBI and PnMV on plant height and morphology during commercial production, ii. - elucidate the mechanism of PoiBI induced height reduction in a current commercial cultivar, iii. - determine the effectiveness of intraspecific hybridisation for developing shorter poinsettias and iv. - introduce new genes for reduced height into the commercial cultivar population via interspecific hybridisation. Additionally, studies on ploidy, cytology and *in vivo* pollen germination were also performed to assist in the development of dwarf poinsettias.

Chapter 2. Review of literature

Taxonomy and cytology in *Euphorbia* subgenus *Poinsettia*

The genus *Euphorbia* includes approximately 2000 species (Oudejans 1990). Subgenus *Poinsettia* encompasses twenty four species and these are taxonomically the closest relatives of *Euphorbia pulcherrima* Klotzsch. (Mayfield 1997). The subgenus is uniquely characterised by cyathial glands that are deeply cupped and lack petaloid appendages. Coloured bracts often surround the inflorescence. Subgenus *Poinsettia* is split into two alliances, namely *Euphorbia dentata* and *Euphorbia* subgenus *Poinsettia sensu stricto*. Eighteen species within the subgenus are native to Mexico and the rest originated from either Brazil, Argentina or Florida. Mayfield (1997) hypothesised that the species in subgenus *Poinsettia* form a monophyletic group nested within the large and diverse subgenus *Agaloma*. Species within the *Poinsettia sensu stricto* alliance can be considered more closely related to *E. pulcherrima*. The closest relative to *E. pulcherrima* is *E. cornastra*. Both have large glands, a woody habit, three rayed umbels and 2n = 28 chromosome number. *E. cornastra* grows to a height of two metres and *E. pulcherrima* reaches ten metres (Mayfield 1997). Species in the subgenus are listed in Appendix 4.

The chromosome number for most species in subgenus *Poinsettia* are n = 14 or 28, (2n = 28 or 56) and for *E. pulcherrima* 2n = 28 or 56. The somatic chromosome number of poinsettia cultivars is either 28 or 56 (Ewart and Walker 1960). Those with 2n = 28 are presumed to be diploids (Rinehart pers. comm. 1997, Sander pers. comm. 1999, Trees pers. comm. 1999). This presumption has not been conclusively proven (Ewart and Walker 1960, Bempong 1967, Bempong and Sink 1968a and b). Poinsettias with 2n = 42 and 35 chromosomes have been generated through crossing (Bempong 1967) and some aneuploid chromosome counts have been recorded as well (Kumar and Subramaniam 1986).

Market analyses

The predominant sales period for poinsettias commences in November and extends to a few weeks after December 25th, in most countries (Anon.1996b). These ornamental potted plants are produced in a variety of forms and are used for decoration during the Christmas period (Table 2.1, Figure 2.1).

Table 2.1. Common poinsettia plant forms (Ecke *et al.* 1990, Wilkerson *et al.* 1997)

Plant types	Pot diameter (mm)	Plant height (mm)	Description
Personal poinsettia	plug	<100	Not branched. Available exclusively from the Paul Ecke Ranch.
Mini	60 - 80	70 - 130	Popular in Europe. Generally non-branched.
Pixie	100	250 - 300	Popular size. A single branched plant per pot.
Pinched (branched)	130 - 150 or 200	†	Most popular form. 150 mm is most widely grown. A single branched plant per pot.
Single stem	130 - 150 or 200	†	3 - 4 non-decapitated plants per pot. This results in one inflorescence per plant.
Baskets	250 - 500	various	3 - 5 plants are used in each hanging or centrepiece basket.
Trees	150 - 300	900 - 1250	A 600 - 900 mm trunk attached to a 300 - 350 mm flowering head.
Bushes	various	various	These are usually left over stockplants that have been allowed to flower.
Tubs	250 - 350	750 - 1200	Approximately 20 - 30 inflorescences are produced on this form

† Consumer surveys have reported overall product height should be from 2.5 -3 times the pot height for these plant forms (Rinehart pers. comm. 1997, Trellinger 1998).

Figure 2.1. Poinsettia plant forms at various stages of production. Top left: branched mini; top right; pixie, centre; branched plant grown in a 150 mm pot, bottom left; tree, bottom right; bush

Bech and Rasmussen estimated world production at 200 million (M) plants in 1996. In 1993, Anon. (1994b) estimated production of approximately 130 M. Plants with branches accounted for approximately 80% of total production and 5 - 8 M plants were produced as miniatures (Anon. 1994b). The branched plant form grown in 150 mm pots (or slightly smaller) is the most prevalent (Table 2.2).

Table 2.2. Global sales information for poinsettias

Country	Units (M)	Major product form (percent of red bract market)	Major red cultivars	Percent of red cultivar production for country
United States	57.3*	150 mm	Freedom	approx. 50%
	65.0[†]	(59%)	Supjibi	approx. 10%
			Dark Red Hegg	approx. 5%
Germany	40.0[††]	-	Peterstar	leading variety
	26.0[†]		Sonora, Lilo	
Netherlands	19.0[††]	-	Angelika and	-
	15.7[†]		Goldfinger	
Italy	18.0[††]	mostly 130 mm	Freedom and	Together = 95%
			Peterstar	
Scandinavia	17.0[††]	120 - 130 mm	Lilo	90%
		(60%)	Peterstar	
United Kingdom		120 - 130 mm	Red Sails, Freedom,	30%
		(40%)	Lilo and Jolly Red	
Philippines		mostly 150 mm	Supjibi	
Japan	5.0[†]	150 mm	Freedom	50%
		(55%)	Red Sails	10%
Denmark, Norway, Sweden, Finland	13.5[†]			
Australia/New Zealand	1.0 - 2.0[†]	‡150 mm	‡Freedom	

All data obtained from † Anon. 1996b except †† Anon. 1994b, * Hamrick 1995 and ‡ White pers. comm. 1997.

The European market is accustomed to a compact plant form, which is now being accepted in the U.S.A. (Anon. 1996b, Ecke 1996). Although some cultivars are acknowledged as growing to a shorter finished height (Ecke 2000, Fischer 2000), the percentage of market share for this plant type is not readily available.

The United States is the largest market for poinsettias in both unit sales and wholesale value. For instance, in 1998, approximately 60 M pots were sold for a wholesale value of U.S.A. $212 M (by operations with sales greater than $100,000; USDA 1999). In the U.S.A., a greater number of poinsettias are sold than any other ornamental flowering potted plant (Nell *et al.* 1995) and they account for 31.4% of the flowering potted plant category (Hamrick 1995). Red bracted plants account for 74% of total poinsettias sold (Anon. 1996b, Higgins 1998) and this statistic is reflective of consumer preference (Behe *et al.* 1997, Behe 1999). The Freedom series of cultivars comprise 55% of the U.S.A. crop (Ball 1997).

Marketing studies in the U.S.A. showed that although poinsettia production is increasing, wholesale prices remain stagnant (Martens and Pyle 1993, Scullin pers. comm. 1998). Within this market, red bracted, branched cultivars, grown in 150 mm pots are the primary products. Estimates of consumer preferred plant height for this product range from approximately 1.5 to 2.0 times the pot height. Therefore the total height, including the pot height is 375 - 450 mm. Consumer surveys conducted by The Paul Ecke Ranch (a major grower and marketer of poinsettias) showed that a height of 460 mm is the most desirable (Rinehart pers. comm. 1997). Researchers such as Moe *et al.* (1992) and Trellinger (1998) and White (Nursery Manager, Oasis Horticulture pers. comm. 1997) aim for a total height of 360 - 380 mm (pot and plant). However, to date, this desirable height is frequently attained via the use of chemical growth retardants. Cultivars developed to obtain desired height in the absence of growth retardant application are desired by the market and can be expected to acquire a market share through decreased cost of production. These cost savings may result in lower consumer prices and/or increased grower profitability.

Propagation

Poinsettias are propagated via both *in vivo* (seed, grafting, root and shoot cuttings) and *in vitro* (micropropagation, adventitious shoots and somatic embryogenesis) methods at present.

In vivo propagation

Seeds

Seeds are generally produced by breeders. Seed lines are not used commercially at present. Seedlings can be transplanted four weeks after sowing. A juvenile 'seedling' growth phase lasts for approximately 6 - 8 weeks (Siraj Ali *et al.* 1990a) prior to a 'mature' growth phase.

Grafting

Grafting is a time consuming and labour intensive method of propagation. It is primarily used to transfer poinsettia branch inducing phytoplasma (PoiBI) to non-infected plants (Lee *et al.* 1997). Approach grafting (Hartmann *et al.* 1990) is often used to achieve this transfer (Dole and Wilkins 1988). This entails growing two plants in one pot, removing with a knife a matching section of stem on both plants to produce identical cambium patterns and binding the cut surfaces together. When the graft has healed, the appropriate scion and stock are cut to produce a scion/stock combination as one plant. Three separate stages exist in the formation of a graft. i. - cohesion of the stock and scion, ii. - callus proliferation at the graft interface and iii. - vascular re-differentiation across the interface. Graft incompatibility tends to increase with widening taxonomic distance between species. It may result from the breakdown of hormonal actions or the release of toxic products by the stock (Nass *et al.* 1997). Interspecific or intergeneric grafting of poinsettias (other than with *E. cyathophora* - Dole *et al.* 1993) has not been reported in the available literature.

Vegetative cuttings

Root propagation can been performed (Stewart and Pryor 1961), but is not utilised commercially. Shoot cuttings are widely accepted as the ideal method of commercial production and this method of propagation is detailed further under commercial propagation practices.

In vitro propagation

Several methods have been reported for *in vitro* propagation of poinsettias. These include micropropagation, of which there are a number of reports (De Langhe *et al.* 1974, Roest and Bokelman 1980, Paludan and Begtrup 1986, Preil 1994a and b, Bech and Rasmussen 1996), and meristem culture, where plants produced have been shown to be genetically stable and could be free of infective/pathogenic organisms such as viruses and phytoplasmas (Bech and Rasmussen 1996). Shoots and roots may also be derived adventitiously from callus (De Langhe *et al.* 1974) and protocols for somatic embryogenesis from callus are also available (Preil *et al.* 1982, Preil *et al.* 1983, Kleffel *et al.* 1986, Preil and Beck 1991, Rugini *et al.* 1993, Preil 1994a and b, Lee *et al.* 1997). *In vitro* techniques are not in commercial use for propagation at present.

Commercial production stages and techniques

Obtaining an ideal poinsettia plant suitable for sale requires optimal conditions and protocols for establishment and development of each stage from the initiation of propagation from parent plants to anthesis. Commercial production constitutes vegetative (clonal) shoot propagation from cultivars grown as stockplants. Poinsettias need to be grown according to specific production procedures to produce an acceptable crop. Plants may be grown and allowed to flower under natural conditions of decreasing daylength or under artificial conditions.

Stockplant establishment

Stockplants are the parental plants, which are grown under conditions similar to the flowering commercial pot plant progeny derived from them. Stockplants are developed from apical shoot cuttings and are grown under a long photoperiod environment, which prevents flowering. A long photoperiod can be attained through supplementary night-time lighting. A minimum light intensity of approximately 2 µmol m^{-2} s^{-1} at plant level is sufficient and can be produced with incandescent light globes placed above the plants and switched on between 10 pm and 2 am (Ecke *et al.* 1990). This lighting can be intermittent, with only 7.5 minutes of light applied once every 30 minutes within this period (Ecke *et al.* 1990). This protocol may be applied during propagation and vegetative growth, when flowering is not desired.

During stockplant growth, frequent decapitation ('pinching') of apical meristems (and varying numbers of associated apical leaves) is practiced to avoid premature floral initiation ('splitting'). Premature floral initiation may occur under long photoperiod conditions when a cultivar specific long day node number is acquired (Evans *et al.* 1992a). Premature floral initiation is typically characterised by the production of three vegetative shoots emerging below an aborted primary cyathium. This problem can be avoided by the removal of meristematic material, which reinstates the juvenile growth phase (Evans *et al.* 1992a, Siraj Ali 1990b).

Vegetative propagation via cuttings from stockplants

In most cultivars, apical shoots suitable for propagation as cuttings, reach maturity on stockplants approximately six weeks after the last apical meristem decapitation. These mature shoots ensure rapid development of uniform roots (Williams *et al.* 1993, Anon. 1994a).

Cuttings (which are 50 - 80 mm long and 6 - 10 mm in diameter; Anon. 1994a) are removed with a sharp knife or by hand. The cut ends are treated with a rooting hormone (Indole-3-butyric acid at 2500 mg/l, Ecke *et al.* 1990) and placed into a propagation substrate. This may consist of a variety of media, including compressed sphagnum peat moss, rockwool, combinations of vermiculite, perlite, sand and peatmoss, and commercial units such as Jiffy® plugs, Grodin® blocks or Oasis® root cubes. Propagation media requires an oxygen diffusion rate of 0.45 $\mu g\ cm^{-2}\ min^{-1}$ for successful root development (Gislerod 1994). The optimum pH for rooting is between 6.0 and 7.0 (Anon. 1994a). Once planted, cuttings are placed in a long photoperiod and intermittent water mist is applied (subjectively determined by the propagator in accordance with the prevailing environmental conditions) at an optimum media temperature of 22 - 24°C (Hall 1992). Mineral fertiliser is sometimes applied directly to the rooting medium (or via the mist) approximately 10 days after the start of propagation to replace nutrients that are leached from the leaves. Over application of nutrients (reaching toxic levels) is deleterious (Anon. 1994a) and therefore should be avoided. Fertiliser containing 100 - 150 mg/l nitrate as a source of nitrogen with or without potassium, can be applied at weekly intervals to the rooting medium until the cuttings are planted (Anon. 1994a). Electrical conductivity of the water should be less than 1 $mS\ cm^{-1}$ (Anon. 1994a).

Light intensity of approximately 400 $\mu mol\ m^{-2}\ s^{-1}$ should be provided during the propagation period (Ecke *et al.* 1990) for all except the light sensitive cultivars (Anon. 1994a). Trellinger (1998) suggests the use of a light intensity of 120 - 140 $\mu mol\ m^{-2}\ s^{-1}$ at least until the cuttings have developed roots. However, growth regulators may be required to suppress stem elongation under such low light intensities. Misting is gradually decreased over a period of two to four weeks during the root development phase to facilitate acclimatisation (hardening) prior to planting.

Growth of planted cuttings

Cuttings with roots are planted into a standard plastic pot (which can be of various sizes) filled with suitable media formulated for the continued growth of the plant. Potting media (growth media) ingredients are designed to provide air spaces and water holding capacity, while maintaining structural stability. A typical peat/perlite media (1:1) has a total porosity of 75%, free porosity of 24% and moisture retention of 50% (Ecke *et al.* 1990). Components such as sphagnum peat, perlite, sand, composted pine bark, coir fibre and vermiculite are often used. Cuttings with roots are planted into moist growing medium so that the growing

medium minimally covers the rooting medium (Jiffy® plug, etc). The planted cuttings are watered thoroughly after being potted (Anon. 1994a) and are maintained under a long photoperiod for approximately four weeks in total. Hourly water misting of the foliage is often practiced for 7 - 10 days post-planting to assist in acclimatisation. Light intensity at this stage is required to be approximately 600 μmol m^{-2} s^{-1} (Anon. 1994a). Full light intensity of approximately 1200 μmol m^{-2} s^{-1} is necessary for optimal growth after the plants have acclimatised (Tayama *et al.* 1990).

A soluble fertiliser containing minor elements at a rate of 150-200 mg/l can be applied within one week from planting. A fungicidal drench can also be applied at this stage (Anon. 1994a). Continued growth of poinsettias requires increased nutrition, which can be obtained through the irrigation supply (fertigation) or by granular application to the potting mix. Recommended elemental concentrations for a constant liquid fertiliser program are dependent upon numerous factors and are presented in Ecke *et al.* (1990). Osmocote®, a controlled release fertiliser (Sierra poinsettia mix 12-12-15 plus minor elements or other formulations) can be applied to provide continued nutrition. Appendix 2 lists the rates and analysis of Scotts Osmocote®Plus. Poinsettias are most responsive to media surface applications soon after potting (Anon. 1996c). Osmocote® 19-6-12 applied at 5.89 kg/m^3 by incorporation into the medium is recommended (Tayama 1988).

Plants grown in 150 mm pots that will remain in the same position for the duration of the growing period should be positioned on a lattice pattern of 325 x 350 mm centres (8.5 pots per square metre - Ecke *et al.* 1990). Pot spacing early in the production cycle encourages horizontal branching, which is detrimental to plant form and packaging of the finished product (Erwin 1993b). If plants are to be transferred to a short photoperiod environment for flower initiation and development, pots are placed in direct edge contact without interpot gaps, (Sidebottom pers. comm. 1997).

Development of uniform branched plants

Apical meristem decapitation (often referred to as 'pinching') is performed 10 - 14 days after planting cuttings with roots. Decapitation encourages axillary shoot growth from the remaining nodes and removes the 'history' of the cutting, re-initialising the generative state of the plant (i.e. juvenility) thereby promoting uniformity (Siraj Ali *et al.* 1990b, Snipen *et al.* 1999). Non-decapitated plants are less uniform in comparison. Apical decapitation is

necessary for the production of multi-branched plants (Ecke *et al.* 1990). Five or more axillary shoots are required on decapitated plants to be acceptable for sale. Plants that do not achieve this criterion are sold at a lower price or are discarded (Faust and Heins 1996). Wilkins (pers. comm. 1998) suggests that at least 7 - 8 leaves/nodes should be left on the plant at the apical decapitation stage. During this period, humidity must be kept at a higher level than at later stages, with ideal temperatures of approximately 27°C during the day and 20°C at night (Ecke *et al.* 1990).

Rapid and uniform axillary shoot development occurs when all tissues (such as immature leaves) that contribute to apical dominance are removed (Erwin 1993a). Either a 'hard' or a 'soft' decapitation (with leaf removal) will accomplish this goal (Berghage *et al.* 1989). Decapitations are rated according to the amount of stem tissue and the types of leaves removed. 'Hard' is defined as removal of the apical meristem including stem (20 - 30 mm) and leaf tissue associated with all immature leaves. 'Soft' is defined as removal of the apical meristem including stem (approx. 5 mm) and leaf tissue associated with leaves less than or equal to 20 mm long. 'Soft with leaf removal' is defined as 'soft' with removal of all immature leaves but not the associated stem tissue (approx. 5 mm). After decapitation, a minimum period of 10 days is required prior to floral induction (Wilkins pers. comm. 1998).

Induction and development of flowering

Ideally, at this stage, plants are well established in their containers, and axillary branches have commenced growth below the point of decapitation (Ecke *et al.* 1990). To permit flowering, plants need to be subjected to either a natural shortening photoperiod less than the critical photoperiod required for floral induction or an artificial short photoperiod environment of approximately 10 h duration. The critical photoperiod for flower initiation in most commercially important cultivars (under natural conditions) is approximately 12 hours and 20 minutes (Ecke *et al.* 1990). In Sydney, Australia, this photoperiod is reached on about the 15th of March (Anon. 1996a). Six to ten consecutive short days are required before the reproductive structures are visible (Grueber and Wilkins 1994). The recommended average temperature from the start of flower initiation through to the end of bract development is 20°C (Tayama *et al.* 1990).

Flowering will proceed normally if temperatures are maintained between 15.5°C and 21°C (Larson *et al.* 1988). If the temperature exceeds 21°C, a shorter photoperiod is required to

enable normal floral development (Larson *et al.* 1988). Ecke *et al.* (1990) and Hall (1992) suggested that temperatures above 21°C (night) and 24°C (day) cause a delay in normal flower initiation and development. Satisfactory flower initiation may be obtained at night temperatures up to 28°C if a 9 h photoperiod is provided (Shanks 1980). When an artificial short photoperiod is provided, ten hours of light is the standard requirement (White pers. comm. 1997). Extraneous light, even less than 0.4 μmol m^{-2} s^{-1}, is enough to inhibit normal flower bud initiation (Ecke *et al.* 1990). Thus, when artificially inducing a short photoperiod, covers must provide a complete inhibition of light during the dark phase.

The time taken in weeks from the start of floral inductive conditions (short photoperiod) to anthesis is referred to as the response period. Some cultivars are deemed suitable for sale when sufficient bract development and colouration is reached even prior to anthesis. In these cases, the response period reported would be for sufficient bract colouration enabling sale of the product. The response period ranges from seven to eleven weeks among current commercial cultivars (Grueber and Wilkins 1994).

Factors influencing plant height during commercial production

Plant height is influenced by various factors such as agronomy, genotype, environment, physiology and infectious agents such as viruses and phytoplasmas. Height is controlled by cell division and cell elongation. A reduction in either of these is reported to induce dwarfness or compactness in many plants (Brown *et al.* 1994).

In poinsettia, the stem elongation pattern, leading to a certain plant height, is described by internode number, length and position on a reproductive plant (Berghage and Heins 1991). Erwin (1993b) lists the following factors as the components of height for decapitated plants i. - primary stem length (below decapitation point), ii. - internode number on an axillary shoot and iii. - individual internode length on an axillary shoot. For example, cv. Freedom has 5 - 7 leaves in each axillary bud immediately after decapitation. If the plant was decapitated to 7 - 8 nodes, then at the completion of production, a commercially acceptable plant with 12 to 15 nodes below the inflorescence will be obtained (Wilkins pers. comm. 1998).

Agronomic factors

Height is directly related to both node number and internode length, which are dependent upon growth. Growth is influenced by several important factors. For example, when a crop is

grown under artificial short photoperiod, long photoperiod growth duration should not exceed six weeks. Similarly, for a natural season crop, the growth period should not exceed eight weeks (Anon. 1995) as this will result in increased node number and crop height. Apical decapitation methods influence height and were discussed previously (Berghage *et al.* 1989, Erwin 1993a).

The optimum application of fertiliser is important to growth and any over application should be avoided (Anon. 1995). However, Ludolph (1992) suggested that high soluble salt concentrations in the potting media could be used to control plant height by limiting root water uptake, and therefore reduce stem elongation. This method is not recommended for ornamental plants at present, due to an increased risk of root diseases (Ludolph 1992).

In potting media, over-watering encourages weak, thin and elongated growth (Williams 1992). Alternatively, moisture stress will result in short plants by reducing turgor pressure (Erwin *et al.* 1992) and this leads to unacceptably small bracts (White and Holcomb 1974). The restriction of root growth in a container is also a growth control measure, via limiting water supply and nutrition (Shanks 1980). Mechanical stimulation such as shaking, bending or brushing via air movement or objects has been used to control plant growth in several ornamental species with some success (Erwin *et al.* 1992, Garner and Langton 1997, Garner *et al.* 1997).

The intimate knowledge of poinsettia growth and development has led to the use of 'graphical tracking' to monitor plant height according to desired growth progression (Fisher 1995, Fisher and Heins 1995). This method enables prediction of plant height according to a standard curve. Although growth of decapitated plants can be predicted with confidence, non-decapitated plants exhibit greater variability and predictions are less accurate.

Genetic factors

Utilising cultivars, which are compact due to the expression of one or more dwarfing genes, is rated as the most effective means of controlling height for ornamental plants (Ludolph 1992) including poinsettias (Tayama *et al.* 1990). Therefore, crosses conducted among short cultivars are a potential method of producing shorter poinsettias. Reports of genetic inheritance studies of height are limited in ornamental species, especially in poinsettias. Internode number (determined by leaf unfolding rate) and internode length are of critical

importance to the production of short, vigorous poinsettias. Therefore, determination of genetic mechanisms involved in expression and the mode of control of these two parameters could enable the development of dwarf poinsettias. For example, in *Chrysanthemum morifolium*, studies of the genetic control of internode length and leaf unfolding rate (a measure of internode number per unit time) have given estimates of broad and narrow sense heritability of these traits and have lead to the successful identification of suitable progeny for further development (Langton and Dixon 1984).

Numerous dwarf mutants have been produced in many plant species (Broertjes and Van Harten 1988). Characterisation of dwarf plants often reveals the involvement of gene(s) concerning GA synthesis or metabolism (Reid 1986, Amirsadeghi *et al.* 1998). Application of GA to plants will often restore the normal phenotype, although some genes are GA insensitive.

In the Euphorbiaceae, published investigation of genetic dwarfness is limited to *Ricinus communis* (Singh and Yadav 1982, Reid 1986, Reddy and Sathaiah 1997).

In addition to dwarfing genes that reduce internode length, genetic variability for factors indirectly related to height can be used to select shorter plants. For example, variability exists within the cultivated poinsettias for response period (Larson *et al.* 1999a and b, Larson and McCall 1999a, Ecke 2000, Fischer 2000). Earlier responding varieties may acquire fewer nodes to be acceptable for sale, and hence, result in reduced height. The response period could be influenced by several factors i. - the critical day length for floral initiation, ii. - the node production rate and iii. - sufficient floral development to enable sale of the product (e.g. transitional bract number, sufficient bract expansion, etc). Variability exists within cultivated poinsettias for critical daylength requirement (Grueber and Wilkins 1994, Wilkins pers. comm. 1998) and this ranges from 6 - 9 days under an artificial short photoperiod of 8 hours. Variability also exists for vigour (Faust *et al.* 1997, Larson *et al.* 1999a and b, Rinehart pers. comm. 1997) and is probably a function of the node production rate. Approximately 0.2 leaves are produced per day at constant 21°C temperature (Wilkins pers. comm. 1998). Differences exist among cultivars for the development of leaves, transitional bracts and bracts. These differences account for most of the distinctness between cultivars in response period (Grueber and Wilkins 1994).

Heat tolerance is a key factor required to prevent delays in floral initiation and development and loss of branching. Variability within the commercial cultivar population exists for this character (Barrett *et al.* 1998, Tayama *et al.* 1990, Faust and Heins 1996). Utilisation of relevant cultivars with such variability may allow the production and flowering of plants at higher than recommended temperatures with reduced internode elongation. Partitioning between root and shoot growth (harvest index) may be important in controlling plant height. This has been investigated extensively in cereals and a response to selection for plant architecture can be expected in ornamentals (De Jong 1991).

In addition to individual gene(s), differing ploidy levels can influence plant height and heat tolerance. For example, the diploid cv. Angelika is vigorous and tall, whereas the tetraploid cv. Supjibi is shorter with improved heat tolerance, post harvest qualities, thicker stems and larger bracts that extend horizontally rather than droop in comparison to cv. Angelika (Tayama *et al.* 1990). Ling *et al.* (1997) used RAPD (Randomly Amplified Polymorphic DNA) markers to show cv. Supjibi was very closely related to cv. Angelika, suggesting that cv. Supjibi is a tetraploid form of cv. Angelika. This was verified by treating cv. Angelika plants with colchicine and deriving tetraploid plants. The derived plants were indistinguishable from cv. Supjibi (Trees pers. comm. 1999).

Genetically modifying plants by incorporation of DNA constructs into the plant genome can produce dwarf plants. Identification and isolation of dwarfing genes is required prior to production of dwarf plants through genetic manipulation (Amirsadeghi *et al.* 1998). Several gene sequences are available for transformation, with a potential consequence of reducing plant height (e.g. Bruhn 2000). For example, phytochrome gene(s), (Blowers 1994, Tennessen 1998 and Zheng *et al.* 1999) antisense gibberellin synthesis gene(s), rol genes A, B and C from *Agrobacterium rhizogenes* (Rugini *et al.* 1993, Senior *et al.* 1995) and others associated with plant hormones such as IAA are available. These may be inserted into the plant genome via *Agrobacterium tumefaciens*, electroporation or microprojectile bombardment (Larkin *et al.* 1994). Published literature on transforming poinsettia is limited to preliminary work by Rugini *et al.* (1993) and results of a project conducted by Blowers (1994).

A complex, genetically controlled mechanism that can influence plant height is inbreeding depression (IBD). IBD is characterised by a continued failure to survive and reproduce during the life cycle (Sage *et al.* 1994). Initially, parents may produce a reduced seed set, or partially developed seeds. Seeds produced may have lower germination, or germinate and then rapidly

die. Plants eventually produced may express deleterious effects such as mosaic leaves, or stunted growth. Plants grown to maturity may exhibit a lack of reproductive structures (Harding *et al.* 1981). The rapid development of IBD in poinsettias was reported by Sink (pers. comm. 1997) when breeding poinsettias for commercial applications.

There is substantial evidence that IBD is caused by accumulation of recessive deleterious mutations in plants and animals (Willis 1999). Elimination of deleterious alleles responsible for IBD is necessary to overcome the effects of IBD. This is easier if deleterious recessive mutations impose a single large identifiable effect (Husband and Schemske 1996, Willis 1999). If IBD is caused by a large number of recessive mutations that impose a small reduction in fitness, purging IBD through selfing is difficult (Willis 1999).

Environmental factors

Environmental factors affecting plant height include temperature, light, CO_2 percentage, water availability and nutrient status. CO_2 is rarely manipulated for poinsettia production and water availability and nutrient status are greatly influenced by management practices and were discussed previously. Temperature and light factors are discussed below.

Temperature

The average daily temperature or the cumulative total heat to which the plant is exposed (Tayama *et al.* 1990) determines the rate of leaf unfolding in poinsettia (Erwin 1993a). Increased rate of leaf unfolding leads to increased leaf/node number and therefore height within a given period. To offset potential increases in height, planting can be performed later than normal to accommodate rapid growth (i.e. reduced growth period). A typical poinsettia cultivar has a base constant temperature for leaf unfolding of ~7°C, the maximum is at approximately 25°C (Berghage and Heins 1991, Erwin 1993a) when approximately one leaf unfolds every 4 to 5 days (Tayama *et al.* 1990). At 32°C, the rate of leaf unfolding gradually decreases and at a constant temperature of 37.5°C, plant death occurs (Erwin 1993a). Increased average temperatures between the dates of apical decapitation and flower initiation will result in more leaves unfolding prior to flower initiation and hence more nodes on a lateral shoot, leading to increased height (Berghage and Heins 1991). Internode length is greatly reduced after floral initiation as internodes approach the inflorescence (Berghage and Heins 1991) with the exception of when average night temperatures exceed 24.5°C. Delay in

flowering results in further node development and an increase in overall plant height (Erwin 1993a).

Internode length is influenced by the difference between average day and night temperatures (DIF) (Erwin 1993a). By manipulating DIF, it is possible to control leaf unfolding and internode elongation. The control of these processes by DIF is via reduction in cellular elongation, not division (Myster and Moe 1995). Internode elongation increases with increasing DIF within the 10 - 30°C temperature range (Erwin 1993a). Internode elongation is the least at zero DIF, greater at negative DIF and greatest at positive DIF (Erwin 1993a). Most plants respond to a change in day/night temperatures within 24 hours (Myster and Moe 1995). During artificial short days, response to DIF increases (Erwin 1993a). In general, plants grown at zero DIF are the earliest to flower (Moe *et al.* 1992) and the shortest.

Decreasing temperature below the greenhouse ambient temperature during the early 2 - 3 hours of the morning (DROP) reduces internode elongation (Erwin 1993a). For DROP to be effective, the temperature must be reduced prior to exposure of plants to light (Erwin 1993a). High light intensity during the photoperiod is essential to the effective use of DIF or DROP (Erwin 1993a, Grindal and Moe 1997) and this limits application of these methods of height control. The use of DIF and DROP is also limited by the large differences in response between varieties (Moe *et al.* 1992, Sauer and Hintze 1997, Eames and Clifford 1999). Besides affecting internode length and plant height, DIF also affects post-harvest quality (Moe *et al.* 1992), leaf and shoot orientation, chlorophyll content, lateral branching, and petiole and flower stalk elongation (Myster and Moe 1995). Negative DIF delays flowering significantly in poinsettia while DROP on the other hand affects flowering marginally (Myster and Moe 1995). Therefore, DROP is preferred over DIF for reducing plant height due to reduced adverse effects.

Light

Internode elongation is influenced by the proportion of red and far red light intercepted by the phytochrome pigment in plants. Phytochrome monitors daylength and is important in photomorphogenesis (Tayama *et al.* 1990, McMahon 1998). Phytochrome exists in two inter-convertible forms, one of which preferentially absorbs red light (R, 660 nm), while the other absorbs far red light (Fr, 730 nm). A high R : Fr ratio reduces stem elongation and produces short, well branched plants, while a high Fr: R ratio promotes elongation (Tayama *et al.* 1990). This is demonstrated on stockplants grown under low light intensity. These plants

produce cuttings with long internodes, large leaves and thin stems. Plants therefore need to be decapitated at a height greater than desired to produce the required number of axillary branches (Tayama *et al.* 1990).

Increased space between potted plants generally results in reduced height (Ecke *et al.* 1990). Inadequate spacing results in branching inhibition and rapid elongation of the first internode (Williams 1992, Erwin 1993a). Additionally, high humidity results in internode elongation through lack of air circulation (Williams 1992). Long photoperiod lighting using incandescent lamps (high in Fr) also results in stem elongation, compared to fluorescent or high pressure sodium lamps (higher R) (Ludolph 1992). Floral initiation and development under artificial short days (approximately 9 hours day length) produces shorter plants than those grown under naturally decreasing day length (Anon. 1996c).

Plant height can be reduced by as much as 30% by growing plants under photo-selective plastics, which change the light spectrum (McMahon 1998). Eames and Clifford (1999) found that the most successful filter for controlling plant height was the far-red type. Further work is needed to optimise this filter system, especially under conditions of low light intensity.

Physiological factors (growth regulators)

The interaction between gibberellic acid, growth retardants, light and temperature is very important to plant height. Gibberellins, auxins, cytokinins, abscisic acid and ethylene are the five accepted classes of plant growth regulating hormones (Moore 1984). Interactions between hormones (complementary, antagonistic and often complicated) are responsible for normal plant growth (Moore 1984).

Gibberellic acid and GA-inhibitors

There are numerous forms of gibberellins (about 40 are known to occur in plants) and they affect other physiological processes besides stem elongation (Moore 1984). The primary gibberellin that controls shoot elongation in higher plants appears to be GA_1 (Halmann 1990). GA is produced in young, actively photosynthesising leaves and roots (Moore 1984). Numerous GA mutants are characterised (Ross *et al.* 1997). Experiments with foliar application of GA_3 on poinsettias showed that node number did not significantly increase in treated plants over that of controls, but internode length increased dramatically (Bernuetz 1995, unpublished data). Likewise, Mynett and Wilkonska (1989) achieved stem elongation

in poinsettias by application of 250-750mg/l GA_3. Moore (1984) explained that GA stimulates cell elongation in most plants, usually through an increase in internode elongation due to increased water uptake. Dry weight is thus often unaffected. Evans *et al.* (1992b) found that foliar application of GA_3 inhibited ontogenetic aging of the meristem, increasing the number of nodes produced prior to long day floral initiation (splitting). Application of GA_1, GA_3, GA_4 and GA_7 increased mean internode length similarly.

Anti-gibberellin growth regulators

Growth regulators (GRs) limit internode elongation by blocking various steps in the GA metabolism pathway (Halmann 1990, Albrecht *et al.* 1992). Therefore, it is expected that the effects of over application of growth regulating chemicals could be reversed by application of GA (Cox 2000).

It is believed that all GRs have a similar action within the plant but the crop responses differ (Albrecht *et al.* 1992). The use of GRs is critical to the production of a high quality poinsettia crop even in cultivars that are naturally of short height (Barrett 1993, Erwin 1993b). GRs are most frequently used during propagation to control height (due to high temperatures, humidity, close spacing and low light intensity - Anon. 1994a). Most commonly used GRs include A-rest® (ancymidol), B-nine® (daminozide), Bonzi® (paclobutrazol), Cycocel® (chlormequat) and Sumagic® (uniconazole-p) (Williams 1995). The effectiveness of GR application depends on the method of application, plant genotype, current and expected future temperatures, pH, light, moisture, chemical concentration and evaporation rate, wettability, season, growth medium, age and stage of development, mode and uniformity of application (Ecke *et al.* 1990). There are concerns regarding the effects (adverse) of these chemicals on the environment (Berghage and Heins 1991, Martens and Pyle 1993). The potential hazard of GRs to human health has resulted in restrictions on their use and availability (Erwin *et al.* 1992, Myster and Moe 1995). For example, paclobutrazol is classed as an S5 poison in Australia (Anon. 1991) and in 1992 Cycocel® was the only growth regulating chemical registered for application in Germany (Ludolph 1992).

Interaction of gibberellins, growth regulators, light and temperature

Photoperiod, irradiance, light quality and GRs are thought to interact with DIF (Myster and Moe 1995). Evans *et al.* (1992b) proposed that DIF influenced stem elongation through alteration of endogenous GA levels. He suggested that higher temperature could increase endogenous GA levels, which result in increased internode elongation and delayed floral

initiation. Myster and Moe (1995) concurred with this view and suggested that endogenous GA content should be low in negative DIF treated plants. Data from *Campanula isophylla* supported this hypothesis (Myster and Moe 1995).

The effect of temperature on stem elongation may be via phytochrome, or direct action on GA metabolism (Grindal and Moe 1997). Inactivation of GA_1 by the irreversible addition of a hydroxyl group to the C-2 position of the gibberellin molecule is one possible way that plants may reduce GA_1 production under negative DIF conditions (Grindal and Moe 1997).

Plants grown under a positive DIF respond similarly to growth under a low R : Fr light ratio (or high Fr light). Likewise, a negative DIF response is similar to the high R : Fr ratio (or high R light) response (Myster and Moe 1995). GR application overcomes positive DIF stem elongation effects (Myster and Moe 1995). As such, the effect of GR application may be considered similar to plants grown at negative DIF and/or high R : Fr light ratio.

Auxins

Auxins are produced in the apical meristem and are responsible for inhibition of lateral bud growth on stems (Moore 1984). In poinsettia, Indole-3-acetic acid (IAA) is involved in bract abscission, epinasty, (Gilbert and Sink 1970, Gilbert and Sink 1971, Reid *et al.* 1981) and apical dominance resulting in axillary bud inhibition (Weiss and Shillo 1988). The young expanding leaves (YELs) on vegetative poinsettias are the major source of axillary bud inhibition, followed by the apical bud (which includes the meristem, primordial and small unfolded leaves) (Weiss and Shillo 1988). Auxin concentration is highest (on a whole organ basis) in YELs compared to the apical bud. Accordingly, removal of YELs by decapitation down to the fully expanded leaves results in faster axillary bud growth and therefore branching (on cv. Annette Hegg Brilliant Diamond plants) than removal of the apical bud alone (Weiss and Shillo 1988). On reproductive plants, bracts and cyathia (which contain high levels of auxins) are the sites of inhibition.

Cytokinins

Cytokinins (CK) are found in root tips and young leaves, and do not appear to be readily transported (Moore 1984). They promote cell expansion and initiate growth in inactive lateral buds, or buds inhibited by apical dominance (Moore 1984, Kaminek *et al.* 1987). In poinsettia, an increase in branch number by axillary bud stimulation (in non-decapitated free branching and restricted-branching plants) by application of CKs was reported by several

authors (Carpenter *et al.* 1971, Milbocker 1972, Ching 1985, Semeniuk and Griesbach 1985, Kaminek *et al.* 1987, Witaszek 1989). Application of CKs directly to the bud produces greater branching than spray application to the whole plant, which may induce leaf chlorosis (Carpenter *et al.* 1971, Semeniuk and Griesbach 1985).

Abscisic acid and ethylene

Abscisic acid (ABA) and ethylene are considered inhibitors of plant growth (Moore 1984). Ethylene is a volatile gas that is involved in epinasty and senescence of plant tissues. Ethylene could be involved in poinsettia epinasty (Sacalis 1978, Reid *et al.* 1981, Woodrow and Grodzinski 1987).

Influence of pathogens

Pests and diseases often have an adverse effect on plants by introducing blemishes, causing distorted growth and adversely affecting the health of the crop, usually decreasing height. *Sphaceloma poinsettiae* (poinsettia scab) is a fungal pathogen expressing symptoms similar to artificial application of gibberellic acid on heavily infected branches (Ecke *et al.* 1990). These branches may grow twice as long as normal. Infection with most pathogens can be avoided by growing plants under strict hygienic practices where chemical application may also be necessary.

Influence of infective agents

Generally, plant breeders attempt to eliminate all pathogens such as bacteria, fungi and viral agents prior to releasing a new cultivar. However, two types of infectious agents are frequently present in poinsettia cultivars received directly from breeders. These are viruses and phytoplasmas.

Most viruses cause plant dwarfing and stunting. Their effects usually result in a reduction in growth regulating substances within the plant, manifested through an increase in growth inhibiting substances (Agrios 1997). Phytoplasmas frequently induce symptoms that may influence plant height (e.g. little leaf and witches brooms).

Poinsettia Mosaic Virus (PnMV)

Characteristics

PnMV is tentatively placed in the tymovirus group (Fulton and Fulton 1980, Lesemann *et al.* 1983). PnMV is reported to frequently infect poinsettias (Koenig and Lesemann 1980, Paludan and Begtrup 1986, Bellardi *et al.* 1995). PnMV was identified in 45 commercial cultivars obtained from several countries and breeders by Bech and Rasmussen (1996) and was present in all 21 cultivars developed in the U.S.A. and Europe (Lee *et al.* 1997).

Symptoms

Generally, PnMV infected plants display an angular mosaic on leaves when plants are grown at or below 20°C (Ecke *et al.* 1990). PnMV infected plants are also frequently infected with another virus named poinsettia cryptic virus (PnCV), therefore the 'mosaic disease' can be considered a mixed virus infection (Koenig and Lesemann 1980, Bellardi *et al.* 1995, Bertaccini *et al.* 1996). Symptoms of this infection are prominent during winter and include floral and leaf deformations (Fulton and Fulton 1980, Paludan and Begtrup 1986), chlorotic mottle, leaf mosaic, vein yellowing and stunting, decreased vigour, reduced quality and production yield decreases from 6-10% (Bellardi *et al.* 1995, Bertaccini *et al.* 1996). Symptomless infections are also very common. Although PnMV and PnCV are often found in a mixed infection, PnCV alone does not display symptoms (Koenig and Lesemann 1980, Koenig *et al.* 1986, Ecke *et al.* 1990, Schoenfelder pers. comm. 1999).

Bech and Rasmussen (1996) demonstrated that inoculation of virus free plants with PnMV infected sap resulted in morphologically similar plants, except for the presence of an angular mosaic on leaves. Ecke *et al.* (1990) suggest that PnMV is not commercially detrimental because infected stockplants produce cuttings and plants equivalent to those free of PnMV. Van Der Meij (pers. comm. 1999) suggested that PnMV did not have an effect on branching, flowering or dwarfness during his investigations.

Transmission

Viruses may be transmitted via seed, grafting, mechanical methods or insect vectors. Seed transmission of PnMV between poinsettias is unlikely (Fulton and Fulton 1980 - unpublished data, Koenig and Lesemann 1980). Additionally, seed transmission from infected *Euphorbia cyathophora* plants (taxonomically related to poinsettias) did not occur (Fulton and Fulton 1980). Further studies are necessary to confirm seed transmission if any.

Grafting successfully transmits PnMV (Fulton and Fulton 1980). Mechanical inoculation results in successful transmission but the efficiency is low (Fulton and Fulton 1980, Bech and Rasmussen 1996). Plants subjected to leaf rubbing with PnMV sap extract display visual symptoms of infection after four weeks (Pfannenstiel *et al.* 1982). Leaf and root contact between infected and non-infected plants did not transmit the virus (Pfannenstiel *et al.* 1982). Simulation of removing cuttings from stockplants by hand, by removing cuttings alternately from infected and non-infected plants, also failed to transmit PnMV. Whitefly (*Trialeurodes vaporariorum*) and two-spotted mites (*Tetranychus telarius*) did not transmit PnMV (Fulton and Fulton 1980). Pfannenstiel *et al.* (1982) concluded that virus transmission between plants during normal propagation was unlikely and the maintenance of virus-free stock could be accomplished without difficulty.

Detection

PnMV is detected using various methods such as ELISA (Enzyme Linked Immunosorbent Assay; Fulton and Fulton 1980), ISEM (Immunosorbent Electron Microscopy; Paludan and Begtrup 1986, Bertaccini *et al.* 1996), agar gel double diffusion tests (Koenig and Lesemann 1980, Bertaccini *et al.* 1996) sap inoculation to *Nicotiana benthamiana* (Lesemann *et al.* 1983, Bellardi *et al.* 1995) and GLAD (Gold Labelled Antibody Decoration; Bellardi *et al.* 1995). Visual detection may be accomplished by observing plants for the angular leaf mosaic (Ecke *et al.* 1990). Because *N. benthamiana* is not susceptible to PnCV, a pure PnMV infection may be obtained in this species (Lesemann *et al.* 1983).

Within the Euphorbiaceae, the only host of PnMV reported in the available literature is *E. fulgens*. *E. fulgens* is also a host to PnCV (Bellardi and Bertaccini 1989).

Poinsettia cryptic Virus (PnCV)

Characteristics

PnCV is tentatively placed in the cryptic virus group (Koenig and Lesemann 1980).

Symptoms

In poinsettias, PnCV is often found in a mixed infection with PnMV, and this mixed infection is often postulated as the cause of several symptoms. These symptoms were described previously under poinsettia mosaic virus. However, cryptic viruses are generally considered symptomless (Boccardo *et al.* 1987) and in addition, PnCV alone does not produce any

symptoms in infected poinsettia plants (Koenig and Lesemann 1980, Koenig *et al.* 1986, Ecke *et al.* 1990, Koenig pers. comm. 2000, Schoenfelder pers. comm. 1999). Preil (pers. comm. 1998) states PnCV has no negative effects on poinsettias grown in Europe under optimal greenhouse conditions.

Transmission

Cryptic viruses in general are considered as readily transmissible through seed and are not transmissible via grafting (Boccardo *et al.* 1987, Ghabrial *et al.* 1998). Further studies are required to clarify graft transmissibility of this virus.

Studies of the seed transmissibility of PnCV have not been reported (Boccardo *et al.* 1987). Koenig and Lesemann (1980), Koenig *et al.* 1996 and Koenig (pers. comm. 2000) have suggested that PnCV is transmitted via seed, but data was not presented. Further studies are required to determine if the virus is seed transmissibile.

Plants can be freed from PnCV through *in vitro* cell suspension culture and therefore, PnCV may not be a typical cryptic virus (Boccardo *et al.* 1987).

Detection

PnCV may be detected via ISEM (Paludan and Begtrup 1986, Bellardi *et al.* 1995, Bertaccini *et al.* 1996), GLAD (Bellardi *et al.* 1995) and ELISA. ELISA was difficult and is not recommended (Van Der Meij pers. comm. 1999, Schoenfelder pers. comm. 1999). In poinsettias containing both PnCV and PnMV, the titre of PnCV has been reported to be 1,000 times lower than that of PnMV (Lesemann *et al.* 1983).

Poinsettia Branch Inducing Phytoplasma (PoiBI)

Characteristics

Phytoplasmas (previously mycoplasma-like organisms) are unculturable, cell wall-less bacteria that inhabit the phloem vessels of plants (Lee *et al.* 1997). Poinsettia branch inducing phytoplasma (PoiBI) is identified as a member of the 16S rRNA group III, to which western-X and related phytoplasmas also belong (Lee *et al.* 1997). Western-X phytoplasma is a serious pathogen infecting stone and pome fruit trees and is not currently present in Australia. Due to the similarities between PoiBI and western-X phytoplasmas, it is thought that PoiBI

could infect and spread through other crop species (either directly or through mutation), causing diseases and crop losses.

Symptoms

Common phytoplasma infection symptoms include stunting, dieback, leaf yellowing, reduced leaf size ('little leaf'), axillary bud proliferation ('witches brooms'), phyllody of floral organs (petals and sepals resemble leaves), virescence (greening of petals) and floral gigantism ('big bud') (Davis *et al.* 1997). Floral organs expressing these symptoms are frequently sterile.

In commercially produced poinsettia cultivars, PoiBI is common, but has only recently been detected (Lee *et al.* 1995, Bertaccini *et al.* 1996). Prior to its detection, this agent (then unknown) was termed the 'graft transmissible branching agent' (Lee *et al.* 1997). Several authors characterised the transmission and morphological effects of this agent (Stimart 1983, Dole, Wilkins 1988, Dole and Wilkins 1991, Dole *et al.* 1993, Bech and Rasmussen 1996, Ruiz-Sifre *et al.* 1997). The symptoms of infection have been postulated to include an increase in branch number (Bech and Rasmussen 1996), reduced stem diameter (Dole and Wilkins 1988, Bech and Rasmussen 1996), changes in leaf shape (from oak-leaf shaped to elliptical), shorter internodes, less intensive bract colour, earlier anthesis, earlier cyathia abortion, lower fertility (seed set after pollination) and reduced growth (Preil 1994b). Dole and Wilkins (1988 and 1994) consider that anthesis date and bract colour remain unchanged.

A study by Bech and Rasmussen (1996) showed a 20% reduction in height was due to infection with PoiBI. Reduction in height was through a decrease in internode length. Measurements were made on approach-grafted plants four months after grafting with plants still attached. There are no published studies that address whether statistical differences exist between PoiBI infected and non-infected plants, especially in the absence of PnMV or during standard commercial production practices. Further study is required.

The symptoms of phytoplasma infection suggest a disturbance of the hormonal balance of the plant (Lee and Davis 1992). According to Lee, PoiBI triggers the formation of axillary branches in infected poinsettias by influencing two growth regulating hormones (types not mentioned), which result in the plant growing outward rather than upward (Suszkiw 1998). Similar situations have been noted in other species. For example, in phytoplasma infected plants of *Solanum melongena* (eggplant), increased levels of IAA and cytokinins were found in all infected tissues compared to healthy controls (Das and Mitra 1998). Pertot *et al.* (1998)

found that endogenous IAA concentrations were greater in *Catharanthus roseus* plants infected with clover phyllody phytoplasma.

Transmission

Transmission of phytoplasmas by various homopterous insect vectors, grafting and the parasitic plant *Cuscuta* sp. has been demonstrated (McCoy *et al.* 1989, Gunderson *et al.* 1996, Davis *et al.* 1997). In commercially grown poinsettias, transmission by insects, seed and during normal culture practices is not considered possible (Dole *et al.* 1993). Approach grafting is the most common method used for transmission. Vascular connections between grafted plants are not required to transmit PoiBI. The primary symptom of successful transmission is the development of shoots on the lowermost stem nodes 10 days after grafting (Ruiz-Sifre *et al.* 1997).

Detection

Phytoplasmas can be detected by a number of methods including serological assays, ELISA, dot blot immunoassays, immunofluorescence microscopy and ISEM (Lee and Davis 1992). However, the most sensitive method of detection at present involves the use of PCR (polymerase chain reaction; Davis *et al.* 1997). PoiBI has been detected by PCR, either by direct PCR using universal primers, nested testing or via western-X specific primers (Bertaccini *et al.* 1996, Lee *et al.* 1997, Gibb pers. comm. 1997 respectively).

Development of infection free poinsettias

Several methods have been successful for the removal of PnMV and PnCV from infected poinsettias. These include heat treatment, suspension culture, meristem culture and somatic embryogenesis (Preil *et al.* 1982, Paludan and Begtrup 1986, Preil 1994a and b, Bech and Rasmussen 1996, Lee *et al.* 1997). Phytoplasmas are limited to phloem vessels of plants and are not present in meristems. They are heat labile at 37°C (Lee and Davis 1992). As such, the removal of PoiBI by heat treatment (Bech and Rasmussen 1996), suspension culture (Dole and Wilkins 1988, Preil 1994b), meristem culture (Bech and Rasmussen 1996) and somatic embryogenesis (Lee *et al.* 1997) has been successful, as expected.

In vitro culture

Roest and Bokelman (1980) obtained adventitious shoots and roots from stem explants placed onto a media consisting of MS basal salts (Murashige and Skoog 1962) 0.8% agar, 3%

sucrose, 2g/l myo-inositol, 1.0 mg/l BA, 0.1 mg/l NAA and pH adjusted to 5.8. Paludan and Begtrup (1986) placed excised meristems (0.25 mm) of several poinsettia cultivars onto various media with an aim of obtaining PnMV and PnCV free plants. Most media enabled the development of shoots from the meristems. Approximately 54 - 95% of plants derived from shoots tested negative to PnMV. Additionally, 42 - 90% of shoots tested negative to PnCV based on two to three repeated negative ISEM tests. Bech and Rasmussen (1996) excised meristems (0.25 mm) from apical shoots of cv. Freedom red to obtain PnMV free plant material. Superior *in vitro* growth was obtained using MS basal salts, 1.0 mg/l BAP, 0.2 mg/l IAA and other additives as used by Preil *et al.* (1982 - i.e. 3% sucrose, 0.6% agar and pH 5.8). Shoots established from meristems could be transferred to rooting media after 4 - 5 months. Twenty randomly chosen meristem derived plants were tested for PnMV and all were negative.

Heat treatment

Treatment of whole plants in a heated cabinet at temperature greater than 37°C followed by excision of apical mini-cuttings can be used to remove PoiBI and PnMV from the cultivar V10 Amy red (Guy pers. comm. 1998). Heat treatment has also resulted in PnMV and PoiBI free plants in three published reports (Pfannenstiel *et al.* 1982, Paludan and Begtrup 1986, Bech and Rasmussen 1996).

Selecting for plant height – practical considerations

Selection of dwarf plants requires an understanding of the mechanisms that influence plant height. Plant height is influenced by factors such as the growing environment, management practices and genotype, and these have been discussed previously (under factors influencing plant height under commercial production). Provided the growing environment and management practices are uniform across the experimental area, plant height in poinsettias is then affected by node number and internode length, which are in turn determined by the genotype. Measurement of both of these characters would provide data to enable selection of shorter plants. It is imperative to select dwarf plants that possess a rapid node production rate, because slow growing dwarf plants are not desirable. Dwarfs that are slow growing could result in an extension of the production period. One method to determine growth rate is the time taken in days to produce a node (T/N). Selecting plants with a low T/N value and a low height is desirable. Other characters such as branch number, stem diameter and response period could affect plant height and should be measured.

To increase selection efficiency, a marker trait (molecular or phenotypic) can be used in an attempt to improve a target trait such as plant height (Eaton and Lapins 1970, Paprstein 1998, Harding *et al.* 1991). Selection can also be based on a juvenile phenotypic character, which correlates with the adult phenotype (Reid 1986, Waycott *et al.* 1995). If the correlation is high, the population can be culled early in the growth cycle, thus increasing efficiency. For example, application of GA to wheat seedling populations possessing GA insensitive dwarfing gene(s) allows phenotypic selection of these dwarf genotypes (Brown pers. comm. 1997).

Plants in a population under selection should be grown according to commercial production methodology. Selections therefore, will be more representative of a commercial product and suited to the growth environment. Selection under the commercial growing environment is especially important if the response to environment varies strongly with genotype (De Jong 1991). For example, selection of poinsettias for production in Australia during the major production phase (August – December) necessitates selection during this period or later (mid to late summer) because heat tolerance is a major criterion. A second objective is to select for ornamentally desirable traits at the same time as plant height, so that improvements in both objectives can be made.

Commercially grown poinsettias differ from plants derived from seeds in that a germination period is not applicable and they are frequently infected with PoiBI, PnMV and PnCV. To ensure uniformity after a non-uniform germination period, all seedlings could be decapitated to re-instate juvenility and enable new growth from an approximately equal starting point. It may be considered that introducing this phytoplasma/virus complex (or at least PoiBI only) would be beneficial to selection, especially considering PoiBI could contribute to reduced plant height. However, grafting every seedling in a population (potentially thousands of plants) would be labour intensive and costly. In addition, a high proportion of plants would be culled, as only the best are selected. It is postulated that introducing PoiBI after selection would be the most suitable method for developing dwarf poinsettias, even though a genotype x PoiBI interaction could be present. It may also be desirable to maintain some uninfected stockplants for future breeding because PoiBI infection has been postulated to reduce seed set. In addition, grafting poinsettias to obtain PoiBI would not enable the selection of plants that are genotypically prolific branchers. High branching plants could be desirable in future studies, especially if somatic embryogenesis is to be considered as a method of large scale propagation.

Seedling juvenility must be overcome prior to selection (De Jong 1991). Juvenility influences flowering and rooting ability, leaf characteristics, leaf length, internodal characteristics and phyllotaxy in poinsettia (Siraj Ali *et al.* 1990a). The transition from juvenile to mature plants occurs between six and eight weeks post-germination (Siraj Ali *et al.* 1990a). Therefore, poinsettias should be selected after this period to ensure the mature phenotype is being considered.

Reid (1986) suggests selection of dwarf plants from a population involves growing a large number of plants because optimum methods of reducing unwanted plants are not available to improve efficiency. Efficient selection methods have been utilised to select plants grown *in vitro* for resistance to various toxins, pathogens and herbicides. Some researchers have attempted selection of dwarf genotypes *in vitro* by addition of supra-optimal concentrations of growth hormones (Lane *et al.* 1982, Geier 1983) but this was not successful.

Once plants are selected for desired attributes at the single plant stage, replicated trials need to be conducted in the envisaged production environment and/or in isoclimatic zones to validate selections and obtain a better understanding of genotype x environment interactions. It is also important to select dwarf plants that perform acceptably year - round in Australia, not only during the major production period, because poinsettias are grown throughout the year in Australia.

Breeding poinsettias

Background

The first concerted effort to breed poinsettias involved cross and self pollinations of the cultivars available in 1949 (the seedling cultivar Oakleaf, Ecke white and mutants of both). These cultivars were very close to wild poinsettias. Approximately one seed was produced from ten pollinations. Few seedlings appeared normal, most were stunted, distorted and weak (Hartley 1995). Self pollination was not successful for the production of inbred lines, so all further generations were produced via sibling crosses. One generation was produced annually. The best progeny (showing improvements in horticulturally important traits) were selected and used as parents for subsequent breeding.

Prior to the 1960s, it is likely that almost all red bracted poinsettias in the commercial crop grown in the U.S.A. were somatic mutations of the cv. Oakleaf (Stewart and Pryor 1961).

This trend continued until 1979, when it appeared that commercial breeders had continued to use only the material provided to them from the USDA (Stewart *et al.* 1979).

During the 1960s, several cultivars were released from private companies. Series of mutants were then selected from these cultivars over time (Appendix 5). The majority of commercial cultivars developed until about 1988 were derived via somatic mutation (Appendix 5), thus limiting genetic diversity within the cultivated population. Currently, all major breeding companies have intraspecific hybridisation projects in progress. Since 1990, numerous cultivars have been released but records of their parentages are difficult to obtain. However, to obtain Plant Breeders Rights (in Australia) or a plant patent (U.S.A.) information relating to the development of the cultivar must be provided and this database can be utilised to obtain parentages of some cultivars. Recent publications have aimed at identifying differences between poinsettia cultivars via molecular methods, to obtain an understanding of genetic relations and for use in cultivar identification (Ling *et al.* 1997, Starman and Abbitt 1997, Starman *et al.* 1999).

Sink (pers. comm. 1997) suggests that the reason mutation breeding and the selection of naturally occurring mutants is practiced in poinsettias is because the crop is heterozygous, polyploid and suffers rapid inbreeding depression. Additionally, seed production is difficult and at low frequency in breeding work.

Factors influencing successful intraspecific hybridisation

Ploidy

Ploidy level has particular consequence on crossability within many species. In poinsettia, commercially available plants are either $2n = 28$ or $2n = 56$. If crosses are to be performed between these two ploidies, the efficiency of production and any special techniques (such as embryo rescue) need to be understood. The $2n = 28$ chromosome number is taken as the diploid condition in poinsettia, but this has not been thoroughly proven (Ewart and Walker 1960, Bempong 1967, Bempong and Sink 1968a, Sink pers. comm. 1997). Ewart and Walker (1960) suggest the haploid chromosome number is 7, not 14, based on the observation of quadrivalents at diakinesis, and subsequent movement of bivalents to the poles in anaphase 1 in $2n = 28$ plants (as confirmed by Pai 1960). Bempong and Sink (1968a) supported this reasoning based on their discovery of seven sets of four similar chromosomes present in two

2n = 28 cultivars. However, others do not agree that 2n = 28 plants are tetraploid (Rinehart pers. comm. 1997, Sander pers. comm. 1999, Trees pers. comm. 1999).

Poinsettias possessing 2n = 56 chromosomes are termed tetraploids, however, they may actually be octaploids. Tetraploids have been produced from diploids (Stewart 1951) and they generally possess (in comparison with diploids) shorter internode lengths (Sander pers. comm. 1999, Trees pers. comm. 1999), increased stomatal size, pollen grain size, cyathia size, fruit and seed size, bract thickness and broadness and stem diameter (Stewart and Pryor 1961). The tetraploid cultivars are thought to have arisen via somatic doubling (Emsweller and Uhring 1960, Ecke and Matkin 1976).

Poinsettias possessing 2n = 42 chromosomes are termed triploids, but could be hexaploids. Production of triploids was attempted by Ewart and Walker (1960), Bempong and Sink (1968b) and Milbocker and Sink (1969a). Only the latter two authors succeeded in producing 2n = 42 plants, always using the diploid as the female. The efficiency of production was low (approx. 0.2%) and was attributed to a lack of endosperm development (Milbocker and Sink 1969b). Triploid plants have also been developed via endosperm culture of *Emblica officinalis* (Sehgal *et al.* 1994) and *Mallotus philippensis* (Sehgal and Abbas 1996) both members of the Euphorbiaceae.

Further work by Bempong (1967) involved hybridisation between 2n = 28 and 2n = 42 chromosome plants. He successfully produced 2n = 35 chromosome plants that were slow growing and required an extended growth period to flower. Aneuploid chromosome counts have also been reported (Mayfield 1997).

Seed production

Seed production is dependent upon a number of factors. Plant genotypes, number of pollination repetitions, temperature and light contribute towards efficient seed production. Seed production in poinsettias whether by cross or self pollination is inherently low. The trilocular ovary may contain a maximum of three seeds and generally an average of two seeds per ovary is obtained (Rinehart pers. comm. 1997). Seeds require approximately 5 months to reach maturity (Ewart and Walker 1960). Efficiency of seed production can be up to 88% when crosses are performed between 2n = 28 chromosome plants (Milbocker and Sink 1969b). However, 24% of seeds obtained in this study failed to germinate due to the lack of an embryo or incomplete embryo development. Semeniuk and Stewart (1960) showed

average seed set from several crosses of diploids was 62% when the plants used for crossing were grown at 21°C.

When performing pollinations, Ewart and Walker (1960) emasculated female flowers daily, and applied pollen several times to ensure fertilisation. Bempong (1967) recommends the method of Pai (1960) for self pollinating poinsettias; to hand pollinate several times within a period of three days. Methods to ensure efficient seed production in poinsettias were still the subject of research in 1997 (Craig 1997).

Temperature deviations above or below 21°C affect seed set in poinsettia. The greatest yield of seed in self and cross pollinations of Ruth Ecke and White Ecke (both diploids) was obtained at a constant temperature of 21°C. Of a possible 162 seeds, 101 (62%) were produced at 21°C, 21 (13%) at 26.5°C, 12 (7.5%) at 15.5°C and 1 each (0.6%) at 10°C and 32°C (Semeniuk and Stewart 1960). These authors suggest that normal deviations in temperature in the field could be of sufficient duration and magnitude to reduce seed set severely. They consider the period after fertilization and during early development of the embryo as most sensitive to temperature influences. Several authors have used a temperature of 21°C during seed set studies in poinsettias (Shanks 1980, Stewart 1960, Bempong 1967). All of these studies have shown that seed can be produced under natural shortening day length conditions, however, there are no reports of seed production under artificially applied short days in the available literature.

The phytoplasma (PoiBI) causing branching in poinsettia (Lee *et al.* 1997) may be a hindrance to seed production. Preil (1994b) attributes low fertility and earlier cyathia abortion to infection, but data was not presented.

Relationship between pollen type, compatibility and breeding system

Pollen type similarities between species could indicate taxonomic relatedness and be useful in selection of species for interspecific hybridisation. The behaviour of pollen is crucial to the success of pollination. Pollen types tend to follow general rules of behaviour.

Euphorbia is one of few angiosperm genera that possess species with both bi- (II) and tri- (III) nucleate pollen types (Webster and Rupert 1973). Brewbaker (1967) described four species in the genus *Poinsettia* (prior to its inclusion in *Euphorbia*) as having trinucleate pollen. Sink (1963) reported binucleate pollen in eight commercial poinsettia cultivars. Treatment of *E.*

pulcherrima pollen with the DNA fluorochrome 4',6-diamidino-2-phenyl-indole (DAPI) showed trinucleate pollen, that possessed an autofluorescent exine (Corriveau and Coleman 1988). The differences in pollen cytology need to be considered prior to selecting parental plants for crossing. In addition, pollen type could influence ability to store pollen and the type of incompatibility reaction, if any. Brewbaker (1967) summarised the general physiological differences between bi and tri-nucleate pollen as follows (self incompatibility is abbreviated as S-I).

Pollen cytology	Binucleate	Trinucleate
Viability in vitro	Good	Poor
Storage longevity	Good	Poor
Site of S-I inhibition	Style, ovary	Stigma
Type of S-I control	Gametophytic	Sporophytic

East (1940) refuted a report by Trelease (1897) suggesting *E. pulcherrima* is self-incompatible. Self incompatibility was reported in two *Euphorbia* species, *E. cyparissias* and *E. milli* (= *splendens*) (Webster and Rupert 1973). In the latter, inhibition occurred at the ovary (East 1940, Brewbaker 1957). According to the classification using pollen to ovule ratios (Cruden 1977) poinsettias would be xenogamous (outbreeding).

Interspecific hybridisation for introduction of novel height genes

Wide hybridisation is a valuable breeding tool for introgression of traits from one species or genus to a different receptive species/genus. Two methods of wide hybridisation are possible. Somatic hybridisation has been reported in plants, but its use is limited to plants that exhibit protoplast regeneration from cell culture (Larkin *et al.* 1994). Interspecific pollination is the second option. Interspecific hybridisation (via pollination) with *E. pulcherrima* has been performed on a very limited basis. Pollinations between *E. pulcherrima* and *E. heterophylla* were unsuccessful in producing seed (Sink pers. comm. 1997). Likewise, crossing *E. heterophylla*, *E. variegata* and *E. pulcherrima* did not result in seed set (Percy-Langcaster 1935). Success with interspecific hybridisation often depends upon the relatedness of the species involved (taxonomy), and may specifically include chromosome number, nuclear and cytoplasmic compatibility. To attempt to overcome these problems, interspecific crosses should be performed between a range of genotypes and in both directions, as some combinations may be more compatible than others, or unilateral barriers may be present (Pickersgill 1993). Incompatibilities between the genomes, cytoplasm or embryo and

endosperm may account for F1 non-viability. Some of these factors may be overcome by application of flower and fruit setting hormones such as auxins and gibberellins, as well as embryo culture (Pickersgill 1993).

Cross pollination between two species may result in a variety of outcomes, ranging from a lack of pollen germination through to normal seed production. The placement of pollen, timing of application and environmental factors, are important in determining the success of pollination. When incompatibility barriers are present at this stage, mentor pollen, early pollination, large changes in temperature, cut style pollination, direct pollination of the ovules or ovary or *in vitro* pollination may be used to effect fertilisation (Pickersgill 1993).

Embryo rescue to facilitate development of interspecific F_1 progeny

Sexual interspecific hybridisation methodology is similar to that of sexual intraspecific hybridisation. However, under interspecific hybridisation, developing ovaries often abort prior to the development of mature seeds. Early removal and *in vitro* culture of the developing embryo is often needed to obtain the F_1 hybrid. If the embryo has reached the heart or later stages its chances of successfully reaching maturity under *in vitro* culture are increased as it is larger, has obtained polarization which leads to root and shoot differentiation, and has switched from heterotrophy to autotrophy (Pickersgill 1993). Removal of embryos before this stage is usually difficult, and often results in fatal damage. These globular embryos are undifferentiated and are heterotrophic, relying on the endosperm to provide nutrients. Ovule culture is recommended for very small embryos. The most common cause of abortion of hybrid seeds is due to the failure of normal endosperm development, abnormalities in hybrid embryos occurs less frequently (Pickersgill 1993). Media for embryo rescue range from basal MS (Murashige and Skoog, 1962) types to highly modified recipes, and may need to be changed during the growth of the embryo.

Factors influencing ongoing breeding with F_1 interspecific hybrids

F_1 interspecific hybrids need to be assessed for fertility if further breeding work is to be carried out (e.g. backcrossing or selfing). Recombination between the chromosomes of both species in the hybrid is useful for further breeding. The presence of barriers to recombination often hinders breeding. To overcome this problem chromosome doubling in the F_1 hybrid may be used, or the same method may be applied to the parental species prior to crossing. Colchicine, oryzalin and other chromosome doubling agents may be used to achieve this goal. Only one or two traits may be required from the donor species, requiring only the smallest

possible segment of the donor species to be incorporated, usually through repetitive backcrossing (Thomas 1993). To acquire these traits without other linked deleterious traits in a commercial cultivar is difficult and takes several years (Pickersgill 1993).

Use of mutation breeding for developing poinsettia cultivars and dwarfs

Mutation breeding is effectively the use of plants that have undergone gene mutation, artificially induced or otherwise, for breeding purposes. In vegetatively propagated crops, mutation breeding is recommended when further improvement of an outstanding cultivar is desired. Single or multiple gene mutations may occur, which allows the majority of an outstanding genotype to survive unaltered (Broertjes and van Harten 1988). More than 1,700 induced mutant varieties involving 154 plant species have been officially released, including numerous dwarfs (Maluszynski *et al.* 1995). Many poinsettias have been produced via induced and/or spontaneous mutation (refer to Appendix 5).

Most mutational events behave like recessives, in about 95-99% of cases, mutations appear to convert a dominant allele to recessive (Brock 1978). For these to be recognised, cross or self pollination followed by examination of the F1 and F2 progeny should be undertaken. Irradiation of homozygous dominant genotypes is not recommended, since most heterozygous mutants cannot be recognised in the resulting population (Horn *et al.* 1986). As such, it is desirable that the cultivar or line used has the ability to produce viable self pollinated seed. Poinsettias show a high rate of mutation (Hammer 1997). It is common knowledge amongst poinsettia breeders that the use of mutation breeding facilitates the production of colour series of poinsettias, from an initial red plant. Love (1966) implies that poinsettias have favourable conditions for mutant induction because they exhibit periclinal chimerism, differences in ploidy, heterozygosity and a tendency for chloroplast mutation. Poinsettias have been treated with X-rays as cuttings with roots (Love 1966, Potsch 1966) or as cell cultures (Preil *et al.* 1983, Kleffel *et al.* 1986). Gamma irradiation has also been used on cell cultures (Rugini *et al.* 1993). Derera (1997 pers. comm.) successfully developed a dwarf, variegated poinsettia through gamma irradiation of vegetative cuttings of the cv. Diva.

Proposed poinsettia ideotype

Listed below are a series of characteristics that are considered desirable for a poinsettia cultivar. Ecke (1990) and Gross (1993) consider these characteristics important in a poinsettia cultivar.

- minimal or no requirements for chemical growth retardant application
- early flowering
- heat tolerance and the ability to initiate and develop flowers at temperatures above 21°C
- desirable bract and leaf colours, cyathia number and size
- good post harvest performance (bract, leaf and cyathia retention and low bract fading)
- self-branching
- cyathia and bracts closely packed in the inflorescence
- lack of pollen for reduced incidence of *Botrytis* and reduced post-harvest quality
- ability to withstand being covered with a sleeve and be transported without leaf and bract drooping (epinasty)
- bruise resistant, flexible bracts
- tendency to not prematurely flower
- rapid rooting and good root system development
- resistance to common fungi such as *Botrytis, Pythium, Rhizoctonia* and *Thielaviopsis*
- stockplants that produce suitable numbers of cuttings
- low temperature growth and growth at night temperatures <16°C

These characteristics need to be selected in a breeding program to develop commercially viable dwarf poinsettias.

Chapter 3. Studies on phytoplasma (PoiBI) and viruses (PnMV, PnCV) and their effects on plant height

General introduction

Viruses are known to induce dwarfness in many plant species (Agrios 1997). In poinsettias, the viral 'mosaic disease' caused by poinsettia mosaic virus (PnMV) and poinsettia cryptic virus (PnCV) has been suggested to decrease vigour and yield (Bellardi *et al.* 1995, Bertaccini *et al.* 1996). Poinsettia branch inducing phytoplasma (PoiBI) induces branching in poinsettias (Lee *et al.* 1997), may reduce internode length (Preil 1994b) and decrease plant height (Bech and Rasmussen 1996) under certain growth (non-commercial) conditions. The effect of these infective agents on plant height and morphology, either alone or in specific mixed infection, during commercial production is not reported in the published literature. Therefore, a possible means of inducing dwarfness in poinsettias is to infect plants with viruses and/or phytoplasmas. This chapter reports the results of such investigations conducted in Australia under a commercial growth environment.

Several preliminary investigations were required prior to performing experiments to determine the effects of PnMV, PnCV and PoiBI on plant height. Initially, a survey of a range of current commercially grown poinsettias was conducted to determine if the infective agents are currently present in Australia. In addition, whether cultivars were infected with one, two or all three infective agents was also ascertained. Additional investigations of i. - seed transmissibility ii. - graft transmissibility and iii.- the host range of PnCV, PnMV and PoiBI among species related to poinsettia were performed.

Development of genotypes with different 'infection types' of viruses and phytoplasmas was necessary to undertake these studies. Production of these plants enabled the study of individual and combined effects of PnMV, PoiBI and PnCV on plant height. Plants lacking infection agents were also developed for investigations of plant height to ascertain the true genotypic influence.

The final experiment reported in this chapter investigated the effects of PoiBI infection on endogenous phytohormone concentrations, because PoiBI produced symptoms suggesting alteration of endogenous hormone levels.

Experiment 3.1. Determination of presence and transmission of PnMV, PoiBI and PnCV in current commercial cultivars and related species present in Australia

Introduction

Determination of infection status of PnMV, PoiBI and PnCV prior to implementing infective agent removal techniques for development of 'infection types' is imperative as some cultivars may not be infected with one or more infective agents. Cultivars grown in Australia have been obtained from breeders in other countries, in particular the U.S.A. and Europe. In Australia, PnMV infection of poinsettias has been reported (Guy 1985, Gordon *et al.* 1996), but PoiBI and PnCV have not been recorded to date.

Bech and Rasmussen (1996) reported the presence of PnMV infection in 45 current cultivars obtained from various countries and breeders. Lee *et al.* (1997) reported PnMV in 21 current cultivars grown in the U.S.A.. Van Der Meij (pers. comm. 1999) considers that PnMV is present in virtually all poinsettia cultivars. Therefore, it was expected that PnMV would be present in current cultivars grown commercially in Australia. The 'mosaic disease' is considered to be a mixed infection of PnMV and PnCV (Koenig and Lesemann 1980, Bellardi *et al.* 1995, Bertaccini *et al.* 1996) hence, it is likely that numerous commercial cultivars are infected with PnCV as well, although reports on PnCV infection are limited. Koenig (pers. comm. 2000) suspects most poinsettia cultivars are infected with PnCV.

Lee *et al.* (1997) showed that PoiBI was present in a mixed infection with PnMV in 20 current commercial cultivars exhibiting free-branching available in the U.S.A. (Lee *et al.* 1997). Therefore, it is likely that most plants exhibiting free-branching could be infected with PoiBI, PnMV and possibly PnCV.

This experiment aimed to identify the presence of these infective agents in cultivars/lines grown/sold in Australia and identify their modes of transmission between poinsettias and between poinsettias and related species to enable the future development and maintenance of uninfected stockplants.

Experiment 3.1(a). Survey of Australian poinsettia cultivars for assessment of infection status of PnMV, PoiBI and PnCV

Materials and methods

Growth conditions (standard propagation practices)

Stockplants of germplasm used (Table 3.1.1) were maintained pest and disease free and grown under a long photoperiod by application of supplementary night time lighting (>2 µmol m^{-2} s^{-1} incandescent light) for 4 h from 10 pm. Plants were drip irrigated and 3 - 4 month slow release Osmocote®Plus fertiliser was applied to the potting mix surface at a rate of 4.0 kg/m^3 of potting mix (Appendix 2). Stockplants were apically decapitated regularly, approximately once every four weeks to prevent premature floral initiation.

Apical cuttings, 60 mm in length were removed from stockplants with a sharp knife. Cut ends were dipped for 5 seconds in 1500 mg/l IBA solution prior to insertion into moistened Jiffy ® propagation plugs. Once planted, cuttings were placed under a continuous fog or intermittent water mist for 3 - 4 weeks. Long photoperiod lighting was applied as described previously for stockplants. Mean temperature was maintained between 18°C and 30°C. Cuttings with roots were acclimatised to the greenhouse environment within a week by a gradual reduction in humidity. These cuttings were planted into 150 or 200 mm pots filled with NFP potting mix (Appendix 1). Osmocote®Plus 3 - 4 month slow release fertiliser was applied as described previously at a rate of 4.0 kg/m^3 of potting mix, after planting. Cuttings were allowed to grow into plants under a long photoperiod to maintain vegetative growth.

Plant materials

All plants used in this study are listed in Table 3.1.1. Two plants of each cultivar/line were grown.

Table 3.1.1. Plant material tested for infection status

Cultivar or seedling	Breeder	Year of release	Infective agents tested and test methods				
			PnMV		PoiBI		PnCV
			ELISA	Visual	PCR	Visual	ISEM
Annette Hegg Hot pink	Hegg	1974[2]	✓	✓	n.t.	✓	n.t.
Annette Hegg Top white	Jacobsen	1974[2]	✓	✓	n.t.	✓	n.t.
Flaming sphere	Paul Ecke Ranch	1950[2]	✓	✓	✓	✓	n.t.
Flaming sphere fb‡	Paul Ecke Ranch	1950[2]	✓	✓	n.t.	✓	n.t.
Freedom marble	Paul Ecke Ranch	1993[1]	✓	✓	n.t.	✓	n.t.
Freedom red	Paul Ecke Ranch	1990[1]	✓✓	✓	✓✓	✓	✓✓
Freedom white	Paul Ecke Ranch	1992[1]	✓✓	✓	✓	✓	n.t.
Henrietta Ecke	Paul Ecke Ranch	1927[2]	✓	✓	n.t.	✓	n.t.
Monet	Paul Ecke Ranch	1994[5]	✓	✓	n.t.	✓	n.t.
Pepride	Jacobsen	1994[4]	✓	✓	n.t.	✓	n.t.
Peterstar white	Jacobsen	1989[3]	✓	✓	✓	✓	n.t.
Pink peppermint	Paul Ecke Ranch	1989[2]	✓✓	✓	n.t.	✓	n.t.
Single red	Unknown	Unknown	✓	✓	n.t.	✓	n.t.
Success red	Paul Ecke Ranch	1993[4]	✓	✓	n.t.	✓	n.t.
Supjibi	Gross	1988[2]	✓	✓	n.t.	✓	n.t.
V10 Amy red	Gutbier	1976[2]	✓✓	✓	✓✓	✓	✓✓
V10RxL1rb seedling*	Bernuetz	1995	✓✓	✓	✓✓	✓	n.t.
V10RxL2fb seedling*	Bernuetz	1995	✓✓	✓	✓✓	✓	n.t.

[1]Angus 1996, [2] Ecke *et al.* 1990, [3] Ecke 1996, [4] Hunt 2000, [5] Approximate date, patented in 1994 - U.S.A. Patent and Trademark Office. * Identical genotype produced by author, except rb is restricted branching and fb is free-branching (see 'grafting to transmit PnMV and PoiBI'). ‡ Free-branching form of cv. Flaming Sphere developed by author. n.t.: not tested, ✓: tested once, ✓✓: tested twice after approximate six month interval.

Virus and phytoplasma testing procedures

All plants (Table 3.3.1) were visually screened for PnMV infection by observing mottling symptoms on young leaves produced during growth at 19°C ± 1°C for several weeks. Plants exhibiting mottling symptoms on the recently unfolded leaves were scored as infected. Infection status was also tested by removing three leaves from each plant and subjecting this sample to the ELISA method. ELISA tests were performed according to the Laboratory ETIKET Manual Protocol for PnMV using purified anti-virus immunoglobulins and alkaline-phosphatase conjugates from Agdia, Indiana (Cross pers. comm. 1997). At least one plant was tested per cultivar since prior visual observations at a commercial nursery during winter (June - August) in Australia had indicated that PnMV infection was present in most cultivars. Some plants were tested twice for PnMV via ELISA. These tests were performed at approximate six

by several authors and exhibited visual symptoms. A negative poinsettia control for PnMV was not available.

Two cultivars (Freedom red and V10 Amy red - Table 3.1.1) were tested for PnCV according to the ISEM (Immunosorbent Electron Microscopy) method described by Derrick (1973), with modifications (Srivastava pers. comm. 1999). Three leaves were collected from each plant to be tested and taken to the testing facility (Elizabeth Macarthur Agricultural Institute, Menangle NSW). Purified PnMV specific antibody with no cross reaction with PnMV was obtained (donated by M. Schoenfelder) and diluted 1:500 in 0.1M Sorensen's buffer. Fifteen 1 µl drops of the diluted antibody were placed on a piece of dental wax in a moist Petri dish. Copper grids (400 mesh in size) coated with parlodion/carbon were floated upside down on the diluted antibody for 1 hour at 37°C. Each grid was then washed for 10 minutes in Sorensen's buffer. A leaf sample (10 mm) was ground using a mortar and pestle in 1 ml of Sorensen's buffer and then poured into a 1.5 ml microfuge tube and centrifuged at 2000 rpm for 2 minutes. The supernatant was extracted and fifteen 1 µl drops of this supernatant were placed onto a fresh piece of dental wax in a moist petri dish. Antibody coated grids were placed over the leaf extract drops upside down at 21°C for 1 - 2 hours. Each grid was lifted with tweezers and gently washed with 20 drops of water, followed by two drops of uranyl acetate stain. Grids were blotted dry and examined under a Philips 208 transmission electron microscope. All testing was performed at Elizabeth Macarthur Agricultural Institute, Menangle NSW by Mr. M. Srivastava. Repeated tests were performed at greater than six-month intervals. A positive PnCV control derived from infected poinsettia leaf extract (virus particles) was supplied by M. Schoenfelder. A suitable negative control (derived from poinsettia leaf extract) was not available, because the methods of transmission of PnCV were not known as to date and therefore, all poinsettias need to be excluded. Therefore, the closely related species *E. cornastra*, which was derived via seed and had not been in contact with poinsettias was first tested to ascertain its infection status. Since it was shown to be negative, it was used as a negative control.

Plants from seven cultivars were tested (Table 3.1.1) for PoiBI infection via the PCR procedure described by Schneider *et al.* (1999). Three leaves from each plant to be tested were excised and sent to the testing facility (Northern Territory University, Darwin NT). Two primer pairs, namely fP1 (Deng and Hiruki 1991) and rP7 (Schneider *et al.* 1995) were used to amplify sections of the ribosomal operon including the 16S rRNA gene, spacer region and beginning of the 23S rRNA gene. Restriction enzyme digestion of the product from a sample

of cv. V10 Amy red was performed with *Alu*I and *Rsa*I for restriction fragment length polymorphism (RFLP) analysis. Sequencing of the 16S/23S spacer region from cv. V10 Amy red phytoplasma RNA was also performed and the sequence was compared for similarity to the corresponding western-X sequence (Smart *et al.* 1996). All testing was performed at Northern Territory University by Dr. K. Gibb, Ms. S. De La Rue and Ms. A. Padovan. Some plants were tested twice for PoiBI via PCR. These tests were performed at approximate six month intervals. Visual screening for PoiBI infection was performed by observing free-branching symptoms (Figure 3.1.3). The two seed derived plants V10RxLfb and V10RxLrb were used as positive and negative controls respectively.

Grafting to transmit PoiBI and PnMV

Grafting was conducted to transfer PoiBI and PnMV to one seed derived line and one cultivar. The line used was V10RxL, a seed derived line not infected with PnMV or PoiBI. The cultivar used was Flaming Sphere (infected with PnMV, but not PoiBI). Three graft combinations were made. First, clones of V10RxL were grafted to cv. Single red (infected with PnMV) with restricted branching. Second, clones of V10RxL were grafted to cv. V10 Amy red with free branching (infected with PnMV and PoiBI). Third, clones of cv. Flaming sphere (PnMV infected) were approach grafted to cv. V10 Amy red (infected with PnMV and PoiBI). The approach grafting method required that a vertical section of the stem on both plants to be grafted was cut open (approx. 20 - 30 mm long and deep enough to cut through the cambium). The two cut surfaces from relevant plants were placed facing each other and the graft union was sealed with Parafilm® M laboratory film.

Results

PnMV testing

All cultivars and lines tested were infected with PnMV as determined by ELISA testing (Table 3.1.2). Initial visual appraisal (Figure 3.1.1) was effective 72% of the time in selecting infected plants. The rest (28%) were considered symptomless infections. Controls tested either positive or negative to PnMV as expected.

PnCV testing

The two cultivars tested, Freedom red and V10 Amy red, were infected with PnCV. PnCV infected plants did not show any symptoms, as reported previously (Koenig and Lesemann 1980, Ecke *et al.* 1990). Controls tested either positive or negative to PnCV as expected.

Figure 3.1.1. Symptoms of PnMV infection in poinsettias

Table 3.1.2. PnMV, PoiBI and PnCV test results for cultivar/line

Cultivar or seedling	Infective agent presence/absence and testing technique				
	PnMV		PoiBI		PnCV
	ELISA	Visual	PCR	Visual	ISEM
Annette Hegg Hot pink	+	+	n.t.	+	n.t.
Annette Hegg Top white	+	-	n.t.	+	n.t.
E. cornastra (PnCV -ve control)	n.t.	n.t.	n.t.	n.t.	-
Flaming sphere	+	+	-	-	n.t.
Flaming sphere fb‡	+	+	n.t.	+	n.t.
Freedom marble	+	-	n.t.	+	n.t.
Freedom red (PnMV +ve control)	+,+	+	+,+	+	+,+
Freedom white	+,+	+	+	+	n.t.
Henrietta Ecke	+	+	n.t.	-	n.t.
M. Schoenfelder extract (PnCV +ve control)	n.t.	n.t.	n.t.	n.t.	+
Monet	+	+	n.t.	+	n.t.
Pepride	+	+	n.t.	+	n.t.
Peterstar white	+	+	+	+	n.t.
Pink peppermint	+,+	-	n.t.	+	n.t.
Single red	+	+	n.t.	-	n.t.
Success red	+	-	n.t.	+	n.t.
Supjibi	+	+	n.t.	+	n.t.
V10 Amy red	+,+	+	+,+	+	+,+
V10RxL1rb seedling* (PoiBI -ve control)	+,+	-	-,-	-	n.t.
V10RxL2fb seedling* (PoiBI +ve control)	+,+	+	+,+	+	n.t.

* Identical seedling genotype produced by author, except rb is restricted branching and fb is free-branching. ‡Free-branching form of cv. Flaming sphere developed by author by grafting cv. Flaming sphere to cv. V10 Amy red. n.t.: not tested, + : tested positive, - : tested negative, double + : tested positive twice after approximate six month interval.

PoiBI testing

In the seven cultivars/lines tested, PCR testing results correlated with the visual assessments of infected and non-infected plants. Symptoms of infection were characterised by shoot growth from many or all buds on the main stem of a vegetative cutting. Mature plants were compact with numerous branches, whereas non-infected plants had fewer branches and were taller (Figure 3.1.3). Only seven cultivars/lines were tested via PCR to verify visual symptoms, as testing a large sample was expensive and deemed unnecessary due to obvious symptoms of infection.

RFLP analysis of the cv. V10 Amy red fP1/rP7 PCR product via *Alu*I and *Rsa*I digestion verified that PoiBI was different to other phytoplasmas present in Australia, and had a restriction pattern identical to western-X disease (refer to Schneider *et al.* 1999). Sequencing data revealed the 16S/23S spacer region was identical (100%) to the western-X disease phytoplasma spacer region sequence. Only one phytoplasma genotype was found in cv. V10 Amy red. This may have been due to the nature of the PCR system, where the phytoplasma with the highest titre is usually preferentially amplified (Schneider *et al.* 1999). Controls tested either positive or negative to PoiBI as expected.

Two distinct phenotypes were observed several weeks after grafting V10RxL clones. V10RxL1rb (restricted branching) resulted from grafting to Single red. This plant was indistinguishable from the original clone and repeatedly tested negative to PoiBI (PCR), but positive to PnMV. V10RxL2fb (free-branching) resulted from grafting to free-branching cv. V10 Amy red and exhibited increased branching. This plant repeatedly tested positive to PoiBI (PCR) and PnMV (ELISA). Additionally, cv. Flaming Sphere became free-branching after grafting to cv. V10 Amy red. These results indicated successful transmission of PoiBI and that this phytoplasma, not PnMV, was the cause of branching symptoms and changes in morphology as demonstrated by Lee *et al.* (1997).

Multiple testing (PnCV, PnMV, PoiBI)

Cultivars V10 Amy red and Freedom red were tested for the presence of all three infective agents and both cultivars were infected with all three.

Figure 3.1.2. PnCV particles revealed by ISEM (x 62,500)

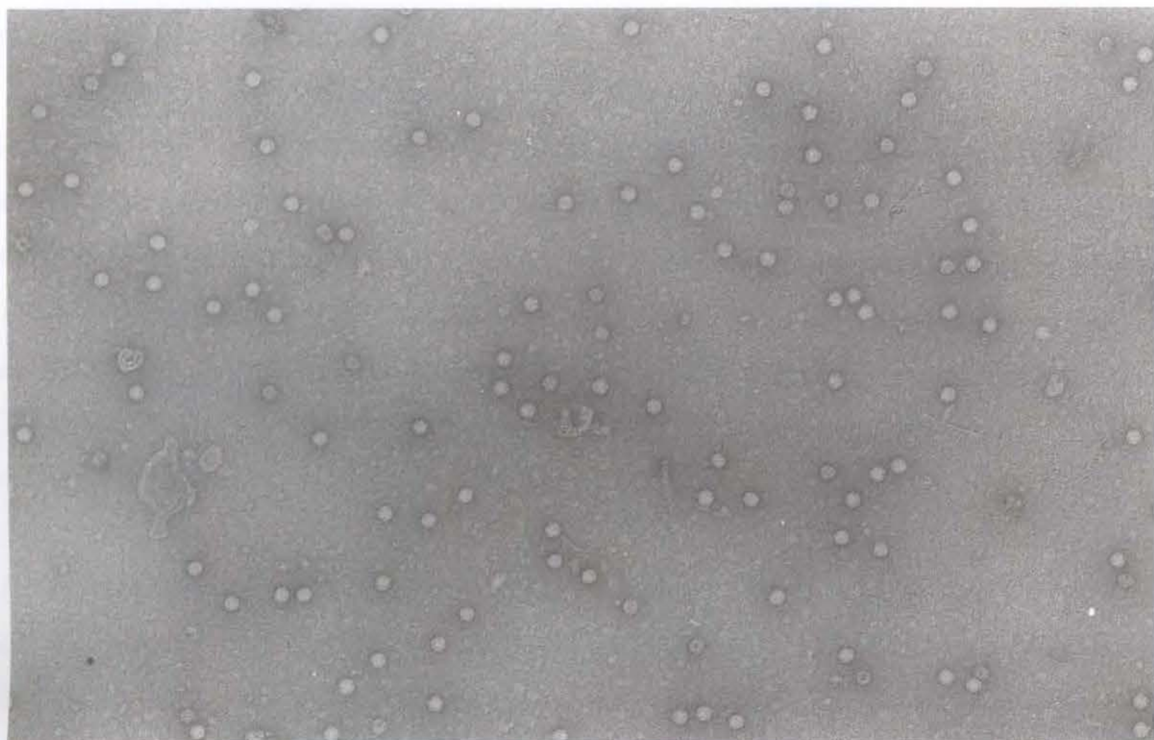


Figure 3.1.3. Symptoms of PoiBI infection in infected, highly branched (right) and non-infected, restricted branching (left) cv. Freedom red stockplants

Discussion

This experiment was performed to determine the PnMV, PoiBI and PnCV infection status of current and older cultivars and several species.

All commercial cultivars tested (current or earlier released) were infected with PnMV as evidenced by ELISA testing. These results are in agreement with other reports, which showed that PnMV is present in many commercial cultivars in other countries (Koenig and Lesemann 1980, Paludan and Begtrup 1986, Bellardi *et al.* 1995, Bech and Rasmussen 1996 and Lee *et al.* 1997). ELISA testing results often concurred with visual symptoms (angular mosaic). Therefore, visual symptoms could be used to identify most infected poinsettia plants (72%) although symptomless infections were present (28%).

All current commercial cultivars tested were shown to be infected with PoiBI either via PCR or via visual symptoms. This is the first report of the presence of this phytoplasma in Australia. The results of some of this testing were presented in a joint paper (Schneider *et al.* 1999). The visual branching symptoms of PoiBI infection were distinct and obvious and therefore led to the use of visual screening rather than PCR due to the efficiency and low cost of visual observation relative to PCR. Older cultivars such as Flaming sphere, Henrietta Ecke and Single red were not infected, but cv. Flaming sphere displayed the typical branching symptoms resulting from PoiBI infection after grafting to cv. V10 Amy red containing PoiBI. The results from grafting to obtain PoiBI also verified the effectiveness of the approach grafting technique.

Lee *et al.* (1997) detected a secondary phytoplasma in their studies, which was found in two from twenty cultivars tested. Abad *et al.* (1997) identified four genotypes present in poinsettia. Bertaccini *et al.* (1996) found more than one phytoplasma present in samples tested, either in association with PnCV and PnMV or with PnCV alone. In this study, only one phytoplasma was found in the cultivar V10 Amy red.

PnCV was present in both of the two commercial cultivars tested (Freedom red and V10 Amy red). This is the first report of occurrence of this virus in Australia.

Experiment 3.1(b). Investigation of transmission of PnMV, PoiBI and PnCV

Materials and methods

i. - Assessment of seed transmission of PnMV, PoiBI and PnCV from infected poinsettia parents to self pollinated progeny

Mature plants of cultivars V10 Amy red and Freedom red, both infected with PnMV, PoiBI and PnCV were grown at 21°C ± 1°C under a 10 h photoperiod with light intensity of 300 $\mu mol\ m^{-2}\ s^{-1}$. To establish if these infective agents were transmissible via seed, manual self-pollinations were performed on receptive stigmas. Seeds produced were collected and sown in a greenhouse into 70 ml tubes filled with NFP potting mix. Lights positioned above the plants provided a long photoperiod (>2 $\mu mol\ m^{-2}\ s^{-1}$ for 4 h from 10 pm) to prevent premature floral initiation. Once seedlings reached the two-leaf stage, they were planted into 200 mm diameter plastic pots and grown according to standard growing practices (Experiment 3.1(a)) in a commercial nursery. Several months after germination the seed derived plants were tested/observed for PnMV (visual), PoiBI (visual) and PnCV (ISEM) infection as described previously in Experiment 3.1(a). Positive controls were plants that tested positive in Experiment 3.1(a). Negative controls were plants that tested negative in Experiment 3.1(a).

Table 3.1.3. Poinsettia seedling plants derived via manual self pollination of two cultivars infected with PnMV, PoiBI and PnCV

Freedom red (+p/+mv/+cv)	V10 Amy red (+p/+mv/+cv)
98-474.1	98-241.1
98-474.2	98-631.1
98-683.1	
99-279.2	
99-279.3	

ii. - Assessment of graft transmission of PnMV, PoiBI and PnCV between cv. V10 Amy red and *Euphorbia cornastra*

To assess and confirm if PnMV, PoiBI and PnCV could be transmitted via grafting, approach grafting was performed (as described in Experiment 3.1(a)) between branches of poinsettia cultivar V10 Amy red and *Euphorbia cornastra*, a species closely related to poinsettia. The cultivar V10 Amy red was infected with PnCV, PnMV and PoiBI (refer to Experiment 3.1(a)), whereas the plant of *E. cornastra* was verified as being non-infected with PnMV (via

ELISA) and PnCV (ISEM). The *E. cornastra* plant was also not considered to host PoiBI infection as it was derived from seeds, which are considered not to carry this infective agent. Therefore, if the product of grafting *E. cornastra* shows infection by any of the infective agents mentioned above then it would be highly likely that they would be graft transmissible. The grafted *E. cornastra* plant was tested for infection with PnMV (ELISA), PoiBI (visual) and PnCV (ISEM) several months after grafting as described in Experiment 3.1(a).

iii. - Assessment of graft transmission of PnMV and PoiBI between cv. V10 Amy red and other related *Euphorbia* species

To ascertain transmission of PnMV and PoiBI between poinsettias and other related species (other than *E. cornastra*), plants of three species were obtained (Table 3.1.4) and grown according to standard practices applicable for poinsettias. All plants shown in Table 3.1.4 were tested for PnMV (both ELISA and visually) and PoiBI (visually) and were considered not infected. Plant materials were sent to appropriate testing personnel and tests were conducted as detailed previously (Experiment 3.1(a)).

Approach grafts were performed, after testing for infective agents, between the species listed in Table 3.1.4 and poinsettia cv. V10 Amy red. All plants were tested for PnMV infection (ELISA) and PoiBI infection (visual) several months after grafting to cv. V10 Amy red.

Table 3.1.4. Euphorbia species used for grafting to cv. V10 Amy red

Species	Initial propagule	PnMV (ELISA)	PoiBI (Visual)
Euphorbia cotinifolia	vegetative clone (cutting from a parent plant)	-	-
Euphorbia fulgens	vegetative clone (cutting from a parent plant)	+	-
Euphorbia leucocephala	vegetative clone (cutting from a parent plant)	-	-

+ : tested positive, - : tested negative.

Results

i. - Assessment of seed transmission of PnMV, PoiBI and PnCV from infected poinsettia parents to self pollinated progeny

Table 3.1.5 presents results of poinsettia seed transmission studies for PnMV, PoiBI (both assessed visually) and PnCV (assessed via ISEM). PnMV and PoiBI did not appear to transmit via seed from infected parents to progeny. Symptoms of infection by each agent was not seen among seedlings derived from infected parents. This result concurs with the

suggestions of Fulton and Fulton (1980), Koenig and Lesemann (1980 for PnMV) and Dole *et al.* (1993 for PoiBI). PnCV was shown to be transmitted (ISEM) to two (one seedling from cv. Freedom red and one from cv. V10 Amy red) out of seven seedlings, although visual symptoms of infection were not present, concurring with previous suggestions (Koenig and Lesemann 1980, Koenig *et al.* 1986, Ecke *et al.* 1990, Koenig pers. comm. 2000, Schoenfelder pers. comm. 1999). Transmission of PnCV via seed has not been previously demonstrated.

Table 3.1.5. Results of assessment of transmission of PnMV, PoiBI and PnCV from infected poinsettia parents to progeny derived via manual self pollination

Seedling derived via manual self pollination	PnMV (visual)	PoiBI (visual)	PnCV (ISEM)
98-474.1	-	-	-,-
98-474.2	-	-	-
98-683.1	-	-	-
99-279.2	-	-	-
99-279.3	-	-	+
98-241.1	-	-	-
98-631.1	-	-	+

+ : tested positive, - : tested negative, double + or - : tested twice after approximate six month interval.

iii. - Assessment of graft transmission of PnMV, PoiBI and PnCV between cv. V10 Amy red and *Euphorbia cornastra*

Grafting *E. cornastra* with cv. V10 Amy red resulted in *E. cornastra* becoming infected with PnMV and PnCV as ascertained by ELISA and ISEM testing respectively.

PnMV infection of *E. cornastra* was recently reported by Floeistad and Blystad (1999). The results of this study concur with their report. PnCV was not previously reported in this species, and these results therefore indicate a possible extension of the host range of this virus to *E. cornastra*. Typical visual symptoms for any virus infection were not observed.

Symptoms of PoiBI infection were present in *E. cornastra*. Plants were characterised by increased branching, reduced stem diameter and shorter internode lengths (Figure 3.1.4), similar to PoiBI infected poinsettia cultivars. Positive and negative controls tested as

expected. Further PCR verification could be used to confirm the visual symptoms. Transmission of PoiBI to species other than *E. pulcherrima* has not been previously reported.

Figure 3.1.4. *E. cornastra* plants displaying (left) non-infected control and (right) changed morphology after approach grafting to cv. V10 Amy red infected with PnCV, PnMV and PoiBI

iv. - Assessment of graft transmission of PnMV and PoiBI between cv. V10 Amy red and other related *Euphorbia* species

None of the three species investigated displayed typical symptoms of PoiBI infection (e.g. increased branching). Therefore, PoiBI was probably not transmitted. PnMV, however, was transmitted to *E. leucocephala,* and E. *fulgens* was infected with PnMV prior to grafting. *E. fulgens* has been reported as a host of PnMV (Meyer 1988, Bartkowski and Meyer-Kahsnitz 1990) and PnCV (Bellardi and Bertaccini 1989). The susceptibility of *E. leucocephala* to PnMV was not previously reported. Confirmation of infection here extends the host range of this virus. All three graft combinations exhibited breakdown of the graft union, perhaps due to graft incompatibility.

Table 3.1.6. Graft transmission of PoiBI and PnMV from infected poinsettia cv. V10 Amy red to *Euphorbia* species

Species grafted to cv. V10 Amy red	PnMV (ELISA)	PoiBI (Visual)
Euphorbia cotinifolia	-	-
Euphorbia fulgens	+	-
Euphorbia leucocephala	+	-

+ : tested positive, - : tested negative.

Discussion

This experiment investigated the transmission of PnMV, PoiBI and PnCV in poinsettia via seed, and PnMV and PoiBI in several species via grafting.

PnCV was shown to be both graft and seed transmissible in this study. This is also the first report of graft transmissibility of this virus. Grafts were performed between infected poinsettias and non-infected *E. cornastra* plants rather than on non-infected poinsettias. This is because infected poinsettias have been shown to possess a very low titre of PnCV particles in comparison to PnMV (Lesemann *et al.* 1983), and poinsettias derived from seeds could be infected due to seed transmission of this virus (Experiment 3.1(a)). Therefore, use of poinsettias could lead to misleading results. Considering that PnCV was transmitted via grafting to a species other than *E. pulcherrima*, namely *E. cornastra*, it is highly likely that it is transmissible within the same species as well. Transmission via grafting is considered unusual for cryptic viruses (Boccardo *et al.* 1987, Ghabrial *et al.* 1998). Therefore, PnCV may not be a 'typical' cryptic virus.

PnMV was found to be graft transmissible, but not seed transmissible as per visual observations of poinsettia leaves for angular mosaic. Previous studies have reported that PnMV is not seed transmissible between poinsettias, therefore this method of transmission was not investigated in depth (e.g. ELISA testing).

This study provides evidence that all three infective agents (PnMV, PoiBI and PnCV) are present in Australia in current commercial cultivars. It is likely that this situation will persist if breeders continue to use PnCV infected stock for pollinations and PnCV, PnMV and PoiBI infected rootstocks for grafting because transmission is possible by these methods. Further

studies are necessary to determine if seed transmission of PnCV occurs in only one direction (i.e. from the male or the female only) or in both directions.

The presence of viruses in ornamental plants is generally considered detrimental. The 'mosaic' disease, which causes decreased vigour and production yield in poinsettias, (Bellardi *et al.* 1995, Bertaccini *et al.* 1996) has been attributed to the presence of PnMV and PnCV. Plants infected with PnCV alone have never been shown to have visually identifiable symptoms (Koenig and Lesemann 1980, Koenig *et al.* 1986, Ecke *et al.* 1990, Koenig pers. comm. 2000, Schoenfelder pers. comm. 1999). Therefore, it is likely that PnMV, the other component of 'mosaic' disease (Bellardi *et al.* 1995, Bertaccini *et al.* 1996) could be the sole agent responsible for the previously described symptoms. Reports of the effects of PnMV on plant morphology are conflicting (no effect - Ecke *et al.* 1990, Bech and Rasmussen 1996, Van Der Meij pers. comm. 1999, some effect - Fulton and Fulton 1980, Paludan and Begtrup 1986, Bellardi *et al.* 1995, Bertaccini *et al.* 1996). Therefore, the effects of PnMV need to be investigated further.

Several authors have shown that PnMV and/or PnCV free poinsettias can be obtained through somatic embryogenesis (Lee *et al.* 1997), meristem culture (Bech and Rasmussen 1996) or heat treatment (Bech and Rasmussen 1996) of infected plants. However, plants carrying PoiBI but not infected with viruses were not reported from these methods. It could be that PoiBI is frequently removed by the same methods that facilitate removal of PnMV and PnCV. PoiBI is essential to the production of free-branching plants that are necessary for increased vegetative cutting production from stockplants and for finished plant form. Lee *et al.* (1997) showed that PnMV free, PoiBI infected cultivars could be constructed by separating PoiBI from PnMV in *Vinca* by the use of a *Cuscuta* sp. as a bridge for transmission. (However, PnCV testing was not conducted - Lee pers. comm. 1998).

Addition of PoiBI to poinsettias could additionally aid in the development of dwarf plants. Therefore, the effects of PoiBI and PnMV on plant height require further investigation. Investigation of PnCV would not be a major priority, although virus free poinsettia cultivars would be of benefit to the poinsettia industry (Bradel *et al.* 2000).

PoiBI is closely related to western-X phytoplasma, which is a serious pathogen of fruit trees. Extensive studies have shown that western-X phytoplasma is not present in Australia (Schneider *et al.* 1999). Because PoiBI shares a close relationship with western-X

phytoplasma, some quarantine authorities may consider PoiBI as a possible threat to the stone fruit industry in this country. However, PoiBI has been present in Australia for over 15 years due to cv. V10 Amy red being grown by at least one nursery for this period (White pers. comm. 1997). Symptoms of PoiBI infection and the presence of PoiBI in other species in Australia have not been reported (except in this thesis where grafting transmitted the infective agent to *E. cornastra* only), indicating that PoiBI has not widely spread. This could be due to lack of suitable insect vectors that are required to transmit the phytoplasma in nature. It may be that such vector(s) are not present in Australia. The transmissibility of PoiBI via insect vectors may also be reduced, because it has been graft transmitted by poinsettia breeders for a long period. Due to the lack of selective pressure for natural progression through the biological cycle, phytoplasmas may have low or no insect vector transmissibility (McCoy *et al.* 1989). Additionally, PoiBI may be specific to *E. pulcherrima* and *E. cornastra* as it did not induce infection symptoms in other *Euphorbia* species that were approach grafted to cv. V10 Amy red.

In addition to the previous studies in poinsettias the infection status of several species, prior to and after grafting to PnMV and PoiBI infected cv. V10 Amy red was investigated. These studies revealed a new host for PnMV, *E. leucocephala*. A new host was found for PnCV, *E. cornastra*. *E. cornastra* showed symptoms of PoiBI infection and also became infected with PnMV. Verification of PoiBI presence via molecular methods could be performed to confirm the visual symptoms in this species.

As an additional note, a seedling derived plant of *Euphorbia lathyrus* was found to be infected with PnMV (via ELISA) after grafting to cv. V10 Amy red.

Experiment 3.2. Production of virus (PnMV, PnCV) and phytoplasma (PoiBI) – free poinsettias

Introduction

Studies conducted in Experiment 3.1 showed that commercially available poinsettias could be infected with PoiBI, PnMV and PnCV. Both PoiBI and PnMV infection can be detected by visual observation or via PCR and ELISA respectively. However, PnCV infection is symptomless and several authors have reported no effects of this virus on plant morphology (Koenig and Lesemann 1980, Koenig *et al.* 1986, Ecke *et al.* 1990, Koenig pers. comm. 2000, Schoenfelder pers. comm. 1999). Therefore, it was assumed that its influence on plant height is almost non-existent. In addition, low titre of PnCV coupled with symptomless infection would not lend itself to easy and cost effective testing. Due to these factors, PnCV was not assigned a top priority in this study.

This experiment was designed to produce plants devoid of PoiBI and/or PnMV and if possible PnCV as well, thus creating different infection types. Absence of all infective agents was also considered as a 'type'. PnCV infection was tested last and only in selected plants whose titre could be increased over time to enable conclusive detection.

Table 3.2.1. Possible infection types of PoiBI and PnMV

PoiBI	PnMV	Description	Code based on infection type
-	-	PnMV and PoiBI free	-p/-mv
+	-	PoiBI infected, PnMV free	+p/-mv
-	+	PnMV infected, PoiBI free	-p/+mv
+	+	Both PoiBI and PnMV infected*	+p/+mv

* Highly prevalent in commercial cultivars. + : tested positive, - : tested negative.

Meristem culture (Bech and Rasmussen 1996), somatic embryogenesis (Preil *et al.* 1982, Lee *et al.* 1997) and heat treatment (Bech and Rasmussen 1996) have been demonstrated to remove all three infective agents in poinsettia.

The aims of this experiment were i. - to produce all four infection types of PnMV and PoiBI in at least one cultivar and ii. - develop infection free plants of several cultivars to quantify 'true' height as expressed by contributing gene/s without the intervention of PnMV or PoiBI.

Materials and methods

Donor plant growth conditions and experimental overview

Cultivar stockplants (Table 3.2.2) were grown according to standard commercial practices, and maintained insect and disease free. These were the source material for *in vitro* culture. Ten cultivars, six infective agent removal techniques and three initiation media were utilised and are described in Table 3.2.2. Media components are presented in Table 3.2.3.

Sterilisation of explant materials

Shoot tips (approximately 50 mm long) were harvested from stockplants and defoliated in the greenhouse. These shoot tips were transported to the laboratory for meristem culture and micropropagation. Cyathia were collected prior to anthesis and ovaries were collected approximately 2 weeks after anthesis. Plant materials were placed in air-tight containers with 3% NaOCL (sodium hypochlorite) solution. A drop of Tween 20 was added as a surfactant and the container was sealed and shaken vigorously for 10 seconds approximately once every minute for a total of 10 minutes. Plant materials were then rinsed three times with sterile distilled water, and allowed to remain in the final wash until cultured, to prevent dehydration.

Media selection and *in vitro* growth conditions

Different media (Table 3.2.3) were selected based either on the author's prior experience or on published previous work (Roest and Bokelman 1980, Lee *et al.* 1997). Explants were cultured on the most appropriate known media to obtain the best possible culture growth and development. The culture procedures used for each type of explant are explained later. Growth room temperature was maintained at 25°C ± 2°C. Crompton® 40W RS White fluorescent lights were used to provide a light intensity of approximately 60 - 70 $\mu mol\ m^{-2}\ s^{-1}$ at culture container lid level for 16 h/day.

Table 3.2.2. Cultivars, source of explants for infective agent removal, original infection types, infective agent removal techniques used, initiation media and number of propagules used for the removal of PoiBI, PnMV and PnCV

Cultivar	Source of explants for infective agent removal	Original Infection type§	Infective agent removal technique used	Initiation media‡	No. of propagules
Lemon drop	greenhouse	+p/+mv/n.t.	cyathia culture	MP	5
Supjibi	greenhouse	+p/+mv/n.t.	cyathia culture	AS	3
V10 Amy red	greenhouse	+p/+mv/+cv	cyathia culture	MP	7
V10 Amy red†	greenhouse	+p/+mv/+cv	heat treatment	n.a.	n.a.
Angelika	*in vitro*	n.t./n.t./n.t.	meristem culture	AS	7
Diva	greenhouse	+p/+mv/n.t.	meristem culture	AS	3
Freedom red	*in vitro*	+p/+mv/+cv	meristem culture	AS	10
Freedom white	*in vitro*	+p/+mv/n.t.	meristem culture	AS	3
Lemon drop	*in vitro*	+p/+mv/n.t.	meristem culture	AS	5
Pepride	greenhouse	+p/+mv/n.t.	meristem culture	AS	8
Peterstar white	greenhouse	+p/+mv/n.t.	meristem culture	AS	7
Freedom red	*in vitro*	+p/+mv/+cv	micropropagation	MP	10
Freedom white	*in vitro*	+p/+mv/n.t.	micropropagation	MP	10
Lemon drop	*in vitro*	+p/+mv/n.t.	micropropagation	MP	10
Pink peppermint	*in vitro*	+p/+mv/n.t.	micropropagation	MP	10
Freedom white	greenhouse	+p/+mv/n.t.	ovary wall culture	AS	4
Pink peppermint	greenhouse	+p/+mv/n.t.	ovule culture	SE	7

+p/+mv/+cv = infected with PoiBI, PnMV and PnCV. n.t.: not tested. ‡ Refer to Table 3.2.3 for media descriptions.

§ Infection type determined as described in Experiment 3.1. † Provided by G. Guy. n.a. : not applicable.

Explant initiation and continued growth *in vitro*

Shoot tips

These were used as the source material for micropropagation. Approximately 5 - 10 mm of the shoot tip apex was excised from each stockplant and placed upright into MP medium. The medium was contained in plastic Bunzl® jars with a height of 80 mm and width of 68 mm. Shoots and multi-branched shoot clumps were produced 4 weeks after the start of culture. These shoots were then cut into approximately 10 - 30 mm length pieces and subcultured onto fresh media of the same composition. Subculturing was performed once every 3 - 6 weeks.

Table 3.2.3. ***In vitro*** **culture media composition**

Media type	Use and reference	Components of media‡
MP	Shoot multiplication media (Bernuetz)	4.42g/l MS salts†, 3% sucrose, 0.7% agar, pH 5.8 1g/l casein hydrolysate, 0.3 mg/l 6-benzylaminopurine
SE	Somatic embryogenesis media (Lee *et al.* 1997)	4.42g/l MS salts†, 2% sucrose, 0.7% agar, pH 5.8 1g/l casein hydrolysate, 0.4 mg/l 6-benzylaminopurine, 0.8 mg/l 1-napthaleneacetic acid
AS	Adventitious shoot media (Roest and Bokelman 1980)	4.42g/l MS salts†, 3% sucrose, 0.7% agar, pH 5.8 2g/l myo-inositol, 1.0 mg/l 6-benzylaminopurine, 0.1 mg/l 1-napthaleneacetic acid

‡ Media were adjusted to designated pH prior to autoclaving at $1 kg/cm^2$ at 121°C for 17 minutes.

† Murashige and Skoog (1962) obtained from Sigma-Aldrich Pty. Ltd., cat. no. M5519.

Apical meristems.

Apical meristems (approx. 0.5 - 1 mm) were dissected from shoot tips (harvested either from stockplants in the greenhouse or *in vitro)* and placed with their cut side facing down onto AS medium. Approximately 4 weeks later, meristems that regenerated shoots were transferred to MP medium. If any shoots were present greater than 10 mm long, then they were excised and placed separately into MP medium. Subculturing was performed once every 3 - 4 weeks.

Cyathia.

Each cyathium was cut into four longitudinal sections and placed onto either AS or MP medium. Cyathia with regenerating shoots and internal cyathial structures were dissected 3 - 4 weeks after the start of culture and subcultured onto MP medium. Subculturing was performed several times, every 3 - 4 weeks.

Ovaries and ovules.

Ovules derived from unfertilised ovaries were longitudinally dissected and placed onto SE medium. Explants showing either callus regeneration or embryogenic growth were excised and subcultured onto the same media. Somatic embryos that developed were subcultured onto MP medium after development of the cotyledons. Ovary wall sections were placed onto AS medium. Adventitious shoots developing from the wall tissue were subcultured onto MP medium. Subsequent subculturing occurred at 3 - 4 week intervals onto the same media.

Growth conditions of regenerants in the greenhouse

Regenerated small shoots of approximately 20 - 40 mm length were excised from *in vitro* culture as they developed over an extended period of greater than 8 weeks. Primary shoots were selected and axillary branches were avoided. Basal cut ends were treated with a liquid solution containing 2000 mg/l IBA by rapidly dipping, prior to insertion into Jiffy® propagation plugs. Fongarid® fungicide was applied at weekly intervals commencing one week from the date of propagation. Moist conditions were maintained by continuous generation of water fog using an ultrasonic fog generator (Uni Fogger™). Temperature was maintained at 25°C ± 2°C and an artificial long photoperiod was provided via incandescent lights between 10 pm and 2 am each day. A light intensity of approximately 120 $\mu mol\ m^{-2}\ s^{-1}$ was recorded at noon. The shoot cuttings developed roots, which protruded from the sides of the propagation plug within 3 - 5 weeks. Plants were gradually acclimatised to the greenhouse environment over a 3 - 7 day period by gradual reduction in humidity. Shoot cuttings with roots were then planted into plastic pots and treated in an identical manner to cuttings derived vegetatively from stockplants (refer to Experiment 3.1).

Heat treatment

Heat treatment was performed by G. Guy (Pathologist, F & I Baguley Flower Growers, Victoria). Plants of cv. V10 Amy red were placed inside a heat treatment cabinet maintained between 33°C and 37°C. Small (approximately 30 - 40 mm) apical stem cuttings were periodically removed from these plants and propagated after three weeks from commencement of treatment. Plants were generated from the heat-treated cuttings.

Virus and phytoplasma testing procedures

PnMV

Plants generated via all procedures were tested for PnMV (Table 3.2.4). PnMV testing was performed initially by visual observation of mottling symptoms on leaves. Plants displaying mottling symptoms were considered as infected and were not re-tested. All plants without obvious mottling symptoms were tested using ELISA, with tests repeated at 5 - 6 month intervals (as described in Experiment 3.1).

PoiBI

Visual symptoms of infection were used to screen for presence or absence of PoiBI. Several plants were also tested for PoiBI via a modification of the PCR protocol detailed in Experiment 3.1 (Schneider *et al.* 1999). Modifications included the use of fP1 and Wxint

(Smart *et al.* 1996) primer pairs, which are western-X phytoplasma specific primers and the use of 35 PCR cycles rather than 30 as suggested by Smart *et al.* (1996). This protocol modification increased sensitivity, and identified only those phytoplasmas specific to the western-X group. The fP1/WXint DNA products (approx. 1600bp) derived from two samples were subjected to RFLP testing using *Hpa*II, *Mse*I and *Rsa*I restriction enzymes, to determine similarity to other phytoplasmas within the group (i.e. green valley-X) and to identify any closely related phytoplasmas, if present. PCR techniques were performed by K. Gibb and S. De La Rue at the Northern Territory University, Australia. All plants were visually screened for PoiBI numerous times (approximately weekly) during the following year.

PnCV

Eight plants were tested for PnCV according to the ISEM method described in Experiment 3.1. The interval between repeated tests (upto a maximum of three tests) was approximately 6 months. PnCV testing was performed last, to allow the viral concentration to increase in plants over the longest possible time. *E. cornastra* was utilised as a negative control as in Experiment 3.1.

Results

Shoot regeneration

Shoots regenerated from explants such as meristems, micropropagated shoot tips, cyathia and ovules are shown in Figures 3.2.1 and 3.2.2. Shoots regenerating from meristems and micropropagated shoot tips appeared to develop from pre-formed nodes. Some obvious adventitious shoots originating from cyathia and ovary wall tissue were also noted. Shoots regenerated from ovules were derived via the development and germination of somatic embryos. All shoots underwent axillary branching when cut and placed on MP medium.

Micropropagation led to an approximate doubling of the initial shoot number within 4 weeks. The time from the initiation to the production of the first few (3 - 6) shoots via meristem culture was between 5 - 6 months. The other three *in vitro* methods (cyathia, ovary wall and ovule culture) were slower to produce the first few shoots.

Harvested *in vitro* shoots and cuttings from heat-treated material developed roots within 2 - 4 weeks and grew rapidly into plants once planted in the greenhouse.

Figure 3.2.1. Shoot regeneration from meristems (top) and cyathia (bottom)

Figure 3.2.2. Shoot regeneration from shoot tips (top) and ovules (bottom)

Results of virus and phytoplasma testing of plants developed through infective agent removal techniques

The various infection types of PnMV and PoiBI developed from each infective agent removal technique are presented in Table 3.2.4. Harvesting of plants from *in vitro* culture ceased once sufficient PoiBI and PnMV infection types were produced. One hundred and nineteen plants were produced and tested.

Table 3.2.4. Number of plants produced of each infection type from various infective agent removal techniques

		Number of plants of each infection type produced†			
Cultivar	Infective agent removal technique used	-p/-mv	-p/+mv	+p/-mv	+p/+mv
Lemon drop	cyathia culture		5		
Supjibi	cyathia culture	8			
V10 Amy red	cyathia culture	1	6	1	1
V10 Amy red	heat treatment	3	9		1‡
Angelika	meristem culture	8	3		
Diva	meristem culture		2		
Freedom red	meristem culture	1	9		
Freedom white	meristem culture	8			
Lemon drop	meristem culture	6			
Pepride	meristem culture		9		
Peterstar white	meristem culture	3	5		
Freedom red	micropropagation			1	5
Freedom white	micropropagation		2	1	3
Lemon drop	micropropagation		1		2
Pink peppermint	micropropagation				1
Freedom white	ovary wall culture	11	1		
Pink peppermint	ovule (somatic embryos)	2			

† Infection type abbreviations, +: tested positive, -: tested negative, p = PoiBI, mv = PnMV. ‡ Positive Control (+p/+mv).

PnMV

From the 119 plants tested for PnMV, 23 were visually identified as infected. The remaining 96 were tested via ELISA. ELISA testing revealed 42 more plants with confirmed presence of PnMV. Therefore 65 of the 119 plants developed were infected with PnMV.

PoiBI

Infection with this agent was noted visually in planted cuttings approximately two weeks after planting. Infected plants displayed bud swelling and shoot growth from axillary buds. Sixteen plants were identified as infected via visual observations. Plants without infection symptoms after approximately four weeks never developed branching symptoms. In addition, visual screening of several infected and non-infected plants was validated with PCR testing in all cases.

Heat treatment and all *in vitro* culture methods resulted in removal of PoiBI. Two plants (Freedom red via micropropagation and V10 Amy red from cyathia culture) were generated with PoiBI symptoms, but without PnMV infection. PoiBI presence in these plants was verified via PCR. RFLP testing of the P1/WXint product (approximately 1600bp) identified only one phytoplasma genotype, closely related to green valley-X (Figure 3.2.3). Controls tested as expected.

Figure 3.2.3. RFLP analysis of P1/WXint PCR amplified products digested with *Hpa*II, *Mse*I and *Rsa*I restriction enzymes from cultivars Freedom red (1056) and V10 Amy red (1057 and 450) infected with PoiBI. GVX: green valley-X disease. Marker = 1 kb ladder

PnCV

A total of eight plants were tested for PnCV presence and all were infected. Four of these eight plants were derived from cv. Freedom red. Each plant was a separate infection type of PnMV and PoiBI i.e. one plant each of -p/-mv, -p/+mv, +p/-mv and +p/+mv. The remaining four plants were each a separate infection type PnMV and PoiBI of cv. V10 Amy red, as described previously for cv. Freedom red. Positive and negative controls tested as expected.

Production of PnMV and PoiBI free (-p/-mv) plants

Plants of eight cultivars (Table 3.2.4) were produced devoid of PnMV and PoiBI (-p/-mv) infection. These plants were obtained via all treatments except micropropagation. A total of 51 plants were obtained.

Production of plants with PnMV (-p/+mv) only

These plants were easily produced from most treatments, including micropropagation. A total of 52 plants were produced of this infection type.

Production of plants with PoiBI (+p/-mv) only

Three plants, each from a different cultivar, were developed possessing PoiBI only. These plants did not contain PnMV.

Production of plants infected with PnMV and PoiBI (similar to original parental stock, +p/+mv)

Only two treatments (micropropagation and cyathia culture) resulted in plants with unchanged infection status. A total of 13 plants were produced.

Plant selections for further studies

Plants were selected for further studies based upon repeated infective agent test results (Table 3.2.5). Two cultivars (Freedom red and V10 Amy red) possessed the four possible infection types, and cuttings from these original mother plants were used in future experiments (Experiments 3.3, 3.4 and 4.4). Three other cultivars (Supjibi, Freedom white and Lemon drop) were obtained with the -p/-mv infection type. These plants were used as mother stock to produce cuttings used in Experiment 4.4.

Table 3.2.5. List of plants selected for further studies and their infection status

Cultivar and plant accession number	Infective agent removal technique	Code for infection type‡	PoiBI (PCR)	PoiBI (visual)	PnMV (ELISA)†	PnMV (visual)†	PnCV (ISEM)†
Freedom red 7	meristem	-p/-mv/+cv	n.t.	-	-,-,-	-,-,-	+,+,+
Freedom red 5	micropropagation	+p/-mv/+cv	+	+	-,-	-,-,-	+,+
Freedom red 3	meristem	-p/+mv/+cv	n.t.	-	+,+	-,+	+,+
Freedom red 8	micropropagation	+p/+mv/+cv	n.t.	+	-,+,+	-,+	+
V10 Amy red 2	heat treatment	-p/-mv/+cv	n.t.	-	-,-,-	-,-,-	+,+
V10 Amy red 10	cyathia	+p/-mv/+cv	+	+	-,-,-,-	-,-,-	+,+
V10 Amy red 4	heat treatment	-p/+mv/+cv	n.t.	-	+,+	-,+	+
V10 Amy red 6	cyathia	+p/+mv/+cv	+	+	+,+	+,+	+
Supjibi 1	cyathia	-p/-mv/n.t.	n.t.	-	-,-	-,-	n.t.
Supjibi 3	cyathia	-p/mv-/n.t.	n.t.	-	-,-	-,-	n.t.
Peterstar white 3	meristem	-p/+mv/n.t.	n.t.	-	+	+,+	n.t.
Pepride 1	meristem	-p/+mv/n.t.	n.t.	-	+	+,+	n.t.
Freedom white 7	ovary wall	-p/-mv/n.t.	n.t.	-	-,-	-,-	n.t.
Freedom white 9	ovary wall	-p/-mv/n.t.	n.t.	-	-,-	-,-	n.t.
Freedom white 1	meristem	-p/-mv/n.t.	n.t.	-	-,-	-,-	n.t.
Lemon drop 1	meristem	-p/-mv/n.t.	n.t.	-	-,-	-,-	n.t.
Lemon drop 2	meristem	-p/-mv/n.t.	n.t.	-	-,-	-,-	n.t.

+ = tested positive, - = tested negative, n.t.: not tested, p = PoiBI, mv = PnMV, cv = PnCV. † Repeated scores (+ or -) indicates the results for number of tests repeated at approximate 6 month intervals.

Discussion

The aims of this experiment were achieved as all four infection types were produced in at least one cultivar and -p/-mv plant(s) of five additional cultivars were developed. Two cultivars (Freedom red and V10 Amy red) were developed with all four desired infection types (-p/-mv, -p/+mv, +p/-mv, +p/+mv). In this study, PnCV infection of these plants was confirmed and therefore plants designated -p/-mv could not be deemed 'virus free' in a strict sense.

Plants infected with PnCV and PnMV appeared morphologically identical to plants of the same genotype infected with PnCV alone. The angular mosaic produced on leaves (due to PnMV infection) when plants were grown at <20°C was the only distinguishing feature between these plant types.

It is desirable to produce a source of plant material containing PoiBI alone (lacking PnMV and PnCV) for the development of virus-free 'free-branching' lines (Bradel *et al.* 2000). These lines could lack the deleterious effects attributed to virus infection, such as leaf mosaic, reduced vigour (Bellardi *et al.* 1995, Bertaccini *et al.* 1996) and floral and leaf deformations (Fulton and Fulton 1980, Paludan and Begtrup 1986). However, these symptoms could be due to PnMV infection alone. Poinsettias lacking PnMV, but possessing free-branching symptoms have been reported (Pfannenstiel *et al.* 1982, Bertaccini *et al.* 1996 and Lee *et al.* 1997). The two former authors identified these plants while selecting among a commercial population. Pfannensteil *et al.* (1982) did not test for PnCV however, Bertaccini *et al.* (1996) confirmed that PnCV was present in their plants. Lee *et al.* (1997) produced plants containing PoiBI without PnMV. However, they did not test for PnCV (Lee pers. comm. 1998). Production of a 'pure source' of PoiBI (lacking PnMV and PnCV) was not accomplished in this study, although two sources of PoiBI infection without PnMV were produced. Therefore, there is still no published account of the production of virus-free but PoiBI infected poinsettia(s).

To produce plants infected with PoiBI, but free of PnCV and PnMV, four possible methods can be suggested. These could be conducted in the future and are as follows. i. - apply infective agent removal techniques to +p/-mv/+cv plants already developed and test plants produced for PnCV infection, provided they possess PoiBI infection symptoms, ii. - increase the size of the population derived from infective agent removal experiments using the original infected material, iii. - find a host that permits infection with PoiBI but not PnMV or PnCV or iv. - establish a means to *in vitro* culture PoiBI, whereby 'clean' plants (e.g. some seed derived plants) can be artificially infected, for example, via injection of the PoiBI culture.

The first two suggestions could allow for the production of +p/-mv/-cv poinsettia plants. The first option would be most efficient of these two options because PnMV is already removed. The latter two suggestions (iii and iv) can only produce a 'source' of virus-free PoiBI for future transmission into non-infected seedlings.

A suitable alternative host for PoiBI (but not PnMV and PnCV) could be *Vinca* sp. Lee *et al.* (1997) demonstrated that *Vinca* is not a host of PnMV, but will host PoiBI. However, whether *Vinca* is host to PnCV is yet to be determined. *In vitro* culture of PoiBI itself may allow the separation of PnMV and PnCV, however, reports of phytoplasma culture were not found in the available literature.

This study reports the first evidence of the development of somatic embryos from ovules, and adventitious shoots from cyathia and ovary wall material in poinsettias. Further studies are needed to determine the repeatability of these results.

The plants obtained from this experiment provide the required mother stock for further studies on the effects of PnMV and PoiBI on plant height during commercial production. Additionally, the effects of PoiBI on endogenous hormone levels could now be ascertained in future experiments, as the required negative controls (PoiBI-free plants) are now available.

Experiment 3.3. Assessment of the effects of poinsettia branch inducing phytoplasma (PoiBI) and poinsettia mosaic virus (PnMV) on height and morphology during commercial production of cv. V10 Amy red

Introduction

The infective agents PnMV and PoiBI are known to cause modification of the morphology of poinsettia cultivars. However, PnCV is thought to be symptomless due to lack of visually observable symptoms. The effects of PoiBI and PnMV on height *per se* and the morphology of poinsettia cultivars are not quantified in the available literature, especially under a commercial production environment. Plants of cv. V10 Amy red carrying different 'infection types' of PnMV and PoiBI were developed in Experiment 3.2 (Table 3.2.5). Each 'infection type' represents the presence/absence of PoiBI and PnMV either alone or in combination. Plants with no detected infection agent were also considered as an 'infection type'. These plants were used to further study the effects of infection type on height and other morphological characteristics in this cultivar.

Cultivar V10 Amy red was released in 1976 (Ecke *et al.* 1990) and was grown in Australia prior to 1985 (White pers. comm. 1997). This cultivar is short and has prolific branching. Cuttings were harvested from cv. V10 Amy red stockplants carrying each of the four infection types and grown according to commercially accepted practices. Measurements were recorded during each production phase such as planting, before and after apical decapitation, transition to short photoperiod and date of sale to determine the effects of infection type on height and morphology.

Materials and methods

Plant materials

Infection types of PnMV and PoiBI (Table 3.3.1) were verified for up to two years using methods stated in Experiment 3.2. All plants were infected with the symptomless PnCV.

Table 3.3.1. PnMV and PoiBI infection types of cv. V10 Amy red, with code denoting presence/absence of infective agent

PoiBI	PnMV	Description	Code for infection types
-	-	PnMV and PoiBI free	-p/-mv
-	+	PnMV infected, PoiBI free	-p/+mv
+	-	PoiBI infected, PnMV free	+p/-mv
+	+	Both PoiBI and PnMV infected†	+p/+mv

†Common in commercial cultivars, refer to Experiment 3.1(a). p = PoiBI, mv = PnMV.

Experimental protocol

The following protocol utilised from initiation of stockplants through to floral anthesis is termed 'standard production practice'. This protocol is referred to in all future experiments and is summarised in Table 3.3.2.

Table 3.3.2. Plant developmental stages, number of days from commencement of experiment and temperatures recorded at each stage

			Temperature (°C)	
Stages of plant development	Stage code	Day‡	Min.	Max.
Commence propagation	0	0		
Planting	1	25	16	36
Pre-apical decapitation	2	43	18	29
Post-apical decapitation	3	43	18	29
Start of short photoperiod	4	65	18	29
Final scoring	5	121	18	29

‡ Experiment conducted from February (late summer) - June (early winter).

Stockplant management

Two to four stockplants were developed from cuttings harvested from parental plants of each infection type (Table 3.2.5 lists original plants of cv. V10 Amy red). Conventional propagation and growing procedures were used. Stockplants were regularly apically decapitated to prevent premature flower initiation and to encourage branching. Utensils used for this procedure (and subsequent procedures where contamination could be a possibility) were cleaned using 80% alcohol and wiped with a disposable towel after each decapitation, to avoid any possibility of infective agent transmission. Osmocote®Plus, a 3 - 4 month slow release fertiliser, was applied as a top dressing at the recommended rate (Appendix 2) and plants were manually watered as required. Stockplants were placed approximately 750 mm

apart as measured from pot centres. Contact between plants of different infection types was prevented by this increased spacing. A long photoperiod was provided by using incandescent lights (>2 μmol m^{-2} s^{-1}) between 10pm and 2am. Temperature range during stockplant growth was from 17°C to 36°C. Apical stem decapitation ceased 6 weeks prior to collection of cuttings.

Stage 0. Vegetative propagation

Cuttings of 60 mm length were removed from healthy stockplants with a knife during the early morning. Cuttings were then wrapped in sheets of moist newspaper and transported to the propagation area of a commercial nursery. The cut ends were dipped into 2000 mg/l IBA solution and inserted into expanded Jiffy® propagation plugs (from here on termed 'plugs') placed in a 48 cell tray. Up to twelve cuttings of the same infection type were evenly spaced in each tray and placed in a greenhouse. Excess leaves were removed with a knife to standardise leaf area. Leaves were pushed down to reveal apices of cuttings. Lighting was provided to prevent flowering for 4 h from 10 pm (>2 μmol m^{-2} s^{-1}) by incandescent lights hung above the crop. Greenhouse temperature ranged from 16 - 36°C. Water misting was adjusted as required and gradually reduced during the last week of propagation to facilitate acclimatisation.

Stage 1. Planting

The propagated materials produced roots and were ready for planting after 26 days. Figure 3.3.2 displays several stages of the experiment. Prior to planting, the following measurements were recorded.

- Number of roots protruding outside the plug (score of 1 = 5 or more roots, 2 = 1 - 4 roots, 3 = 0 roots)
- Height (mm) from the top of the plug to the tip of the shoot
- Number of nodes along the stem, visible from the top of the plug to the most apical node attached to a leaf >30 mm in length
- Number of leaves from the top of the plug to the most apical leaf >30 mm in length
- Stem diameter (mm) 10 mm from the top of the propagation plug
- Mean internode length (H/N) was determined by dividing height (mm) by the number of nodes

Cuttings (along with plugs) were planted at the centre of pots (with a diameter and height of 150 mm) ensuring that the top of the plug was level with the top of the potting mix in the pots. Potting mix was 5-10 mm below the pot rim. Each pot was individually tagged and randomly placed within the experimental area (completely random design). The distance between pot centres was 350 mm. Plants were watered manually as required for the first two weeks. Subsequently, a calibrated dripper system was installed, and thereafter all plants were provided with the same volume of water at irrigation. Osmocote®Plus 3 - 4 month duration controlled release fertiliser was evenly applied one week after planting to the potting mix surface of each pot at a rate of 4.0 kg/m^3 of potting mix. A long photoperiod (4 h from 10 pm) was provided as described in vegetative propagation.

Stage 2. Pre-apical stem decapitation

Just prior to apical decapitation at 44 days post-propagation, all parameters that were measured at the commencement of planting were measured again, excluding root quantity. Additionally, the number of branches longer than 10 mm was also recorded.

Stage 3. Post-apical stem decapitation

After recording measurements, (stage 2 - see previous paragraph) plants were apically decapitated with a knife, leaving six nodes on the stem above the top of the plug. At least nine nodes were present on every plant prior to decapitation. Immediately after decapitation, the height from the top of the plug to the tip of the plant was measured.

Stage 4. Transfer to short photoperiod environment

Sixty six days from the date of propagation, plants were measured again for various parameters as at pre-apical decapitation. Following measurements, plants were transported to an area with an artificial short photoperiod of 10 h (7 am to 5 pm) to induce flowering. The randomisation pattern, pot spacing and irrigation network were as described for stage 1.

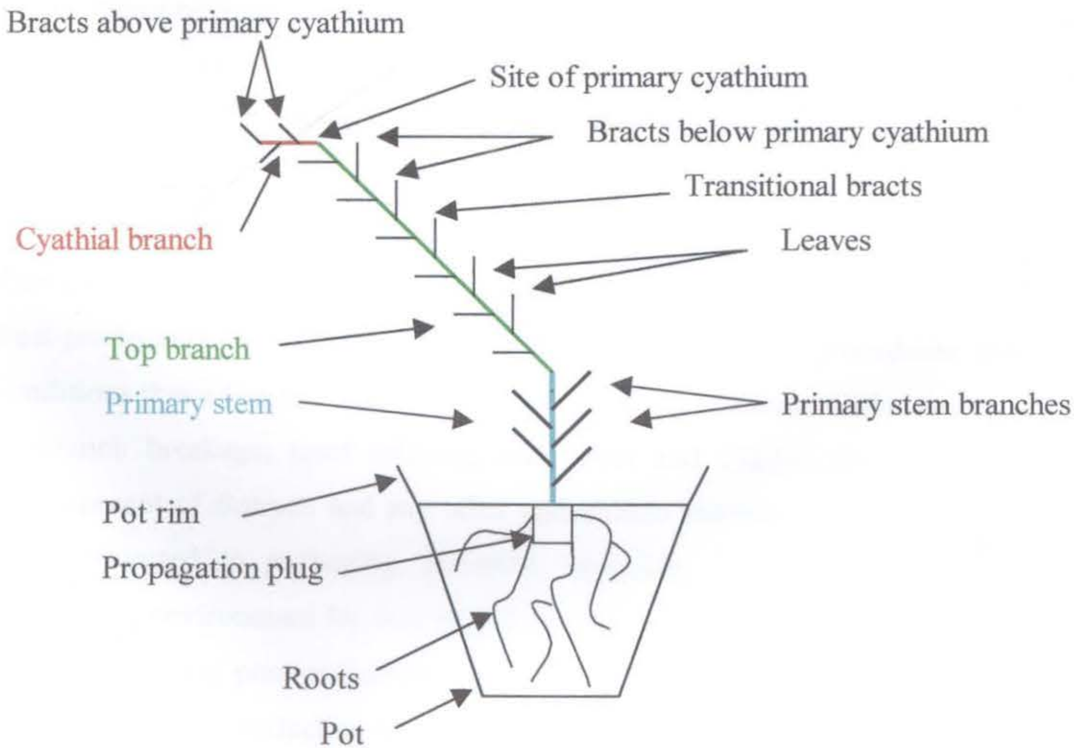
Stage 5. Final scoring

Final scoring was performed at 121 days from the date of propagation. A schematic diagram representing plant morphology at this stage is shown in Figure 3.3.1.

The following parameters were measured for all plants.

- Anthesis (score of 1 = present, 2 = absent) on at least one cyathium per plant
- Height (mm) from the top of the propagation plug/soil surface to the tip of the plant
- Node number on the primary stem, the uppermost shoot and along a single cyathial branch
- Stem diameter (mm) approximately 3 mm from the propagation plug up to and including the youngest bract >30 mm
- Branch number (big branches >50 mm, small branches from 10 mm - 50 mm)
- Width of the largest bract (mm)
- Plant symmetry (visual score, 1 = symmetrical, 2 = non-symmetrical)
- Bract shape and colour
- Leaf colour

Figure 3.3.1. Schematic diagram showing morphology of a poinsettia at Stage 5 indicating site of measurements (leaves, bracts and excess branches removed)



Mean internode length (H/N) was determined by dividing total height (mm) by the number of nodes to the youngest bract >30 mm. Height (mm) from the pot rim to the tip of the plant was also recorded.

The following characteristics were recorded on the uppermost (top) branch (Figure 3.3.1). The area of measurement included the junction of the top branch with the primary stem to the junction with the primary cyathial branches (green colour in Figure 3.3.1).

- Length (mm)
- Number of nodes
- Number of leaves
- Number of transitional bracts (leaves showing anthocyanin colouration up to <50% leaf coverage, determined visually)
- Number of bracts (>50% leaf anthocyanin coverage, determined visually) up to and including one primary cyathial bract. Generally, three bracts emanate below the primary cyathium on all plants, however, in this study only one was taken into account so that node values could be calculated correctly.
- Number of bracts along one cyathial branch after the primary cyathium. Bracts were counted to the youngest bract >30 mm in length.

Of the measurements taken, plant height, node number, mean internode length, stem diameter and branch number were considered as critical measurements relevant to plant height. The remainder of measurements were considered as additional ones that could contribute to general morphology.

Post-production performance testing (preliminary experiment)

Post-production testing was conducted to simulate handling procedures and environmental conditions that a finished plant undergoes from the greenhouse to the consumer. Factors such as branch breakage, bract bruising, leaf, bract and cyathia abscission, bract edge burn, development of *Botrytis* and any other undesirable features are measured. Therefore, plants were subjected to packaging, transport, unpacking and placement in a simulated sale (consumer) environment for four weeks. To obtain a preliminary indication of the effects of infection type on post-production performance, three plants from each infection type were subjected to post-production testing procedures similar to those described by Rinehart (pers. comm. 1998). However, only visual observations were made and photographs were taken due to the preliminary nature of this study. Plants in pots were watered and each plant was separately covered in a nylon sleeve. Six plants covered with sleeves were packed into a cardboard box and then transported by road for 1 h. Boxes were then placed inside a dark room overnight, maintained at 19°C. Plants were unpacked after 24 h from packing and the

sleeves removed. Plants were then randomly placed inside a room at approximately 500 mm spacing as measured from the centre of pots. Temperature was maintained at 25°C ± 2°C and incandescent light at approximately 6 µmol m^{-2} s^{-1} was supplied continuously for 24 h. Plants were watered once every week. Photographs were taken after four weeks.

Data analyses

Data was analysed at each stage of measurement using the MINITAB® statistical software package (MINITAB® for windows release 12.22, Minitab Inc. 1998). Effects of phytoplasma, virus and the interaction of phytoplasma x virus were analysed by conducting analysis of variance (ANOVA) using GLM (General Linear Model). Tukey pairwise comparisons were performed to determine significant differences between means at a 95% confidence interval. Means that were not significantly different were denoted by the same letter when presented in tables.

Results

At planting, irrespective of infection type, 93% of all cuttings had more than five roots per cutting exerted from the propagation plug. The remaining 7% had 1 - 4 roots exerted from the propagation plug. All plants appeared visually uniform for height. Figures 3.3.2 to 3.3.5 show plant development at four stages of the production period.

Critical measurements

Plant height

Means and standard errors for plant height at stages 1 - 5 are shown in Table 3.3.3. ANOVA for the effects PoiBI and PnMV on plant height is shown in Table 3.3.4. Only PoiBI significantly affected plant height (by reduction). This effect was limited to the post-decapitation stage (stage 3). The effect of PnMV on plant height was not significant at any stage (Table 3.3.4).

Figure 3.3.2. Plant morphology and experimental area one week after planting

Figure 3.3.3. Plant morphology and experimental area immediately after apical decapitation

Figure 3.3.4. Plant morphology and experimental area after 4 weeks under short photoperiod environment

Figure 3.3.5. Plant morphology and experimental area after 8 weeks under short photoperiod environment, organised by infection type (Left to right: -p/-mv, -p/+mv, +p/-mv and +p/+mv)

Table 3.3.3. Means and standard errors for height at stages 1 – 5 as influenced by infection type

Stage		1		2		3		4		5	
Infection type	n	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
-p/-mv	19	62.5a	2.1	95.9a	3.4	61.6a	1.4	101a	2.1	235a	4.1
-p/+mv	22	64.1a	2.0	97.4a	2.1	63.0a	2.0	97.2a	2.5	242a	4.0
+p/-mv	21	61.2a	1.9	89.0a	2.4	55.3 b	1.2	104a	2.5	235a	3.1
+p/+mv	21	62.7a	1.7	96.8a	2.0	55.8 b	1.4	103a	2.3	233a	4.6

Means followed by same letter are not significantly different. Stage 1 = planting, 2 = pre-decapitation, 3 = post-decapitation, 4 = short photoperiod, 5 = final scoring.

Table 3.3.4. ANOVA for plant height as influenced by phytoplasma and virus infection at stages 1 – 5

		Mean square				
Source	df	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5
Phytoplasma	1	34.08^{ns}	290.5^{ns}	954.5***	283.8^{ns}	411.4^{ns}
Virus	1	48.05^{ns}	450.0^{ns}	17.604^{ns}	149.59^{ns}	80.95^{ns}
Phytoplasma*Virus	1	0.0473^{ns}	205.0^{ns}	4.1178^{ns}	76.794^{ns}	474.2^{ns}
Residual	79	75.77	127.3	48.08	116.4	331.4

*, **, ***: p <0.05, 0.01, 0.001 respectively. ns: not significant. Stage 1 = planting, 2 = pre-decapitation, 3 = post-decapitation, 4 = short photoperiod, 5 = final scoring.

Node number

Significant effects for node number as influenced by phytoplasma and phytoplasma x virus were identified at stage 1 alone (planting - Table 3.3.6). The mean node number of +p/+mv plants was significantly greater than -p/+mv plants (P = 0.0135) as shown by pairwise comparisons at stage 1 (Table 3.3.5).

Table 3.3.5. Means and their standard errors for node number at stages 1, 2 and 5 as influenced by infection type

Stage		1		2		5	
Infection type	n	Mean	SE	Mean	SE	Mean	SE
-p/-mv	19	6.68ab	0.2	11.9a	0.2	21.3a	0.2
-p/+mv	22	6.46a	0.2	11.7a	0.2	21.2a	0.2
+p/-mv	21	6.67ab	0.2	11.4a	0.2	20.7a	0.3
+p/+mv	21	7.38 b	0.2	11.9a	0.2	20.9a	0.2

Means followed by same letter are not significantly different. Stage 1 = planting, 2 = pre-decapitation, 5 = final scoring. Note: stages 3 and 4 have node number = 6, due to decapitation.

Table 3.3.6. ANOVA for node number as influenced by phytoplasma and virus infection at stages 1, 2 and 5

Stage		Mean square		
Source	df	1	2	5
Phytoplasma	1	4.273*	0.4462^{ns}	3.133^{ns}
Virus	1	1.215^{ns}	0.4771^{ns}	0.127^{ns}
Phytoplasma*Virus	1	4.609*	2.862^{ns}	0.528^{ns}
Residual	79	0.9516	0.9545	1.142

*, **, ***: p<0.05, 0.01, 0.001 respectively. ns: not significant. Stage 1 = planting, 2 = pre-decapitation, 5 = final scoring. Note: stages 3 and 4 have node number = 6, due to decapitation.

Mean internode length

Mean internode length (H/N) was significantly reduced in the presence of phytoplasma at planting (stage 1) and post-decapitation (stage 3) (Tables 3.3.7 and 3.3.8). A virus x phytoplasma interaction was also observed at stage 1.

At planting, mean internode length for +p/+mv plants was significantly shorter compared to -p/+mv plants. This difference was attributed to differences for mean node numbers between these two infection types (refer to Table 3.3.5) as mean heights were not different (Table 3.3.3).

Table 3.3.7. Means and their standard errors for H/N (mm) at stages 1 – 5 as influenced by infection type

Stage		1		2		3		4		5	
Infection type	n	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
-p/-mv	19	9.38ab	0.2	8.03a	0.2	10.3a†	0.2	17.0a	0.3	11.1a	0.2
-p/+mv	22	10.0 a	0.3	8.32a	0.1	10.5a	0.3	16.2a	0.4	11.4a	0.2
+p/-mv	21	9.30ab	0.2	7.79a	0.1	9.21 b	0.2	17.3a	0.4	11.4a	0.2
+p/+mv	21	8.56 b	0.2	8.10a	0.1	9.30 b†	0.2	17.1a	0.4	11.1a	0.2

Means followed by same letter are not significantly different. Stage 1 = planting, 2 = pre-decapitation, 3 = post-decapitation, 4 = short photoperiod, 5 = final scoring. †P-value between -p/-mv and +p/+mv = 0.0506.

Table 3.3.8. ANOVA for mean internode length as influenced by phytoplasma and virus infection at stages 1 – 5

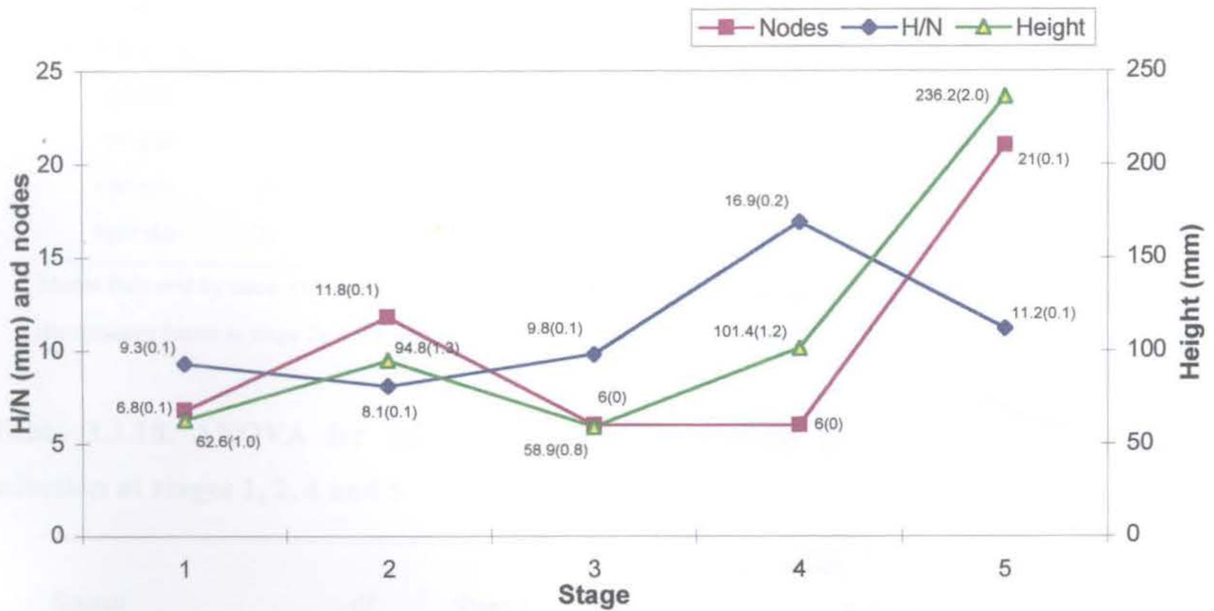
		Mean square				
Source	df	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5
Phytoplasma	1	12.42**	1.113^{ns}	26.16***	7.845^{ns}	0.0000^{ns}
Virus	1	0.0465^{ns}	1.926^{ns}	0.5382^{ns}	4.127^{ns}	0.0095^{ns}
Phytoplasma*Virus	1	9.735**	0.0040^{ns}	0.1181^{ns}	2.092^{ns}	2.399^{ns}
Residual	79	1.374	0.6055	1.349	3.232	1.032

*, **, ***: $p<0.05$, 0.01, 0.001 respectively. ns: not significant. Stage 1 = planting, 2 = pre-decapitation, 3 = post-decapitation, 4 = short photoperiod, 5 = final scoring.

After decapitation (stage 3), mean internode length for phytoplasma infected plants was significantly lower than non-infected plants, although node numbers were an identical 6 nodes for all plants due to decapitation. Reduced internode length for the first six nodes could be due to differences in stockplant morphology. Visual observations showed phytoplasma infected stockplants appeared to possess compact internodes and more branches compared to non-infected types. Because mean internode length differences existed, a significant difference in plant height was recorded at the post-decapitation stage (stage 3). i.e. phytoplasma infected plants were shorter than non-infected plants and the reduction in height was due to shorter mean internode lengths.

Considering that only a few significant differences were observed for height, nodes and H/N (only at stages 1 and 3), means of these parameters for all four infection types were pooled and standard errors were derived for each stage. These pooled results are presented in Figure 3.3.6. Plant height at maturity (stage 5) is the most important factor and at this stage, height ranged from 180 - 271 mm. Acceptable plant height from the pot rim according to industry sources ranges from 225 - 300 mm (Moe *et al.* 1992, Trellinger 1998, Rinehart pers. comm. 1997, White pers. comm. 1997). Therefore, all plants were within or below this accepted range, measured from the propagation plug. When measured from the pot rim, pooled mean height was reduced by approximately 5 mm (from 236 ± 2.0 to 231 ± 2.0 mm).

Figure 3.3.6. Means and standard errors for node number, H/N and height among all plants at the five experimental stages (pooled data)



Leaf number

Leaf number was very highly correlated with node number, as each node is expected to give rise to a leaf. The following correlations were observed r = 0.960*** (stage 1), 0.959*** (stage 2&3) and 0.959*** (stage 4). Therefore, mean node numbers are equivalent to mean leaf numbers and leaf number means at these stages (1, 2, 3, 4) and are not presented (refer to nodes - Table 3.3.5). During the short photoperiod phase (stage 4 - 5) plants became reproductive and leaves changed to bracts. Therefore, leaf numbers for this stage are presented later under 'top branch measurements'.

Stem diameter

Mean stem diameter was significantly influenced by phytoplasma presence at stages 1, 2, 4 and 5. Stages 1 and 2 showed significant effects due to virus presence (Table 3.3.9).

At stages 1 and 2, phytoplasma infection resulted in decreased mean stem diameter. Stages 4 and 5 showed significantly reduced mean stem diameter on phytoplasma infected plants compared to non-infected plants, but no effect of PnMV was seen. The phytoplasma x virus interaction was not significant at any stage.

Table 3.3.9. Means and their standard errors for stem diameter (mm) at stages 1 – 5 as influenced by infection type

Stage		1		2		4		5	
Infection type	n	Mean	SE	Mean	SE	Mean	SE	Mean	SE
-p/-mv	19	4.50a	0.14	5.08a	0.14	6.34a	0.18	8.13a	0.17
-p/+mv	22	4.98a	0.15	5.50ab	0.15	6.64a	0.14	8.57a	0.13
+p/-mv	21	3.93 b	0.09	4.64 b	0.11	5.71 b	0.15	7.59 b	0.12
+p/+mv	21	4.29ab	0.13	4.81 b	0.11	5.64 b	0.14	7.57 b	0.14

Means followed by same letter are not significantly different. Stage 1 = planting, 2 = pre-decapitation, 3 = post-decapitation (same as stage 2), 4 = short photoperiod, 5 = final scoring.

Table 3.3.10. ANOVA for stem diameter as influenced by phytoplasma and virus infection at stages 1, 2, 4 and 5

		Mean square			
Source	df	Stage 1	Stage 2	Stage 4	Stage 5
Phytoplasma	1	8.251***	6.565***	13.60***	12.16***
Virus	1	3.601**	1.787*	0.2568^{ns}	0.8814^{ns}
Phytoplasma*Virus	1	0.0746^{ns}	0.3347^{ns}	0.6917^{ns}	1.097^{ns}
Residual	79	0.3502	0.3410	0.4744	0.4117

Stage 1 = planting, 2 = pre-decapitation, 3 = post-decapitation (identical to stage 2), 4 = short photoperiod, 5 = final scoring. *, **, ***: p<0.05, 0.01, 0.001 respectively. ns: not significant.

Branch number

At planting (stage 1) branches were not present on any plants. By stage 2, (prior to decapitation) some PoiBI infected plants had produced branches (Table 3.3.11, 3.3.12).

Table 3.3.11. Means and standard errors for branch number at stages 2, 4 and 5 and branches >50 mm in length at stage 5, as influenced by phytoplasma and virus infection

Stage		2		4		5		5 (branches >50 mm)	
Infection type	n	Mean	SE	Mean	SE	Mean	SE	Mean	SE
-p/-mv	19	0.000a	0.00	5.32ab	0.19	5.32a	0.20	5.26ab	0.20
-p/+mv	22	0.000a	0.00	4.68 b	0.23	5.41a	0.11	4.95a	0.18
+p/-mv	21	0.762 b	0.22	5.48a	0.15	5.81ab	0.13	5.76 bc	0.15
+p/+mv	21	1.86 c	0.14	5.81a	0.09	6.00 b	0.10	5.90 c	0.12
- phytoplasma*	41	0.00a	0.00	4.98a	0.16	5.37a	0.11	5.10a	0.13
+ phytoplasma*	42	1.31 b	0.15	5.64 b	0.09	5.90 b	0.08	5.83 b	0.10

Means followed by same letter are not significantly different. Stage 2 = pre-decapitation, 3 = post-decapitation (identical to stage 2), 4 = short photoperiod, 5 = final scoring. Maximum of 6 branches attainable due to apical decapitation above 6th node. * = pooled means for plants infected or not infected with phytoplasma.

Significant differences for branch number were found at stages 2, 4 and 5. The main effect of phytoplasma was to significantly increase the number of branches at these stages (Table 3.3.11). A virus x phytoplasma interaction was observed at stages 2 and 4, with a virus effect at stage 2 as well. At stage 5, only phytoplasma significantly affected branch number.

Table 3.3.12. ANOVA for branch number at stages (2, 4 and 5) and branches >50 mm at stage 5, as influenced by phytoplasma and virus infection

		Mean square			
Source	df	Stage 2	Stage 4	Stage 5	Stage 5 (branches >50 mm)
Phytoplasma	1	35.48***	8.583***	6.085***	10.86***
Virus	1	6.205***	0.4675^{ns}	0.4166^{ns}	0.1421^{ns}
Phytoplasma*Virus	1	6.205***	4.840**	0.0488^{ns}	1.054^{ns}
Residual	79	0.3593	0.6247	0.3881	0.5602

*, **, ***: p<0.05, 0.01, 0.001 respectively. ns: not significant. Stage 1 = planting (no branches), 2 = pre-decapitation, 3 = post-decapitation (identical to stage 2), 4 = short photoperiod, 5 = final scoring.

Branches (a mean of 1.3) had emerged from the lowest nodes on PoiBI infected plants at stage 2 (44 days after start of propagation - Table 3.3.11). These branches were not removed by apical decapitation when 6 nodes were left on each plant. At stage 2, the presence of virus and phytoplasma resulted in a significantly greater branch number than phytoplasma alone. Both non-phytoplasma infected plant types (-p/-mv, -p/+mv) did not possess branches at this stage (Table 3.3.11).

At the final scoring, when only branches >50 mm in length were considered, the -p/+mv infection type possessed a greater proportion of small branches (<50 mm in length) than other infection types (Table 3.3.11) indicating that PnMV may influence branch length . The cv. V10 Amy red appeared to be a highly branched genotype that produced similar numbers of branches from the time at which decapitation to 6 nodes commenced regardless of infection type. No correlation was found between height and branch number at stage 5.

Top branch measurements at stage 5

Phytoplasma infection significantly influenced top branch length, leaf number and bract number (below the primary cyathium - Table 3.3.15). Both bract length and bract number were significantly reduced, but leaf number was significantly increased by phytoplasma presence (Table 3.3.13). Considering node number was not significantly different between

infection types, the lower bract number of PoiBI infected plants could be due to increased leaf number. A significant effect of PnMV on top branch length was also found (Table 3.3.15).

Table 3.3.13. Means and standard errors for top branch length (mm) and leaf and bract number below the primary cyathium at stage 5 as influenced by phytoplasma and virus infection

		Length		Leaf number		Bracts below	
Infection type	n	Mean	SE	Mean	SE	Mean	SE
-p/-mv	19	152ab	3.2	5.10ab	0.10	5.79a	0.26
-p/+mv	22	160a	2.7	4.95a	0.10	5.91a	0.16
+p/-mv	21	143 b	2.7	5.76 c	0.14	5.00 b	0.19
+p/+mv	21	149ab	4.0	5.57 bc	0.16	5.24ab	0.14
- phytoplasma*	41	156a	2.1	5.02a	0.07	5.85a	0.15
+ phytoplasma*	42	146 b	2.4	5.67 b	0.11	5.11 b	0.12

Means followed by same letter are not significantly different. * = pooled means for plants infected or not infected with phytoplasma.

Table 3.3.14. Means and standard errors for top branch node, transitional bract and bract above primary cyathium numbers at stage 5 as influenced by infection type

		Node number		Trans-bracts		Bracts above	
Infection type	n	Mean	SE	Mean	SE	Mean	SE
-p/-mv	19	12.5a	0.3	1.58a	0.1	2.79a	0.1
-p/+mv	22	12.5a	0.1	1.59a	0.1	2.73a	0.1
+p/-mv	21	12.0a	0.2	1.19a	0.1	2.76a	0.1
+p/+mv	21	12.4a	0.2	1.62a	0.1	2.52a	0.1

Means followed by same letter are not significantly different. * = pooled means for plants infected or not infected with phytoplasma.

Table 3.3.15. ANOVA for top branch measurements (branch length, node, leaf, transitional bract, bract below the primary cyathium and bract above the primary cyathium numbers) at stage 5 as influenced by phytoplasma and virus infection

Source	df	Mean square					
		Length	Node number	Leaf number	Trans-bracts	Bracts below	Bracts above
Phytoplasma	1	2114**	1.549^{ns}	8.389***	0.6716^{ns}	11.03***	0.2761^{ns}
Virus	1	1041*	1.080^{ns}	0.6022^{ns}	1.004^{ns}	0.6619^{ns}	0.4665^{ns}
Phytoplasma*Virus	1	5.631^{ns}	1.269^{ns}	0.0082^{ns}	0.8978^{ns}	0.0726^{ns}	0.1600^{ns}
Residual	79	211.6	0.9150	0.3506	0.2549	0.7441	0.2097

*, **, ***: $p<0.05$, 0.01, 0.001 respectively. ns: not significant.

Additional measurements at stage 5

At stage 5 (close to anthesis), all plants regardless of infection type possessed at least one cyathium with dehisced anthers. Two parameters could be analysed statistically – largest bract width and plant symmetry (Tables 3.3.16 and 3.3.17).

Table 3.3.16. Means, standard errors and pairwise comparisons for largest bract width (mm) and plant symmetry at stage 5 (including descriptions of leaf and bract colour) as influenced by infection type

		Largest bract width		Plant symmetry		Leaf and bract shape	Leaf colour	Bract colour
Infection type	n	Mean	SE	Mean	SE		Description	
-p/-mv	19	88.6a	2.0	1.10a	0.07	lobed	green	red
-p/+mv	22	80.0 b	1.7	1.09a	0.06	lobed	green	red
+p/-mv	21	106 c	2.5	1.29a	0.10	rounded	green	lighter red
+p/+mv	21	105 c	2.2	1.38a	0.11	rounded	green	lighter red

Means followed by same letter are not significantly different.

Table 3.3.17. ANOVA for the influence of phytoplasma and virus on largest bract width and plant symmetry at stage 5

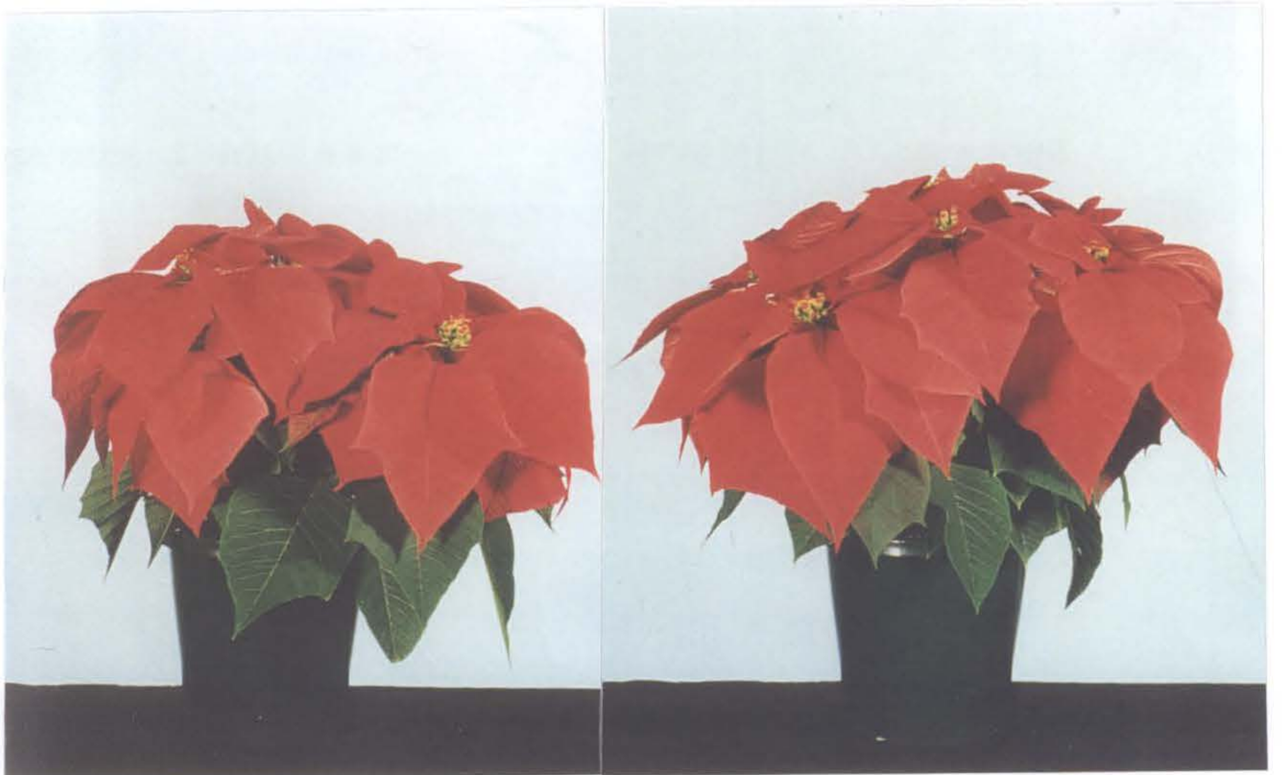
		Mean square	
Source	df	Bract width	Plant symmetry
Phytoplasma	1	9495***	1.145**
Virus	1	441.7*	0.0338^{ns}
Phytoplasma*Virus	1	339.2^{ns}	0.0621^{ns}
Residual	79	95.67	0.3821

*, **, ***: p<0.05, 0.01, 0.001 respectively. ns: not significant.

Phytoplasma infection significantly increased bract width, by approximately 50% over both of the non-PoiBI infection types. Mosaic virus infection significantly reduced bract width on plants not infected with phytoplasma. The reduction was approximately 10%. Plants without phytoplasma infection tended to possess greater symmetry, however comparisons between infection type means did not show significant differences at $p<0.05$. Leaf colour was not influenced by mosaic virus or phytoplasma infection. However, bract colour in phytoplasma infected lines was lighter than non-infected lines. Royal Horticultural Society (RHS) colour charts were not applicable to determine this difference because it was marginal. Bracts on phytoplasma infected plants also had a rippled (rugose) appearance (Figures 3.3.7 – 3.3.8).

Figure 3.3.7. Cultivar V10 Amy red infection type -p/-mv (left) and –p/+mv (right) at stage 5

Figure 3.3.8. Cultivar V10 Amy red infection type +p/-mv (left) and +p/+mv (right) at stage 5



Post-production performance

Examples of plants from each infection type at stage 5 and just prior to post-production testing are shown in Figures 3.3.7 and 3.3.8. The effects of phytoplasma and virus infection after 28 days of post-production testing are visually demonstrated in Figure 3.3.9. Greater bract and leaf numbers are present on phytoplasma infected plants after post-production testing, resulting in improved appearance.

Figure 3.3.9. Effects of infection type on post-production performance of cv. V10 Amy red after 28 days of post-production testing. (Left to right: -p/-mv, +p/-mv, +p/+mv, -p/+mv)

Discussion

The purpose of this experiment was to primarily investigate the individual and combined effects of PoiBI and PnMV on plant height in cv. V10 Amy red. The key observation was that regardless of infection type, at final scoring, mean plant heights were not significantly different (at $p<0.05$). During all other measurement stages, only one small but significant difference for height was found. At this stage (post-decapitation), phytoplasma infected plants possessed reduced height (5.8 - 7.7mm) attributed to a reduction in mean internode length.

These results are in contradiction to those of Bech and Rasmussen (1996) who showed that PoiBI infection resulted in reduced internode length and plant height in cv. Freedom red. However, their studies were not performed in a commercial production environment, and measurements were made on approximately 10 approach grafted plants prior to separation of the graft. Preil (1994b) suggests that PoiBI free plants have reduced internode length, but did not present any data. Reduced internode length was only evidenced at the post-decapitation stage in this experiment. However, stockplants infected with phytoplasma did exhibit reduced internode length. It is likely that differences in height were not recorded in this experiment because of several factors including cultivar genotype, production methodology and time of year.

Cultivar V10 Amy red is genetically a prolific branching, short genotype, when infected with PnMV and PoiBI (Ecke *et al.* 1990). The effects of these two infective agents on a genotypically short cultivar may be reduced, especially during commercial production. Further studies with genetically taller plants need to be undertaken to determine this.

During commercial production, apical decapitation is performed which promotes axillary branching due to removal of apical dominance. Because all plants are decapitated to six nodes, this procedure promotes uniformity. The time of year could have affected the effects of phytoplasma and virus infection, through changes in temperature, light intensity and duration.

Stockplants infected with PoiBI (alone or with PnMV) displayed reduced height, shorter internodes and increased branching. These symptoms were not readily expressed in the commercial production experiment because plants were not grown to produce long stems, or for an extended period (i.e. greater than 16 weeks). Decapitating plants at a higher node

number, or not practicing decapitation could have resulted in differences for plant height between phytoplasma infected and non-infected lines.

Plant height at final scoring was within the limits desired by the poinsettia industry (225 – 300 mm). Plants were measured from the soil/propagation plug surface, and recorded mean heights close to the lower end of the scale. Mean heights recorded from the pot rim were shorter further. These results were achieved without the use of growth regulating chemicals, which are frequently used in the commercial environment during summer/autumn (February – May) when this experiment was performed. These results demonstrate cv. V10 Amy red is a short growing cultivar and may be useful in breeding studies to develop short genotypes that do not require growth regulator application.

Other significant morphological effects of phytoplasma presence included reduced stem diameter, which has been reported by Dole and Wilkins (1988) and Bech and Rasmussen (1996), increased bract size (not previously reported), lighter bract colour (reported by Preil 1994b), lobed leaves and bracts (reported by Dole and Wilkins 1988 and Preil 1994b), bract rugosity (not reported) and increased branch number (reported by Bech and Rasmussen 1996). In contrast to results from Preil (1994b), growth rate was not reduced, internodes were not shorter (except post-decapitation) and anthesis was not earlier.

Morphological effects of PnMV were fewer and less important. PnMV infected plants were almost identical to non-infected control plants for almost all measurements. PnMV did appear to influence certain morphological factors at certain measurement stages, often in a mixed infection with PoiBI, although the significant differences found were only minor. The most important effect of PnMV was to significantly decrease bract width in plants not infected with PoiBI.

Cultivar V10 Amy red was suitable for growth as a commercial product without the presence of PoiBI or PnMV. However, the lack of PoiBI infection reduces the number of cuttings produced from stockplants, which is a commercially significant factor. If PoiBI is considered a threat to the environment (due to its relationship to western-X phytoplasma, a pathogen which is currently not present in Australia), this genotype could be grown without this beneficial organism to a standard equivalent to infected plants. Because V10 Amy red produces a sufficient number of branches after a decapitation to 6 nodes, the production of this genotype via somatic embryogenesis may be viable. At present, a key reason for not using

somatic embryogenesis for commercial poinsettia propagation is that PoiBI is removed during the process.

This study provided evidence that post-production performance is enhanced by phytoplasma presence. Therefore further breeding work for leaf, bract and cyathia retention would be necessary prior to producing this cultivar without phytoplasma. Further studies on post-production performance need to be conducted to quantify the effects of PoiBI and PnMV on bract, leaf and cyathia loss. In figure 3.3.9 all infection types appeared poor after post-production testing. V10 Amy red is known to have poor post-production performance when produced commercially (+p/+mv) and this is one reason why it is not grown extensively at present.

In conclusion, this experiment demonstrates that PoiBI and PnMV either alone or together do not significantly influence plant height in cv. V10 Amy red under commercial production conditions in Australia. These infective agent(s) do, however, affect other morphological parameters such as stem diameter, bract size, leaf and bract shape and branch number. To further characterise the effects of infection type on plant morphology and genotype, another experiment using cv. Freedom red was undertaken (Experiment 3.4). It was expected that this experiment could provide detailed information regarding the effects of PoiBI and PnMV on height and morphology because it was conducted during three different times of the year, and utilised a taller growing cultivar.

Experiment 3.4. Assessment of the effects of poinsettia branch inducing phytoplasma (PoiBI) and poinsettia mosaic virus (PnMV) on height and morphology during commercial production of cv. Freedom red

Introduction

Experiment 3.3 demonstrated that PoiBI and PnMV did not significantly influence plant height in cv. V10 Amy red, except at the post-decapitation stage. Some significant morphological differences were found, especially between phytoplasma infected and non-infected types (e.g. bract colour, bract size and branching).

The current experiment was conducted to determine if phytoplasma and virus symptom expression varied between genotypes. The cultivar Freedom red was chosen because it is commercially important and is also considered taller than cv. V10 Amy red. All four possible infection types of cv. Freedom red were produced in Experiment 3.2 (Table 3.2.5).

To provide a robust measure of the effects of PnMV and PoiBI on plant morphology during commercial production, this study was devised as a series of three experiments conducted at three different times of the year, and spanning nearly all four seasons. Data was analysed individually for each experiment. In addition, data from the three experiments was pooled and analysed. Post-production testing and measurement of root and shoot fresh and dry matter weights were also undertaken in two experiments to further investigate the effects of infection type on morphology.

Materials and methods

Plant materials

The four infection types (-p/-mv, -p/+mv, +p/-mv, +p/+mv) were developed via *in vitro* culture procedures as explained in Experiment 3.2.

Experimental protocol

Three experiments (3.4.1, 3.4.2 and 3.4.3) were performed during different times of the year, and spanning different seasons in Australia. The protocol and measurements taken for all three experiments were identical to Experiment 3.3, except where specifically stated in this experiment.

Table 3.4.1. Stages of plant development and season for experiments

		Experiment 3.4.1 mid winter – late spring	Experiment 3.4.2 late winter – early summer	Experiment 3.4.3 mid spring – late summer
Stages of plant development	Stage	Day	Day	Day
Start of propagation	0	0	0	0
Planting	1	23	30	28
Pre-apical decapitation	2	37	41	41
Post-apical decapitation	3	37	41	41
Start of short photoperiod	4	51	55	54
Final scoring	5	106	105	104

Table 3.4.2. Mean minimum and maximum daily temperatures recorded for each experiment

	Mean daily temperature (°C)					
	Experiment 3.4.1		Experiment 3.4.2		Experiment 3.4.3	
Period	Min.	Max.	Min.	Max.	Min.	Max.
Propagation to planting	18	27	16	25	21	32
Planting to decapitation	23	27	17	40	27	46
Decapitation to short photoperiod transfer	23	27	17	41	16	42
Start to end of short photoperiod	18	27	18	29	18	29

Stockplant management

The stockplant management schedule from Experiment 3.3 was altered to include stockplants being grown in a greenhouse maintained at 25 ± 2°C and provided with automated drip irrigation. This protocol was undertaken to minimise the effects of temperature and varying water application on cuttings harvested from stockplants.

Stage 0. Vegetative propagation

Fongarid® fungicide was applied once at the recommended rate approximately 14 days after the start of propagation, as a precautionary measure against fungal infection of cuttings.

Stage 1. Planting

Cuttings with roots were planted in pots of 175 mm diameter and 145 mm height. This pot size was used because it was expected that some plants grown during the warmest months of the year would become very tall and difficult to handle in smaller 150 mm diameter pots.

Stages 2, 3 and 4. Apical stem decapitation and transfer to short photoperiod

As in Experiment 3.3.

Stage 5. Final scoring

Final scoring was performed between 104 - 106 days from the date of propagation. The definition of a 'transitional bract' was changed to denote a leaf displaying red colouration up to an approximate maximum of 90%. This change enabled easier interpretation of transitional bracts. Bracts were now classified as bracts if they possessed greater than 90% red colouration. Bract width was measured on the three largest bracts on a plant and an average of the three was obtained to increase precision for data analysis.

Post-experiment measurements

Root and shoot weights

Root and shoot weights were measured to further characterise any changes PoiBI and PnMV imparted on cv. Freedom red. After the final scoring, plants of each infection type grown in experiment 3.4.3 were harvested for shoots and roots. Fifteen plants of the -p/-mv and +p/+mv infection types were harvested, 13 of -p/+mv and 17 of +p/-mv were also harvested. Shoots were cut at the base of each plant and placed on an individual plant basis into plastic bags. Each bag was sealed and weighed to obtain the fresh weight. Following measurement of fresh weight, each plastic bag was emptied, and then the shoots repackaged into a paper bag to enable dry weight to be evaluated after heat treatment at 80°C in an oven.

The root mass from each plant was removed from each pot and the potting mix washed off in running tap water. Wet roots from each plant were placed into individual plastic bags and allowed to remain there for approximately an hour, prior to excising the lower corner of the bag to allow any excess water to drain. Thereafter, each bag was weighed and a fresh weight was obtained. Each bag with roots was emptied and then repackaged into a paper bag for drying in an oven to obtain dry weight.

Paper bags containing either roots or shoots were placed into an oven maintained at 80°C for 5 days and afterwards, each bag was weighed to obtain dry weight. In all cases, net weight was obtained by subtracting the weight of the bag from the total weight.

Post-production performance

After the final scoring at stage 5, 6 plants of each infection type grown in experiment 3.4.1 were subjected to post-production testing procedures as described in Experiment 3.3. At 14 days from the start of testing, each pot was watered with approximately 300 ml of tap water. The following measurements were made on every plant at the start of testing and thereafter at 28 days.

- Plant quality (score of 1 = best, suitable for sale and no loss of any appendages through to score of 5 = worst, total defoliation and cyathia loss)
- Number of cyathia greater than 2 mm in diameter
- Total number of leaves, transitional bracts and bracts below the primary cyathium (not including bracts directly subtending the primary cyathium)
- Number of bracts above the primary cyathium that were greater than 30 mm in length. Bracts subtending the primary cyathium, three on each plant, were also included in this count.

From the data obtained, percentage values were derived. These values accounted for differences between infection types at the start of testing and were measured as follows:

- % cyathia loss due to abscission
- % of leaves, transitional bracts and bracts below the primary cyathium that abscised, not including bracts that subtended the primary cyathium
- % of bracts developed above the primary cyathium that abscised, including bracts that subtended the primary cyathium

Data analyses

Commercial production experiments

Data was analysed at each stage using the MINITAB® statistical software package (MINITAB® for windows release 12.22, Minitab Inc. 1998). Effects of experiment, phytoplasma, virus and interactions of these parameters were analysed by conducting Analysis of Variance (AVOVA) utilising General Linear Model (GLM) with sequential (Type I) sum of squares (unequal treatment replication). Tukey pairwise comparisons were performed to determine significant differences between means at a 95% confidence interval. If means were not significantly different, they were denoted by the same letter in tables. The number of plants of each infection type for each experiment is detailed in Table 3.4.3.

Table 3.4.3. Number of plants of each infection type used in three experiments

	Number of plants			
	Experiment 3.4.1	Experiment 3.4.2	Experiment 3.4.3	Total
Infection type				
-p/-mv	18	14	15	47
-p/+mv	16	18	13	47
+p/-mv	18	14	17	49
+p/+mv	19	14	18	51

Root and shoot weights

Root and shoot weight data analyses were performed using the GLM model [phytoplasma, virus, phytoplasma x virus] with sequential (Type I) sum of squares (unequal treatment replication) for plants obtained from experiment 3.4.3.

Post-production testing

Data analyses were performed using the GLM model [phytoplasma, virus, phytoplasma x virus] with adjusted (Type III) sum of squares (equal treatment replication) for 6 plants of each infection type obtained from Experiment 3.4.1.

Results

Commercial production experiments

At planting, cuttings from each experiment had approximately uniform root number. Ninety five percent possessed greater than five roots exerted from the propagation plug, the remaining 5 % recorded 1 - 4. At the completion of the three experiments (stage 5), photographs were taken of plants representative of each infection type and these are shown in Figure 3.4.1.

Figure 3.4.1. Plant morphology at final scoring (stage 5) for (top to bottom) experiment 3.4.1, 3.4.2 and 3.4.3 (infection types left to right are -p/-mv, -p/+mv, +p/-mv, +p/+mv)

Effects of PoiBI, PnMV and experiment on plant height

Effects of PoiBI and PnMV on plant height

Assessment of means and pairwise comparisons for infection type by experiment and as pooled data is presented in Table 3.4.4. PoiBI infected plants were significantly shorter compared to non-PoiBI infected plants, at virtually all stages and all experiments. When analysed as pooled data, PoiBI infected plants were significantly shorter than non-PoiBI infected plants at every stage. Phytoplasma infection and experiment were the main factors affecting plant height at all 5 stages (Table 3.4.5).

Table 3.4.4. Mean heights (mm) and standard errors for experiments 3.4.1, 3.4.2, 3.4.3 and pooled at stages 1 - 5 as influenced by infection type

Experiment	Pooled†		3.4.1		3.4.2		3.4.3	
Infection type	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Stage 1 – Planting								
-p/-mv	67.8a	1.4	72.0a	3.0	67.6a	1.4	62.8ab	1.4
-p/+mv	66.5a	1.4	65.1ab	3.0	68.1a	1.8	65.9a	2.5
+p/-mv	53.9 b	1.2	47.7 c	2.4	55.6 b	1.0	59.0 b	0.7
+p/+mv	57.5 b	1.0	56.1 bc	2.0	54.6 b	1.5	61.2ab	1.4
Stage 2 – Pre-decapitation								
-p/-mv	84.7a	2.2	97.0a	3.7	79.2a	1.9	75.0a	1.8
-p/+mv	85.7a	2.1	98.0a	3.9	80.3a	1.8	78.1a	2.5
+p/-mv	70.1 b	1.2	71.2 b	2.9	64.9 b	1.2	73.3a	0.8
+p/+mv	73.4 b	1.5	81.3 b	2.2	64.4 b	1.3	73.3a	1.9
Stage 3 – Post-decapitation								
-p/-mv	67.1a	1.7	76.9a	3.0	61.8a	1.3	60.3a	1.5
-p/+mv	68.2a	1.7	78.2a	3.0	64.2a	1.6	61.3a	2.5
+p/-mv	54.9 b	1.0	56.1 b	2.3	49.8 b	1.2	57.8a	0.8
+p/+mv	54.9 b	1.0	57.6 b	2.0	49.9 b	1.0	55.9a	1.3
Stage 4 – Short photoperiod								
-p/-mv	75.7a	2.0	82.9a	3.3	61.6a	1.7	80.3a	1.7
-p/+mv	74.1a	1.9	82.4a	3.2	64.0a	1.5	78.1a	3.1
+p/-mv	65.8 b	2.4	60.0 b	2.3	49.6 b	1.1	85.4a	1.5
+p/+mv	66.0 b	2.3	61.9 b	2.0	49.3 b	0.9	83.4a	2.6
Stage 5 – Final scoring								
-p/-mv	347a	14	270a	5.7	313a	9.0	471a	12
-p/+mv	340a	12	273a	5.5	312a	9.4	463a	9.7
+p/-mv	300 b	11	237 b	5.6	275 b	4.9	388 b	9.5
+p/+mv	302 b	9.4	246 b	6.7	278 b	8.9	381 b	7.0

Means followed by the same letter are not significantly different. † Experiments 3.4.1, 3.4.2 and 3.4.3 pooled.

Table 3.4.5. ANOVA for height at stages 1 - 5 as influenced by experiment and phytoplasma and virus infection

		Mean square				
Stage		1	2	3	4	5
Source	df					
Experiment	2	82.57^{ns}	3706***	1853***	10026***	489433***
Phytoplasma	1	6311***	8741***	1863***	5324***	114704***
Virus	1	70.89^{ns}	358.4^{ns}	29.27^{ns}	0.2279^{ns}	5.396^{ns}
Expt*Phyto	2	675.2***	1361***	1164***	3078***	13230***
Expt*Virus	2	29.66^{ns}	135.1^{ns}	20.62^{ns}	48.83^{ns}	830.6^{ns}
Phyto*Virus	1	283.6*	40.35^{ns}	28.12^{ns}	0.1824^{ns}	172.8^{ns}
Expt*Phyto*Virus	2	382.9**	184.2^{ns}	10.93^{ns}	27.03^{ns}	24.62^{ns}
Residual	182	64.44	95.96	64.14	83.27	1021

*, **, ***: p <0.05, 0.01, 0.001 respectively. ns: not significant.

PnMV alone did not significantly influence height at any stage (Table 3.4.5).

Effect of experiment on plant height

Pooled height data for each individual experiment showed plants grew taller with each successive experiment at final scoring - stage 5 (Table 3.4.6). Increases in height may have been due to environmental conditions progressing from cooler, low light intensity days to warmer days with increased light intensity (refer to Table 3.4.2 for temperature data).

Table 3.4.6. Means and standard errors for height (mm) at stages 1 - 5 as influenced by experiment

Stage		1		2		3		4		5	
Experiment	n	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
3.4.1	71	60.0a	1.7	86.5a	2.1	66.8a	1.8	71.4a	1.8	255a	3.4
3.4.2	60	62.0a	1.1	72.8 b	1.3	57.0 b	1.1	56.6 b	1.1	295 b	4.7
3.4.3	63	62.0a	0.8	74.7 b	0.9	58.6 b	0.8	82.1 c	1.1	421 c	7.0

Of interest is the approximate two week period from apical decapitation (stage 3) to the start of short photoperiod (stage 4). Plants in experiments 3.4.1 and 3.4.3 exhibited internode elongation, however plants in experiment 3.4.2 did not (Table 3.4.6 and 3.4.4). This lack of elongation could not be accounted for by the mean day temperature of 41°C experienced in experiment 3.4.2, because experiment 3.4.3 experienced similar high temperatures during this period as well, and did exhibit elongation. The specific days when high temperatures occurred

may have been critical, rather than the mean daily temperature.. High temperatures prevent leaf unfolding and result in death upon continued exposure (Erwin 1993a).

At final scoring, plant height is required to be within 225 – 300 mm. Based on infection type means, the first experiment successfully produced all four infection types between these limits (Table 3.4.4). In the second experiment, only phytoplasma infected plants achieved a mean height within limits. In the third experiment, all infection types exceeded the upper limit. This demonstrated that time of year and PoiBI infection can greatly influence poinsettia height.

Effect of phytoplasma on plant height

Considering PnMV had no significant effect on plant height, plants infected with PoiBI were pooled and plants not infected with PoiBI were pooled for individual experiments. The mean difference between PoiBI infected and non-infected plants for height, increased as experiments progressed from winter to summer (experiment 3.4.1 to 3.4.3, Table 3.4.7).

Table 3.4.7. Differences in mean height (mm) and top branch height between phytoplasma infected and non-infected plants at stage 5 with standard errors

Experiment	Pooled†		3.4.1		3.4.2		3.4.3	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Height (mm)								
- phytoplasma	343.5	9.2	271.1	3.9	312.2	6.5	467.2	7.7
+ phytoplasma	301.2	6.9	241.1	4.4	276.2	5.0	384.7	5.8
Mean difference	42.3		29.9		35.9		82.5	
Top branch length (mm)								
- phytoplasma	215.7	9.1	146.8	3.3	179.3	5.6	341.0	7.7
+ phytoplasma	178.4	6.6	127.0	3.8	143.4	3.7	260.8	5.1
Mean difference	37.3		19.8		35.9		80.2	
Primary stem length (mm)								
- phytoplasma	127.8	1.8	124.3	3.0	132.9	3.2	126.2	2.9
+ phytoplasma	122.8	2.4	114.1	3.6	132.9	3.3	123.9	4.5
Mean difference	5		10.2		0		2.3	

† Experiments 3.4.1, 3.4.2 and 3.4.3 pooled. + = infected with PoiBI, - = not infected with PoiBI.

The mean heights of phytoplasma infected plants from experiments 3.4.1 and 3.4.2 were 29.9 mm and 35.9 mm lower than non-infected plant means (Table 3.4.7). This difference represents a reduction in height of approximately 11% over non infected plants in these two experiments. However, during experiment 3.4.3 where mean heights of all plants were greatest, regardless of infection type, phytoplasma infection resulted in an 82.5 mm reduction in height. This represents a reduction in height of approximately 18% over non-PoiBI infected plants in experiment 3.4.3.

The reduction in plant height between phytoplasma infected and non-infected plants was primarily attributed to a reduction in top branch length (Table 3.4.7). This result was consistent across experiments. Approximately 30% of height reduction in PoiBI infected plants in experiment 3.4.1 was attributed to a reduction in the primary stem length. In experiments 3.4.1 and 3.4.2, the effect of primary stem length on total height was insignificant. Top branch length was also highly correlated with plant height at stage 5 ($r = 0.961^{***}$).

Pooled analysis demonstrated that phytoplasma reduced plant height at all stages from 1 - 5 (Table 3.4.4). At stage 5, the reduction was 42.3 mm (12.3%) over non-infected plants (Table 3.4.7).

Effects of PoiBI, PnMV and experiment on node number and top branch measurements

Node number was analysed for stages 1 and 2 (Tables 3.4.8 and 3.4.9). It was not analysed for stages 3 and 4 because all plants were apically decapitated to 6 nodes. In addition, stage 5 node numbers are not presented, because these are equivalent to node numbers on the top branch presented in Table 3.4.10, with the addition of 6 nodes (to account for the for primary stem) to each mean. Node number at stages 1 and 2 showed the +p/-mv infection type had the lowest mean node number (Table 3.4.8).

Table 3.4.8. Mean node numbers and their standard errors at stages 1 and 2 for experiment as influenced by infection type

Experiment	Pooled†		3.4.1		3.4.2		3.4.3	
Infection type	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Stage 1 – Planting								
-p/-mv	7.40ab	0.19	6.28a	0.27	8.71a	0.16	7.53ab	0.17
-p/+mv	7.55ab	0.19	6.19a	0.23	8.55a	0.17	7.85ab	0.19
+p/-mv	7.04a	0.17	6.06a	0.21	8.00a	0.23	7.29a	0.21
+p/+mv	7.86 b	0.13	7.37 b	0.22	8.36a	0.23	8.00 b	0.20
Stage 2 – Pre-decapitation								
-p/-mv	9.64ab	0.14	9.78a	0.29	9.86a	0.23	9.27a	0.12
-p/+mv	9.83ab	0.19	9.81a	0.16	9.78a	0.17	9.92 b	0.18
+p/-mv	9.22a	0.15	8.78 b	0.32	9.86a	0.23	9.18a	0.13
+p/+mv	9.71 b	0.12	9.79a	0.20	9.64a	0.27	9.67ab	0.16

Means followed by the same letter are not significantly different. †Experiments 3.4. 1, 3.4.2 and 3.4.3 pooled

Table 3.4.9. ANOVA for node number at stages 1 and 2 as influenced by experiment and phytoplasma and virus infection

		Mean square	
Stage		1	2
Source	df		
Experiment	2	61.99***	1.524^{ns}
Phytoplasma	1	0.0643^{ns}	2.966*
Virus	1	9.747***	5.352**
Expt*Phyto	2	3.304*	0.9925^{ns}
Expt*Virus	2	1.368^{ns}	2.498*
Phyto*Virus	1	7.738**	0.839^{ns}
Expt*Phyto*Virus	2	1.274^{ns}	1.777^{ns}
Residual	182	0.7191	0.7577

*, **, ***: $p<0.05$, 0.01, 0.001 respectively. ns: not significant.

The number of nodes and their internodal spacing influences the length of the top branch. Node number was significantly reduced on +p/-mv plants (Table 3.4.10) and may have contributed to a reduction in top branch length on these plants. This reduction was approximately one node, and could be attributed to a significant reduction in bract number, based on pooled data (Table 3.4.10). Correlation between total node number and height was low ($r = 0.165$*).

Table 3.4.10. Means and standard errors for node number, leaf, transitional-bract and bract below the primary cyathium numbers on the top branch at stage 5 as influenced by experiment and infection type

Experiment	Pooled†		3.4.1		3.4.2		3.4.3	
Infection type	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Node number								
-p/-mv	10.7a	0.12	10.3ab	0.21	10.6a	0.20	11.1ab	0.13
-p/+mv	10.7a	0.14	10.9a	0.25	10.3ab	0.19	11.1ab	0.25
+p/-mv	9.9 b	0.16	9.78b	0.22	9.29 c	0.30	10.4a	0.24
+p/+mv	10.5a	0.15	10.6a	0.19	9.50bc	0.27	11.2 b	0.17
Leaf number								
-p/-mv	3.32a	0.13	3.00a	0.14	3.00a	0.28	4.00a	0.14
-p/+mv	3.23a	0.12	3.31a	0.12	2.67a	0.16	3.92a	0.21
+p/-mv	3.75 b	0.14	3.56a	0.20	3.07a	0.16	4.53a	0.21
+p/+mv	3.52ab	0.12	3.42a	0.16	2.93a	0.24	4.11a	0.16
Trans-bracts								
-p/-mv	2.34ab	0.11	2.06ab	0.13	2.57a	0.25	2.47a	0.16
-p/+mv	2.62a	0.08	2.12a	0.08	3.00a	0.11	2.69a	0.13
+p/-mv	2.14 bc	0.10	1.67 b	0.14	2.64a	0.17	2.23ab	0.11
+p/+mv	2.00 c	0.09	1.74ab	0.13	2.43a	0.20	1.94 b	0.10
Bracts below								
-p/-mv	5.00a	0.10	5.28a	0.16	5.00a	0.18	4.67a	0.19
-p/+mv	4.89a	0.11	5.50a	0.16	4.61ab	0.12	4.54a	0.21
+p/-mv	3.96 b	0.13	4.56 b	0.12	3.57 c	0.20	3.65 b	0.24
+p/+mv	4.98a	0.12	5.47a	0.12	4.14 bc	0.18	5.11a	0.20

Means followed by the same letter are not significantly different. †Experiments 3.4.1, 3.4.2 and 3.4.3 pooled.

Table 3.4.11. ANOVA for top branch measurements at stage 5 as influenced by experiment and phytoplasma and virus infection

		Mean square				
Source	df	Length	Node number	Leaf number	Trans-bracts	Bracts below
Experiment	2	477186***	16.01***	25.44***	10.35***	13.81***
Phytoplasma	1	94620***	15.78***	4.217**	7.115***	11.28***
Virus	1	230.9^{ns}	7.983**	0.8036^{ns}	0.0794^{ns}	11.83***
Expt*Phyto	2	15884***	1.843^{ns}	0.1514^{ns}	0.1741^{ns}	1.851*
Expt*Virus	2	1632^{ns}	2.533*	0.6310^{ns}	0.1440^{ns}	1.999*
Phyto*Virus	1	963.8^{ns}	2.834^{ns}	0.5603^{ns}	1.5993*	13.67***
Expt*Phyto*Virus	2	62.99^{ns}	0.2439^{ns}	0.4545^{ns}	0.4831	0.8602^{ns}
Residual	182	796.5	0.7907	0.5456	0.3349	0.4859

*, **, ***: $p<0.05, 0.01, 0.001$ respectively. ns: not significant.

Effects of PnMV, PoiBI and experiment on mean internode length (H/N)

Interpretation of H/N means for pooled experiments showed a clear trend of phytoplasma infected plants possessing shorter mean internode lengths than non-infected plants (Table 3.4.12).

Table 3.4.12. H/N means (mm) and their standard errors at all stages for experiment and infection type

Experiment	Pooled†		3.4.1		3.4.2		3.4.3	
Infection type	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Stage 1 – Planting								
-p/-mv	9.53a	0.39	11.8a	0.68	7.79a§	0.19	8.42a	0.31
-p/+mv	8.98a	0.24	10.6a	0.37	8.98a	0.19	8.42a	0.30
+p/-mv	7.78 b	0.19	7.98 b	0.40	7.01 b§	0.21	8.19a	0.22
+p/+mv	7.39 b	0.15	7.68 b	0.25	6.57 b	0.21	7.71a	0.23
Stage 2 – Pre-decapitation								
-p/-mv	8.82a	0.22	9.99a	0.37	8.08a	0.24	8.10a	0.21
-p/+mv	8.72a	0.20	9.97a	0.33	8.23a	0.15	7.88a	0.25
+p/-mv	7.67 b	0.15	8.16 b	0.24	6.64 b	0.24	8.02a	0.14
+p/+mv	7.62 b	0.14	8.32 b	0.20	6.72 b	0.15	7.58a	0.16
Stage 3 – Post decapitation								
-p/-mv	11.2a	0.29	12.8a	0.51	10.3a	0.22	10.0a	0.25
-p/+mv	11.4a	0.29	13.0a	0.51	10.7a	0.26	10.2a	0.41
+p/-mv	9.10 b	0.17	9.34b	0.38	8.30b	0.19	9.6a	0.13
+p/+mv	9.10 b	0.17	9.60b	0.34	8.32b	0.16	9.3a	0.22
Stage 4 – short photoperiod								
-p/-mv	12.6a	0.33	13.8a	0.55	10.3a	0.28	13.4a	0.28
-p/+mv	12.4a	0.32	13.7a	0.25	10.7a	0.25	13.0a	0.51
+p/-mv	11.0 b	0.40	10.0 b	0.38	8.26b	0.19	14.2a	0.26
+p/+mv	11.0 b	0.38	10.3 b	0.33	8.21b	0.15	13.9a	0.43
Stage 5 – Final scoring‡								
-p/-mv	19.0a	0.76	14.7a	0.38	17.1a	0.47	25.8a	0.63
-p/+mv	18.5a	0.67	14.4a	0.27	17.3a	0.39	25.1a	0.60
+p/-mv	17.8 b	0.61	14.1ab	0.35	16.9a	0.54	22.4 b	0.74
+p/+mv	17.0 b	0.54	13.3 b	0.37	16.9a	0.75	21.0 b	0.38

‡ Total nodes measured on primary stem, top shoot and to last bract >30 mm in length in a single line, does not include every node on the plant. Means followed by the same letter are not significantly different. §(P=0.0534). †Experiments 3.4.1, 3.4.2 and 3.4.3 pooled.

Table 3.4.13. ANOVA for mean internode length (H/N, mm) at each stage as influenced by experiment, phytoplasma and virus infection

		Mean square				
Stage		1	2	3†	4†	5‡
Source	df					
Experiment	2	72.53***	46.20***	51.43***	278.5***	1475***
Phytoplasma	1	147.2***	65.61***	226.7***	147.9***	128.5***
Virus	1	8.230*	0.0642^{ns}	0.8190^{ns}	0.0060^{ns}	16.51*
Expt*Phyto	2	39.64***	10.83***	32.34***	85.49***	53.67***
Expt*Virus	2	1.877^{ns}	1.006^{ns}	0.5700^{ns}	1.356^{ns}	5.253^{ns}
Phyto*Virus	1	0.0000^{ns}	0.0046^{ns}	0.7790^{ns}	0.0050^{ns}	2.685^{ns}
Expt*Phyto*Virus	2	3.188^{ns}	0.1724^{ns}	0.3030^{ns}	0.7510^{ns}	0.3690^{ns}
Residual	182	1.885	0.9063	1.782	2.313	3.979

*, **, ***: $p<0.05$, 0.01, 0.001 respectively. ns: not significant. † all nodes = 6. ‡ Total nodes measured on primary stem, top shoot and to last bract >30 mm in length in a single line, does not include every node on a plant.

This was expected due to similar node numbers across infection types, but significantly different heights. Therefore, decreased plant height in phytoplasma infected plants was attributed to a decrease in mean internode length at each stage.

Effects of PoiBI, PnMV and experiment on stem diameter

Experiment, phytoplasma and virus consistently influenced stem diameter at each stage of development (Table 3.4.15). Four interaction effects were also observed. Mean stem diameter for each infection type generally increased for each subsequent experiment at stage 1. This could be expected due to the time of year when stockplants were growing.

Pairwise comparisons of pooled experiment means for infection type showed that phytoplasma infection lead to reduced stem diameter at all stages (Table 3.4.14). However, an interesting effect of mosaic virus was to significantly increase mean stem diameter in plants infected with phytoplasma at every stage in the pooled analysis. This was interesting because mosaic virus alone (-p/+mv) did not increase stem diameter over PnMV and PoiBI free plants (-p/-mv), therefore suggesting a synergistic effect. Another interesting effect was the correlation between stem diameter at stage 5 and height at stage 5 ($r = 0.595$***). This moderate correlation showed that as stem diameter increased, height also increased.

Table 3.4.14. Stem diameter means (mm) and standard errors at each stage for experiment as influenced by infection type

Experiment	Pooled†		3.4.1		3.4.2		3.4.3	
Infection type	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Stage 1 - planting								
-p/-mv	5.83a	0.13	5.08a	0.14	5.82a	0.17	6.73a	0.11
-p/+mv	5.88a	0.13	5.03a	0.12	6.00a	0.18	6.77a	0.13
+p/-mv	4.63 b	0.11	3.92 b	0.14	4.68 b	0.08	5.35 b	0.11
+p/+mv	5.00 c	0.13	4.45 c	0.11	4.46 b	0.13	6.00 c	0.13
Stage 2 - pre-decapitation								
-p/-mv	6.17a	0.14	5.69a	0.19	5.79a	0.15	7.10a	0.17
-p/+mv	6.30a	0.11	5.87a	0.15	6.11a	0.16	7.08a	0.15
+p/-mv	4.92 b	0.13	4.14 b	0.11	4.57 b	0.09	6.03 b	0.12
+p/+mv	5.31 c	0.14	4.87 c	0.13	4.57 b	0.16	6.36 b	0.13
Stage 4 - short photoperiod								
-p/-mv	7.20a	0.15	6.47a	0.22	7.04a	0.16	8.23a	0.14
-p/+mv	7.20a	0.17	6.16ab	0.18	7.25a	0.22	8.42a	0.21
+p/-mv	5.84 b	0.16	4.78 c	0.15	5.75 b	0.15	7.03 b	0.14
+p/+mv	6.36 c	0.15	5.68 b	0.13	5.64 b	0.14	7.64 c	0.12
Stage 5 - final scoring								
-p/-mv	8.95a	0.11	9.00a	0.16	8.36a	0.17	9.43a	0.14
-p/+mv	9.06a	0.14	8.81a	0.22	8.78a	0.19	9.77a	0.23
+p/-mv	7.11 b	0.16	6.33 b	0.18	6.70 b	0.15	8.29 b	0.15
+p/+mv	7.63 c	0.13	7.13 c	0.14	7.07 b	0.21	8.58 b	0.13

Means followed by the same letter are not significantly different. †Experiments 3.4.1, 3.4.2 and 3.4.3 pooled.

Table 3.4.15. ANOVA for stem diameter at all stages as influenced by experiment and phytoplasma and virus infection

Stage		1	2&3†	4	5
Source	df				
Experiment	2	40.30***	41.45***	69.05***	28.93***
Phytoplasma	1	56.37***	67.70***	65.43***	139.2***
Virus	1	2.366**	4.014**	3.918**	5.837**
Expt*Phyto	2	0.8506^{ns}	0.9753^{ns}	0.8521^{ns}	4.306***
Expt*Virus	2	0.5800^{ns}	0.4836^{ns}	0.5266^{ns}	0.0417^{ns}
Phyto*Virus	1	1.014^{ns}	0.5614^{ns}	2.841*	1.382^{ns}
Expt*Phyto*Virus	2	1.253*	0.8255^{ns}	2.415**	1.462^{ns}
Residual	182	0.2934	0.3450	0.4602	0.4852

*, **, ***: p<0.05, 0.01, 0.001 respectively. ns: not significant. † diameter identical for these two stages.

Effects of PoiBI, PnMV and experiment on branch number

Prior to decapitation, branches were not evident on any plants. Branches emerged from all plants at stage 4 (approximately 2 weeks post-apical decapitation). Experiment, phytoplasma and the experiment x phytoplasma interaction significantly influenced branch number at stages 4 and 5 (Table 3.4.17). Individual experiment and pooled analyses showed phytoplasma infected lines had significantly greater numbers of branches greater than both 10 mm and 50 mm in length at stage 5 (Table 3.4.16). Analysis of branch length showed a proportion of branches are small (<50 mm but >10 mm), and do not contribute to the flowering canopy. High temperatures are known to result in reduced branching. However, reduction in branch number did not occur as experiments progressed from cooler to warmer temperatures (Experiments 3.4.1 → 3.4.3). Branch number and height (at stage 5) were not correlated ($r = 0.73^{ns}$).

Table 3.4.16. Mean branch number greater than 10 mm and greater than 50 mm with standard errors at stages 4 and 5 for experiment as influenced by infection type

Experiment	Pooled†		3.4.1		3.4.2		3.4.3	
Infection type	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Stage 4 - short photoperiod								
-p/-mv	3.94a	0.22	2.22a	0.15	4.71a	0.19	5.27a	0.15
-p/+mv	4.15a	0.19	2.56a	0.13	4.78a	0.19	5.23a	0.12
+p/-mv	5.02 b	0.19	3.94 b	0.34	5.29a	0.22	5.94a	0.06
+p/+mv	5.25 b	0.14	4.68 b	0.28	5.29a	0.22	5.83a	0.09
Stage 5 - final scoring >10 mm								
-p/-mv	4.55a	0.14	3.72a	0.18	4.86a	0.18	5.27a	0.15
-p/+mv	4.64a	0.14	3.75a	0.21	5.00a	0.16	5.23a	0.12
+p/-mv	5.94 b	0.07	6.00 b‡	0.14	5.79 b	0.15	6.00 b	0.00
+p/+mv	6.00 b	0.09	6.26 b‡	0.20	5.64 b	0.13	6.00 b	0.00
Stage 5 – final scoring >50 mm								
-p/-mv	3.90a	0.11	3.28a	0.11	4.07a	0.16	4.47a	0.13
-p/+mv	4.11a	0.11	3.44a	0.13	4.28a	0.16	4.69a	0.13
+p/-mv	5.58 b	0.09	5.39 b	0.14	5.36 b	0.20	5.94 b	0.06
+p/+mv	5.55 b	0.08	5.32 b	0.13	5.43 b	0.17	5.89 b	0.08

Means followed by the same letter are not significantly different. ‡ branch number of 3 x +p/-mv plants and 4 x +p/+mv plants was 7 due to growth from nodes within propagation plug. † Experiments 3.4.1,3.4.2 and 3.4.3 pooled.

Table 3.4.17. ANOVA for branches greater than 10 mm and greater than 50 mm at stages 4 and 5 as influenced by experiment, phytoplasma and virus infection

		Mean square		
Stage and branch size		4 (>10 mm)	5 (>10 mm)	5 (>50 mm)
Source	df			
Experiment	2	112.2***	7.735***	14.35***
Phytoplasma	1	27.94***	89.78***	114.5***
Virus	1	1.250^{ns}	0.2388^{ns}	0.4041^{ns}
Expt*Phyto	2	19.51***	14.96***	2.957***
Expt*Virus	2	2.351*	0.1365^{ns}	0.0455^{ns}
Phyto*Virus	1	0.3988^{ns}	0.0012^{ns}	0.5660^{ns}
Expt*Phyto*Virus	2	0.3568^{ns}	0.2756^{ns}	0.0202^{ns}
Residual	182	0.6306	0.3781	0.2985

*, **, ***: $p<0.05$, 0.01, 0.001 respectively. ns: not significant.

Effects of PoiBI, PnMV and experiment on miscellaneous measurements

Significant differences were found for the effect of experiment, phytoplasma and virus on presence of anthesis, number of bracts greater than 30 mm after the primary cyathium and largest bract width at stage 5 (Table 3.4.20). Results from the pooled analysis showed that anthesis was significantly later for the +p/-mv infection type, and this significant difference was present in two of the three experiments (Table 3.4.18). The number of bracts above the primary cyathium was also significantly lower for this infection type when analysed as pooled data. On an individual experiment basis, the significant differences varied. Bract width was also significantly reduced for the +p/-mv infection type in the pooled analysis.

The leaf shape of phytoplasma infected plants was more rounded than lobed, and bract shape was more rounded and the surface was ruffled (rugose) compared to non-phytoplasma infected plants (Table 3.4.19, Figure 3.4.2). Bract colour was a slightly duller red on +p/-mv plants in each experiment, but only in experiment 3.4.3 was the +p/+mv infection type also dull red. This difference was not discernable in photographs.

Table 3.4.18. Mean number of plants at anthesis, bract number above the primary cyathium and largest bract diameter with standard errors at stage 5 for experiment as influenced by infection type

Experiment	Pooled†		3.4.1		3.4.2		3.4.3	
Infection type	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Anthesis‡								
-p/-mv	1.11a	0.04	1.00 (100%) a	0.00	1.07 (93%) a	0.07	1.27 (73%) a	0.12
-p/+mv	1.13a	0.05	1.00 (100%) a	0.00	1.11 (89%) a	0.08	1.31 (69%) a	0.13
+p/-mv	1.73 b	0.06	1.61 (39%) b	0.12	1.79 (21%) b	0.11	1.82 (18%) b	0.09
+p/+mv	1.29a	0.06	1.00 (100%) a	0.00	1.36 (64%) a	0.13	1.56 (44%) ab	0.12
Bracts above								
-p/-mv	1.66a	0.08	2.06a	0.06	1.71a	0.12	1.13ab	0.09
-p/+mv	1.72a	0.07	2.00a	0.00	1.78a	0.10	1.31a	0.13
+p/-mv	1.04 b	0.05	1.06 b	0.10	1.07 b	0.13	1.00 b	0.00
+p/+mv	1.33 c	0.07	1.84a	0.09	1.07 b	0.13	1.00 b	0.00
Bract diameter (mm)§								
-p/-mv	134a	2.1	145a	3.2	126a	2.6	128ab	2.3
-p/+mv	131a	1.1	143a	2.9	125a	2.8	123a	2.2
+p/-mv	122 b	2.0	123 b	3.7	116a	3.3	126a	2.9
+p/+mv	138a	2.1	147a	3.4	126a	3.4	136 b	2.0

§ Three largest bracts measured on every plant. ‡ Anthesis calculated as percentage of plants reaching anthesis. †Experiments 3.4.1, 3.4.2 and 3.4.3 pooled.

Figure 3.4.2. Effects of infection type on bract morphology at stage 5 (clockwise from top left -p/-mv, -p/+mv, +p/-mv, +p/+mv)

Table 3.4.19. Leaf and bract shape and colour at stage 5 for experiment as influenced by infection type

Experiment Infection type	3.4.1	3.4.2	3.4.3
Leaf shape			
-p/-mv	not determined	lobed	lobed
-p/+mv		lobed	lobed
+p/-mv		less lobed	less lobed
+p/+mv		less lobed	less lobed
Bract shape			
-p/-mv	not determined	pointed, flat	pointed, flat
-p/+mv		pointed, flat	pointed, flat
+p/-mv		rounded, rugose	rounded, rugose
+p/+mv		rounded, rugose	rounded, rugose
Bract colour			
-p/-mv	red	red	red
-p/+mv	red	red	red
+p/-mv	slightly dull red	slightly dull red	slightly dull red
+p/+mv	red	red	slightly dull red

Means followed by the same letter are not significantly different.

Table 3.4.20. ANOVA for presence of anthesis, number of bracts greater than 30 mm after the primary cyathium and largest bract width at stage 5 as influenced by experiment and phytoplasma and virus infection

		Mean square		
Source	df	Anthesis	Bracts above	Bract width
Experiment	2	2.080***	6.776***	4582***
Phytoplasma	1	7.194***	11.52***	389.4^{ns}
Virus	1	2.292***	1.615***	2410***
Expt*Phyto	2	0.1060^{ns}	0.8563**	920.2**
Expt*Virus	2	0.1598^{ns}	0.6100**	358.1^{ns}
Phyto*Virus	1	2.645***	0.6583*	3917***
Expt*Phyto*Virus	2	0.0943^{ns}	1.304***	295.2^{ns}
Residual	182	0.1383	0.1250	146.6

*, **, ***: $p<0.05$, 0.01, 0.001 respectively. ns: not significant.

Effects of PoiBI and PnMV on root and shoot weight

Significant effects were found for virus and phytoplasma x virus on root fresh and dry weights (Table 3.4.22). Root fresh and dry weight was significantly increased only in the presence of phytoplasma and mosaic virus (+p/+mv, Table 3.4.21). The infection type +p/-mv had the lowest root and shoot dry weights, although no significant differences were recorded (Table 3.4.21).

Table 3.4.21. Mean root and shoot fresh and dry weights with standard errors at stage 5 for plants in experiment 3.4.3 as influenced by infection type

		Root fresh weight		Root dry weight		Shoot fresh weight		Shoot dry weight	
Infection type	n	Mean	SE	Mean	SE	Mean	SE	Mean	SE
-p/-mv	15	48.7a	1.3	4.78a	0.12	126a	4.0	16.5a	0.49
-p/+mv	13	46.9a	1.8	4.67ab	0.24	119a	4.0	16.1a	0.64
+p/-mv	17	43.0a	1.4	3.94 b	0.16	124a	4.0	15.2a	0.37
+p/+mv	15	56.0 b	2.1	5.77 c	0.25	131a	3.6	16.0a	0.46

Means followed by the same letter are not significantly different.

Table 3.4.22. ANOVA for root and shoot fresh and dry weight at stage 5 for plants in experiment 3 as influenced by phytoplasma and virus infection

		Mean square			
Source	df	Root FW	Root DW	Shoot FW	Shoot DW
Phytoplasma	1	22.18^{ns}	0.0667^{ns}	340.5^{ns}	6.958^{ns}
Virus	1	557.1**	12.80***	1.000^{ns}	0.724^{ns}
Phyto*Virus	1	817.9***	13.93***	756.9^{ns}	5.782^{ns}
Residual	56	41.62	0.5812	232.0	3.481

*, **, ***: $p<0.05$, 0.01, 0.001 respectively. ns: not significant.

Approximately 90 - 91% of roots and 87 - 88% of shoots were composed of water, regardless of infection type.

Post-production testing

Phytoplasma was the only factor to significantly influence cyathia number and 'leaf, transitional bract, and bracts below the primary cyathium' number at the start of post-production testing (Table 3.4.23). At the completion of testing, significant effects resulted from phytoplasma and the phytoplasma x virus interaction (Table 3.4.24). One significant effect of virus was found on cyathia number.

At the start of post-production testing, mean differences existed between infection types for cyathia number (reduced on phytoplasma infected plants) and an interaction effect was found for 'leaves, transitional bracts and bracts below the primary cyathium' (Table 3.4.24). These differences therefore necessitated the use of percentages for data analyses.

Table 3.4.23. Mean cyathia number, 'pooled leaves, transitional bracts and bracts below the primary cyathium' and 'bracts above primary cyathium' as influenced by infection type (1) prior to post-production testing and (2) mean % loss of the same measurements at 28 days after the start of post-production testing

Parameter	n	Infection type	(1) Mean prior to testing	SE	(2) Mean % loss after 28 days	SE
Cyathia number	6	-p/-mv	47.0a	2.8	75.8a	1.7
	6	-p/+mv	47.2a	3.0	69.4a	4.6
	6	+p/-mv	31.2 b	3.9	46.7 b	5.5
	6	+p/+mv	35.8ab	3.8	76.3a	4.7
Leaves, bracts and transitional bracts below primary cyathium	6	-p/-mv	42.2a	5.4	92.9a	2.3
	6	-p/+mv	47.8ab	5.9	96.4a	1.6
	6	+p/-mv	61.0 b	2.7	50.4 b	1.6
	6	+p/+mv	54.2ab	3.2	41.2 c	2.3
Bracts above the primary cyathium	6	-p/-mv	47.8a	3.0	20.5a	3.5
	6	-p/+mv	47.7a	4.1	23.2a	5.1
	6	+p/-mv	46.2a	4.6	54.5 b	6.0
	6	+p/+mv	58.2a	6.0	32.9a	5.1
Quality†	6	-p/-mv	1.0a	0.00	3.17a	0.17
	6	-p/+mv	1.0a	0.00	3.50a	0.22
	6	+p/-mv	1.0a	0.00	2.00 b	0.00
	6	+p/+mv	1.0a	0.00	1.83 b	0.17

Means followed by the same letter are not significantly different. † quality score from 1 (best) to 5 (worst).

All plants were of equal quality at the start of testing. By the completion of testing, quality was significantly increased on plants infected with phytoplasma (Table 3.4.23, Figure 3.4.3). At completion of testing, phytoplasma infected plants retained a significantly higher percentage of leaves, transitional bracts and bracts below the primary cyathium. An interesting effect of phytoplasma was noticed for the infection type +p/-mv. This infection type had significantly less cyathia loss, but significantly increased bract loss above the primary cyathium.

Table 3.4.24. ANOVA for 'pooled leaves, transitional bracts and bracts below the primary cyathium' and 'bracts above the primary cyathium' for a subset of plants from Experiment 3.4.1 at the start of post-production testing as influenced by phytoplasma and virus infection

		Mean square			
Source	df	Cyathia	Leaves + trans-bracts + bracts below primary cyathium (A)	Bracts above primary cyathium (B)	A+B
Phytoplasma	1	1107**	950.0*	117.0^{ns}	1734**
Virus	1	35.04^{ns}	2.0^{ns}	210.0^{ns}	170.7^{ns}
Phyto*Virus	1	30.38^{ns}	234.4^{ns}	222.0^{ns}	0.2^{ns}
Residual	20	70.93	123.2	125.3	210.1

*, **, ***: p<0.05, 0.01, 0.001 respectively. ns: not significant.

Table 3.4.25. ANOVA for percent cyathia abscission, pooled 'leaves, transitional bracts and bracts below the primary cyathium' abscission and 'bracts above the primary cyathium' abscission for a subset of plants from Experiment 3.4.1 at 28 days after the start of post-production testing as influenced by phytoplasma and virus infection

		Mean square			
Source	df	Cyathia	Leaves + trans-bracts + bracts below primary cyathium (A)	Bracts above primary cyathium (B)	Total A+B
Phytoplasma	1	741.5*	14332***	2869***	920.1**
Virus	1	809.7*	48.50^{ns}	533.9^{ns}	175.0^{ns}
Phyto*Virus	1	1944**	243.8**	895.5*	600.0*
Residual	20	115.2	24.40	151.2	87.52

*, **, ***: p<0.05, 0.01, 0.001 respectively. ns: not significant.

Morphology of plants representative of each infection type at stage 5 (just prior to post-production testing) is shown in Figure 3.4.1 (top). Morphology of plants representative of each infection type after 28 days post-production testing is shown in Figure 3.4.3.

Figure 3.4.3. Morphology of plants representative of each infection type 28 days after post-production testing (infection types left to right are: -p/-mv, -p/+mv, +p/-mv, +p/+mv)

Discussion

The primary aim of this experiment was to determine the effect(s) of PnMV and PoiBI infection types on plant height and morphology during commercial production. The main observation in respect to this aim was that phytoplasma significantly reduced plant height in cv. Freedom red at the completion of production (stage 5) in all three experiments. This is the first such report to quantify the effects of PoiBI on poinsettia height in a commercial production environment.

The mechanism of height reduction concerns reduced mean internode length and its effect is primarily expressed in the top branch. A reduction in height in the top branch accounted for an 11 – 18% reduction in height depending upon time of year. This reduction in height and corresponding increase in branch number indicates disruption of the plants hormonal balance. Post-production testing results support this hypothesis because leaf, transitional bract and bract retention below the primary cyathium was greatest on phytoplasma infected plants 28

days after post-production testing (similar results were observed for cv. V10 Amy red in Experiment 3.3). Leaf and bract loss in poinsettia has been investigated in plants not infected with phytoplasma, and was hypothetically attributed to the concentration of endogenous auxin (Gilbert and Sink 1971). That is, higher levels promote bract and leaf retention. Therefore, it may be possible that plants infected with phytoplasma have an altered auxin concentration or distribution within the plant, resulting in significantly increased leaf, transitional bract and bract retention below the primary cyathium. It could be then expected that cyathia and bract loss above the primary cyathium could also be a result of PoiBI infection. Cyathia loss was however, greater for non-PoiBI infected plants, but bract loss above the primary cyathia was higher for PoiBI infected plants.

Auxin is responsible for apical dominance that inhibits axillary bud growth in poinsettias (Weiss and Shillo 1988). Therefore, a lowering of auxin, or redistribution may be associated with the increased branch production of phytoplasma infected poinsettias. Further studies are required to elucidate the effects of PoiBI on plant morphology. These should investigate endogenous hormone concentrations in phytoplasma infected vs. non-infected plants to determine if alteration of the hormonal balance of auxin and cytokinin is a possible cause of differences in morphology caused by PoiBI infection. Phytoplasma infection also improved post-production performance (increased quality after 28 days simulated consumer environment) through virtue of improved leaf retention. However, the number of cyathia produced initially on phytoplasma infected plants was significantly less than non-infected plants and this effect could be considered negative by poinsettia breeders.

This study also identified several morphological characteristics attributed to phytoplasma infection. Stem diameter was reduced, branch number was increased, bract and leaf shapes were altered and bract colour was slightly reduced in intensity. These results have been observed and discussed previously in Experiment 3.3. However, one observation did not concur with a published report, namely anthesis was later for the +p/-mv infection type (Preil 1994b considers anthesis is earlier) and bract rugosity was observed for all PoiBI infected plants, which has not been previously reported.

PnMV on the other hand did not impact on morphology to such an extent. One effect attributed to PnMV was increased stem diameter, which resulted, in an apparently synergistic manner, in the presence of PoiBI. Increased stem diameter has been associated with a decreased level of axillary branch breakage (Nell and Leonard 1996). Therefore, plants

infected with PoiBI and PnMV may be of greater benefit than phytoplasma alone to combat this problem. Cutting sizes in this experiment could be considered 'narrow' according to the previous authors, however, no stem breakage occurred in this experiment and infection type mean cutting diameters were in the same relative orders across experiments.

PnMV in mixed infection with PoiBI also significantly increased root fresh and dry weight in this study. This small study can be considered as a preliminary investigation, because the method of root washing was not ideal. Many small fibrous roots were lost during the washing process and many adhered to potting mix constituents, making removal difficult. Further studies on root weights should focus on using a potting mixture that separates easily from roots, and all roots should be collected.

The importance of this study and the study utilising cv. V10 Amy red is emphasized by the fact that poinsettia cultivars displaying symptoms of infection with PnMV and PoiBI may be granted Plant Breeders Rights (PBR) in Australia or a Plant Patent in the U.S.A. This is of concern, because quantitative and qualitative measures of plant morphology (including bract colour intensity, response period and growth habit) are major criteria used to determine uniqueness (Starman *et al.* 1999, Costa pers. comm. 2000) and should be based on non-infective agent induced genetic differences. This study has quantified the effects of two infective agents that commonly infect commercially grown poinsettia cultivars, and should be of benefit to these organisations, poinsettia breeders and the poinsettia industry.

Experiment 3.5. Effects of poinsettia branch inducing phytoplasma (PoiBI) on endogenous phytohormone concentrations in cv. Freedom red

Introduction

Phytoplasma (PoiBI) infection leads to a decreased plant height during commercial production of the cultivar Freedom red (Experiment 3.4). Other morphological effects of PoiBI presence include increased branching, reduced internode length, reduced stem diameter and changes in leaf and bract shape. The symptoms of phytoplasma infection suggest alteration/modification of the hormonal balance of the plant (Lee and Davis 1992). Reports are limited regarding hormone changes in phytoplasma infected plants (e.g. Das and Mitra 1998, Pertot *et al.* 1998). Both authors found alterations in endogenous hormone levels when comparing infected and non-infected plants.

This experiment was aimed to develop an initial understanding of the role of endogenous phytohormones in controlling PoiBI induced branching and alteration of morphology in poinsettias.

Materials and methods

Donor plant conditions

Stockplants of PoiBI infected and non-infected cv. Freedom red were developed in Experiment 3.2. PnMV was not present. Stockplants and clones of these plants were repeatedly tested to verify infection type (refer to Experiment 3.2). Temperature was maintained at 25°C ± 2°C during growth. Plants were drip irrigated and Osmocote®Plus 3 - 4 month slow release fertiliser was applied at the recommended rate (Appendix 2). Vegetative growth was maintained by providing incandescent light (>2 μmol m^{-2} s^{-1}) for 4 h from 10 pm.

Vegetative propagation

Uniform, 60 mm long apical cuttings were removed from relevant stockplants with a sharp knife. Excess leaves were removed. Cut ends were dipped for 5 seconds in 1500 mg/l IBA solution prior to insertion into moistened Jiffy® propagation plugs. The planted cuttings were placed into a continuous water fog for 3 weeks and then acclimatised to the greenhouse environment for 1 week by gradually reducing humidity.

Table 3.5.1. Infection types and abbreviations for plant materials

PoiBI presence	PnMV presence	Infection type	Infection type code
-p	-mv	Not infected with PoiBI	-p/-mv
+p	-mv	Infected with PoiBI	+p/-mv

Planting

Twelve cuttings with roots, with/without phytoplasma were planted into 150 mm (diameter and height) pots filled with NFP potting mix. Interpot spacing was 250 mm as measured from the centre of pots. Plants were placed under 2 x 400W diffused sodium lamps providing approximately 350 µmol m^{-2} s^{-1} light intensity between 5 am and 10 pm each day, to maintain long photoperiod and sufficient light for growth. Temperature was maintained at 25°C ± 2°C.

The following measurements were taken.

- Stem diameter (mm) at approximately 10 mm from the top of the propagation plug
- Height (mm) from the top of the plug to the tip
- Number of leaves to the most apical leaf >30 mm in length
- Number of nodes subtending leaves to the most apical leaf >30 mm in length

Mean internode length (H/N) was calculated by dividing height by the number of nodes. Osmocote®Plus 3 - 4 month slow release fertiliser was applied to the potting mix surface at a rate of 4.0 kg/m^3 of potting mix five days after planting. Plants were manually watered and not decapitated.

First sample harvest

At 13 days post-planting, six plants from each infection type were randomly selected. The measurements performed at planting were recorded for these plants. In addition, bud swelling was noted. Selected plants were then excised by cutting the shoots at the top of the propagation plug (potting mix surface). Leaves and petioles were removed with a scalpel. Each sample consisted of two shoot samples placed into a plastic 50 ml screw top tube. The 6 tubes (3 containing two +p/-mv shoots each and 3 containing two -p/-mv shoots each) were sealed and immersed in liquid nitrogen immediately after harvest. Tubes were subsequently removed after approximately 1 h and vacuum freeze dried at -52°C for 3 - 4 days. Freeze dried material was placed in air tight tubes and sent to Dr. C. Beveridge at the University of

Queensland for analysis of endogenous cytokinins and indole-3-acetic acid as listed in Table 3.5.2.

Table 3.5.2. Phytohormones quantified and abbreviations

Phytohormone	Abbreviation
indole-3-acetic acid*	IAA
trans zeatin	t-Z
trans zeatin-riboside	t-ZR
cis zeatin-riboside	c-ZR
dihydrozeatin	DHZ
dihydrozeatin-riboside	DHZR
isopentyl adenine	iP
isopentyl adenosine	iPA
zeatin-7-glucoside	Z7G
zeatin-9-glucoside	Z9G
zeatin-*0*-glucoside	ZOG
trans zeatin riboside-*0*-glucoside	t-ZROG
isopentenyl adenine-9-glucoside, Isopentenyl adenine	iP9G,iP

* auxin, all others are cytokinins.

Second sample harvest

Subsequent to the first harvest, after 13 days, a second harvest was performed as described previously.

IAA extraction, purification and GC-MS-SIM analyses

Methodology used for purification and analyses were as described in Beveridge *et al.* (1994). This involved extraction with 1M ammonium acetate buffer and distilled water at 0 - 4°C. The extract was then filtered and pH adjusted, then dried. Once dried, it was passed through a preconditioned Sep-Pak C_{18} cartridge. The IAA was eluated with MeOH and dried under vacuum. The purified sample was methylated, dried, dissolved with pyridine and silylated. Gas chromatography - mass spectrometry - selected ion monitoring (GC-MS-SIM) analysis was then performed.

Cytokinin extraction, purification and LC-MS-MS analyses

This protocol was obtained from Beveridge (pers. comm. 1999). Vacuum freeze dried tissue samples were ground in liquid N_2 and then extracted in methanol/water (1:1) containing known quantities of deuterated internal standards: [$^2H_5$]-zeatin ([$^2H_5$]-Z), [$^2H_5$]-zeatin-9-

glucoside ([$^2H_5$]-Z9G), [$^2H_5$]-zeatin-*O*-glucoside ([$^2H_5$]-ZOG), [$^2H_5$]-zeatin riboside ([$^2H_5$]-ZR), [$^2H_5$]-zeatin riboside-*O*-glucoside ([$^2H_5$]-ZROG), [$^2H_6$]-isopentenyl adenine ([$^2H_6$]-iP) and [$^2H_6$]-isopentenyl adenosine ([$^2H_6$]-iPA) (Apex Organics, UK). Extracts were centrifuged at 10,000g for 5 min, and supernatants decanted. Pellets were re-extracted twice in methanol/H_2O (1:1) and centrifuged. Supernatants from all extractions were evaporated to an aqueous phase under vacuum and allowed to react with 60 units of alkaline phosphatase (Sigma, St Louis, U.S.A.) for 2 - 3 h at 37°C. Subsequent analysis of ribosides therefore included ribosides and nucleotides. Following the phosphatase reaction, extracts were passed through a C_{18} Sep-Pak cartridge as described by Turnbull *et al.* (1997) and then evaporated to complete dryness under vacuum.

Immunopurification of cytokinins was performed as described by Faiss *et al.* (1997) except that isoprenoid cytokinin immunoaffinity columns were used (OlChemIM Ltd, Olomouc, Czech Republic). The isoprenoid cytokinin columns contain polyclonal affinity purified antibodies against trans-zeatin riboside, isopentenyladenosine and dihydrozeatin riboside. These columns bind cytokinin free bases, ribosides, 9- and 3-glucosides and could also bind 5'-nucleotides of isoprenoid cytokinins should they be present. The samples were re-dissolved in 0.5 to 1 ml PBS buffer (25 mM NaH_2PO_4, 15 mM NaCl, pH 7.2) and passed through a pre-washed (5 ml PBS) preimmune column (OlChemIM Ltd, Olomouc, Czech Republic). The eluate was collected and the preimmune column washed with a further 1 vol PBS, which was added to the initial eluate. Extracts were then passed through a pre-washed (5 ml PBS) isoprenoid cytokinin immunoaffinity column. The eluate collected was passed through the column 4 times and then was kept for *O*-glucoside analysis. Columns were then washed and cytokinins eluted as per Faiss *et al.* (1997). The samples were evaporated to dryness under vacuum.

The pH of samples dissolved in c.a. 2 ml PBS buffer was reduced to pH 5 by the addition of 20 μl 1 M HCl. One unit almond β-glucosidase (Sigma, St Louis, U.S.A.) was added to each sample, followed by an incubation for one hour at 37°C.

LC-MS-MS analyses were performed as described by Prinsen *et al.* (1995) with the exception that Z, ZR, DHZ (dihydrozeatin) and DHZR (dihydrozeatin riboside) and their corresponding glucosides were chromatographically separated from iP and iPA and their glucoside. The stationary phase was a 50 mm polar-linked triple endcapped Zorbax Bonus-RP column (50 mm x 2.1 mm id; 5 μm particle size; Hewlett Packard, Australia). The gradient was 5%

acetonitrile/ammonium acetate (10 mM) (9:1) in ammonium acetate (10 mM) to 100% acetonitrile/ammonium acetate (10 mM) (9:1) over 5.3 minutes. The flow rate was 0.3 $ml.min^{-1}$. The HPLC system (Shimadzu LC-10AT binary gradient system) was connected to an API 3000 triple quadrupole mass spectrometer (PE Biosystems, Thornhill, Canada) with an ion-spray (pneumatically assisted electrospray) interface used in positive ionisation mode (ion spray potential 5500 V; orifice potential 35 V; ring potential 200 V; 30 eV collision energy; dwell time 100 ms). Multiple reactant-ion monitoring (MRM) was used with nitrogen as the collision gas at a pressure setting of 2. Ions monitored were as listed in Table 3.5.2. The $^{2}H_{5}$-Z, $^{2}H_{5}$-Z9G and $^{2}H_{5}$-ZR standards were used to measure DHZ, dihydrozeatin-9-glucodside (DHZ9G) and DHZR, respectively. Calibration curves were all linear and peak areas of DHZ, DHZ9G and DHZR were adjusted to account for ions contributed from Z, Z9G and ZR.

Table 3.5.3. Parent ions and diagnostic transitions used in multiple reaction monitoring (MRM) for the different cytokinins analysed (from Prinsen *et al.* 1995)

Compound	Parent ion	Diagnostic transitions MRM
Z	220	220→136
DHZ	222	222→136
[$^{2}H_{5}$]-Z	225	225→136(137)
Z9G	382	382→220
DHZ9G	384	384→222
[$^{2}H_{5}$]-Z9G	387	387→225
ZR	352	352→220
DHZR	354	354→222
[$^{2}H_{5}$]-ZR	357	357→225
iP9G	366	366→204
iP	204	204→136
[$^{2}H_{6}$]-iP	210	210→136(137)
iPA	336	336→204
[$^{2}H_{6}$]-iPA	342	342→210

Refer to Table 3.5.2 for cytokinin abbreviations.

Data analyses

Data was analysed using the MINITAB® statistical software package (MINITAB® for windows release 12.22, Minitab Inc. 1998). One-way ANOVA (analysis of variance) was used to determine statistically significant differences between PoiBI infected and non-infected plants.

Results

Effects of PoiBI on plant morphology

At the planting stage, phytoplasma infected plants possessed significantly greater mean node number, but reduced mean stem diameter and internode length than non-infected plants (Table 3.5.4). Leaf number and height were not affected. Plants appeared visually similar, except for rounded leaves on infected plants, and lobed leaves on non-infected.

Table 3.5.4 Means, standard errors and mean square values for morphological characteristics of phytoplasma infected and non-infected cv. Freedom red poinsettia plants at the planting stage

Phytoplasma	n	Mean stem diameter (mm)	SE	Mean height (mm)	SE	Mean leaf number	SE	Mean node number	SE	Mean H/N (mm)	SE
-p/-mv	12	5.73	0.17	53.5	0.67	5.73	0.14	5.82	0.12	9.3	0.27
+p/-mv	12	5.21	0.10	53.2	0.54	5.75	0.13	6.92	0.23	7.8	0.24
Mean square		1.546*		0.5000^{ns}		0.2110^{ns}		6.925***		12.46***	

*, **, ***: $p<0.05, 0.01, 0.001$ respectively. ns: not significant.

At the first harvest, mean stem diameter and internode length were significantly reduced on phytoplasma infected plants. Node number was similar for both types. Mean height and leaf number were also significantly reduced for PoiBI infected plants at this stage (Table 3.5.5). Infected plants displayed swollen buds.

Table 3.5.5. Means, standard errors and mean square values for morphological characteristics of phytoplasma infected and non-infected cv. Freedom red poinsettia plants at first harvest (13 days after planting)

Phytoplasma	n	Mean stem diameter (mm)	SE	Mean height (mm)	SE	Mean leaf number	SE	Mean node number	SE	Mean H/N (mm)	SE
-p/-mv	6	5.92	0.27	68.2	0.91	8.17	0.31	8.33	0.33	8.25	0.35
+p/-mv	6	5.17	0.10	59.5	1.1	7.17	0.31	8.50	0.34	7.06	0.30
Mean square		1.688*		225.3***		3.000*		0.0830^{ns}		4.248*	

*, **, ***: $p<0.05, 0.01, 0.001$ respectively. ns: not significant.

At the second harvest, (Table 3.5.6) the same four measurements were again significantly different between infected and non-infected plants (Table 3.5.6). Infected plants had begun to produce shoots from axillary buds (Figure 3.5.1). Phytoplasma infected plants had a mean of four shoots with leaves greater than 10 mm. Non-infected plants remained unchanged.

Table 3.5.6. Means, standard errors and mean square values for morphological characteristics of phytoplasma infected and non-infected cv. Freedom red poinsettia plants at 26 days after planting (second harvest)

Phytoplasma	n	Mean stem diameter (mm)	SE	Mean height (mm)	SE	Mean leaf number	SE	Mean node number	SE	Mean H/N (mm)	SE
-p/-mv	6	7.10	0.10	87.6	1.1	11.4	0.24	11.6	0.24	7.60	0.15
+p/-mv	6	6.33	0.21	76.7	1.4	9.83	0.31	11.0	0.26	7.00	0.10
Mean square		1.603*		326.0***		6.694**		0.9820^{ns}		0.9480**	

*, **, ***: p<0.05, 0.01, 0.001 respectively. ns: not significant.

Figure 3.5.1. Morphological differences between PoiBI infected (right) and non-infected (left) cv. Freedom red poinsettias at 26 days after planting

Effects of PoiBI on endogenous phytohormone concentrations

At the first harvest, infected plants had a significantly lower IAA concentration (P=0.001**), approximately 29% less than non-infected plants (Table 3.5.7). Higher concentrations of t-Z (P=0.025*), t-ZR (P=0.006**) and DHZR (0.034*) were also found in infected plants. The increases as a percentage were approximately 94%, 215% and 233% respectively.

Table 3.5.7. Means, standard errors and mean square values for endogenous phytohormone concentrations of phytoplasma infected and non-infected cv. Freedom red poinsettia plants at 13 days after planting (first harvest)

		Hormone concentrations (pmol/gDW)									
Phytoplasma	n†	Dry weight (g/plant)	SE	IAA	SE	t-Z	SE	t-ZR	SE	c-ZR	SE
-p/-mv	3	0.170	0.01	3593	89	91.0	21	398	46	35.0	10
+p/-mv	3	0.165	0.02	2203	110	177	13	1256	152	42.0	17
Mean square		0.0001^{ns}		2896760**		11008*		1103388**		66.7^{ns}	

		Hormone concentrations (pmol/gDW)									
Phytoplasma	n†	DHZ	SE	DHZR	SE	iP	SE	iPA	SE	Z7G	SE
-p/-mv	3	7.00	0.6	9.00	2.3	19.0	0.67	158	18	6.00	0.67
+p/-mv	3	9.00	1.8	30.0	6.0	19.0	1.4	182	33	6.00	0.56
Mean square		8.167^{ns}		620.2*		1.500^{ns}		864.0^{ns}		0.1670^{ns}	

		Hormone concentrations (pmol/gDW)							
Phytoplasma	n†	Z9G	SE	ZOG	SE	t-ZROG	SE	iP9G, iP7G	SE
-p/-mv	3	0.00	0.0	38.0	13	66.0	15	0.00	0.0
+p/-mv	3	0.00	0.3	60.0	14	79.0	4.7	0.00	0.0
Mean square		0.1670^{ns}		770.7^{ns}		280.2^{ns}		0.000^{ns}	

*, **, ***: p<0.05, 0.01, 0.001 respectively. ns: not significant. † Two plants per replicate.

At the second harvest, phytohormone concentrations were slightly higher in non-infected plants for all hormones except DHZ, iP, Z7G and t-ZROG (Table 3.5.8). However, only Z9G was significantly different. This cytokinin was present at a low level in non-infected plants, but absent from infected plants.

Table 3.5.8. Means, standard errors and mean square values for endogenous phytohormone concentrations of phytoplasma infected and non-infected cv. Freedom red poinsettias at second harvest (26 days after planting)

				Hormone concentrations (pmol/gDW)							
Phytoplasma	n†	Dry weight (g/plant)	SE	IAA	SE	t-Z	SE	t-ZR	SE	c-ZR	SE
-p/-mv	3	0.3390	0.02	4434	203	127	12	348	121	41.0	1.0
+p/-mv	3	0.3025	0.02	3829	356	113	26	214	68	25.5	11
Mean square		0.0080^{ns}		549643^{ns}		294^{ns}		27068^{ns}		240^{ns}	

		Hormone concentrations (pmol/gDW)									
Phytoplasma	n†	DHZ	SE	DHZR	SE	iP	SE	iPA	SE	Z7G	SE
-p/-mv	3	4.33	0.88	13.7	2.7	12.0	3.0	213	101	7.50	1.5
+p/-mv	3	6.67	1.4	11.0	1.1	19.0	4.0	118	20	25.5	18
Mean square		8.170^{ns}		10.70^{ns}		73.50^{ns}		13443^{ns}		324^{ns}	

		Hormone concentrations (pmol/gDW)							
Phytoplasma	n†	Z9G	SE	ZOG	SE	t-ZROG	SE	iP9G, iP7G	SE
-p/-mv	3	2.50	0.5	62.5	13	52.5	5.5	0.00	0.0
+p/-mv	3	0.00	0.0	46.5	30	68.0	25	0.00	0.0
Mean square		6.250*		256^{ns}		240^{ns}		0.00^{ns}	

*, **, ***: $p<0.05$, 0.01, 0.001 respectively. ns: not significant. † Two plants per replicate.

Discussion

This experiment was aimed to provide an understanding of the role of phytohormones in PoiBI-induced height reduction and morphological alteration in cv. Freedom red (i.e. increased branching, reduced stem diameter, changes in leaf shape). The key observation was that PoiBI altered endogenous phytohormone concentrations at the first sampling. Infected plants contained a significantly higher concentration of three cytokinins (t-Z, t-ZR and DHZR) and a significantly reduced concentration of IAA. Cytokinins promote cell expansion and initiate growth in inactive lateral buds, or buds inhibited by apical dominance (Moore 1984, Kaminek *et al.* 1987). In poinsettia, exogenous application of cytokinins has been shown to increase branch number (in non-decapitated free-branching and restricted-branching plants) on numerous occasions (Carpenter *et al.* 1971, Milbocker 1972, Ching 1985, Semeniuk and Griesbach 1985, Kaminek *et al.* 1987, Witaszek 1989). IAA is known to promote apical dominance resulting in axillary bud inhibition in poinsettia (Weiss and Shillo 1988). Therefore, it could be expected that decapitation which results in removal of apical dominance could also lead to lower IAA concentrations in plants. Young expanding leaves

(YELs) on vegetative poinsettias are the major source of axillary bud inhibition. The next greatest source is the apical bud (which includes the meristem, primordial and small unfolded leaves) (Weiss and Shillo 1988). Auxin concentration is highest (on a whole organ basis) in YELs compared to the apical bud.

Kaminek *et al.* (1987) reported that cuttings of a non-branching poinsettia, when planted at an oblique angle, resulted in increased branching. This same result was observed after another study by the author (unpublished) when plants of Angelika free of PoiBI were laid down horizontally for one week and were then stood upright. Plants originally had one stem with no branches. However, shoots had begun to develop from the previously dormant axillary buds after one week of being laid horizontal. Controls remained unbranched. The change of orientation (being laid horizontal) could have resulted in a change of apical dominance and induction of branching when plants were stood upright after one week.

Increases in major cytokinins coupled with a reduction in IAA could be partly responsible for induction of axillary bud growth and subsequent branching in PoiBI-infected poinsettias. The significant differences in plant height measured at all three stages may also be attributed to changes in hormone concentrations.

Changes in phytohormone concentrations affect cell elongation and division. In this experiment it was hypothesised that IAA and cytokinins would most likely be involved due to the presence of increased branching. IAA and cytokinins also influence cell length and division. In this experiment it is likely that altered cytokinin and IAA concentrations resulted in a reduction in mean internode length (H/N) because PoiBI infected plants possessed mean H/N values significantly less than non-infected plants at all three measurement stages.

How PoiBI influences endogenous hormone concentrations, apparently during a short specific period is unknown. PoiBI may produce hormones and elicit an immune response, or change the distribution of phytohormones. Alternatively, PoiBI could trigger gene expression resulting in higher release of phytohormones. Future studies should aim to identify the alterations in IAA, cytokinin and other endogenous hormone concentrations analogous to the relevant growth phases.

It may be possible to achieve a similar effect to PoiBI induced branching and dwarfness in non-infected poinsettias by transformation with a gene coding for cytokinin production,

governed by an inducible promoter. This could provide a means to produce branching in poinsettias at a required time without any infecting phytoplasma. Such a system may be of benefit to the production of different crop types. For example, non-apically decapitated products such as 'straight ups' or standards could be produced without unwanted axillary branching. This system also has application for the development of a somatic embryo vegetative propagation system for commercial production. At present, one of the problems with the somatic embryo system is the loss of PoiBI. By introducing an inducible cytokinin gene or by application of endogenous cytokinin (e.g. spray) production of suitably branched poinsettias could be possible. In addition, poinsettias grown without PoiBI could be seen as a way to avoid potential adverse effects of PoiBI on the environment (if any) due to its relationship with western-X phytoplasma.

An alternative method to induce branching of non-PoiBI infected poinsettias could be to change orientation of the plants for a defined period. This would encourage branching from axillary buds.

Chapter 4. Studies on factors affecting the development of intraspecific hybrids for breeding dwarf poinsettias

General introduction

Plant height in poinsettia is governed by genotype, PoiBI infection and the growth environment. Investigation of inheritance of height in poinsettias is reliant upon the production of genetic recombinants via intraspecific hybridisation. Genetic recombinants allow for accumulation of genes (major and minor) responsible for dwarfness and/or other traits that may influence or contribute to plant height (such as branching and response time). Therefore, by accumulating traits for dwarfness, poinsettias that are shorter than current cultivars could be expected as an outcome.

To breed shorter poinsettias, it is imperative that variability for genes contributing to plant height is present within the selected breeding population. Variability has been demonstrated for height and associated traits within commercial cultivars (Ecke *et al.* 1990, Hammer and Kirk 1992, Larson and Hammer 1997, Larson *et al.* 1998, Larson *et al.* 1999a and b, Ecke 2000, Fischer 2000). However, data was obtained from commercial trials and from breeders' descriptions and is not statistically robust. Commercial trials were not designed to compare plant height in statistically designed experiments (e.g., cuttings were often obtained directly from breeders, and different treatments were applied to different cultivars) and product descriptions from breeders could be subjective. Furthermore, genetic expression of height in commercial cultivars could have been modified by PoiBI, compounding the problem. Experiment 3.4 showed that PoiBI infected cv. Freedom red plants were significantly shorter than non-infected plants when grown according to commercial practices. Therefore, to determine the true genotype of commercial cultivars, PoiBI needs to be removed. Seedlings developed from parents infected with PoiBI do not possess PoiBI since it is not seed transmissible (Dole *et al.* 1993).

PoiBI often exists in a mixed infection with PnMV and PnCV. PnMV did not influence height as shown in Experiments 3.3 and 3.4 and results from Chapter 3 indicated PnMV was not seed transmissible, confirming results of Fulton and Fulton 1980 (unpublished data) and Koenig and Lesemann 1980. PnCV is symptomless (Ecke *et al.* 1990, Bertaccini *et al.* 1996, Schoenfelder pers. comm. 1999) and has not been shown to influence plant height in the available literature. Chapter 3 also demonstrated that PnCV was seed transmissible.

Therefore, seedlings produced may be infected with PnCV, which however, does not influence morphology.

For seedlings to be produced, viable seeds must first be generated from pollinations. Experiment 4.1 deals with factors affecting viable seed production. An understanding of these factors is necessary for efficient production of seeds, and furthermore the production of shorter poinsettias. Following seed production, variability for height was examined in the population developed. Short lines were then selected and examined under the commercial production environment in comparison to current commercially available 'short' cultivars.

These experiments were designed to assess whether genetically dwarf poinsettias devoid of PoiBI could be produced utilising current commercial cultivars as parents. It could be expected that if dwarf plants were obtained without the influence of extra-genetic factors, then genes for dwarfing do exist within the commercial cultivar genepool.

Experiment 4.1. Investigation of factors affecting seed production

Introduction

Production of viable seeds via hybridisation in poinsettias could be governed by factors such as ploidy differences between parents (Ewart and Walker 1960, Bempong and Sink 1968b, Milbocker and Sink 1969a and b) self and cross incompatibility (Bempong 1967) parental fertility, inbreeding depression (Sink pers. comm. 1997) and environmental factors such as temperature, humidity and time of pollination. Understanding the pollination mechanism is imperative for seed production. Low seed set is suggested to be a typical problem as a result of conducting intraspecific crosses (Sink pers. comm. 1997, Rinehart pers. comm. 1997). Low seed set may involve some form(s) of incompatibility (Bempong 1967) and/or inbreeding depression (Sink pers. comm. 1997). A few decades ago, some authors showed that seed production was possible. However, they used cultivars that have been superseded, and are not commercially available at present (Semeniuk and Stewart 1960, Milbocker and Sink 1969a). Therefore, establishment of a working pollination method for crossing current commercial cultivars is a pre-requisite for the development of progeny. These progeny could be expected to produce shorter poinsettias if variability for height is present within this population.

Pollination studies conducted by the author prior to commencement of the current project indicated that producing seeds from current commercial cultivars was a difficult task (Bernuetz unpublished 1995). The cultivars used were V10, V14, V17, Freedom series, Lilo, Hegg series (Diva, Lady, Diva Starlight, Hot pink, Jingle bells III, Dark red, Top white) Celebrate 2, Supjibi, Lemon drop, and Pink peppermint. From 324 random cross and self pollinations performed between plants of various ages under natural daylength in a greenhouse (where temperature was not controlled) only seven seeds were produced. This preliminary research resulted in the production of three seedlings from the following crosses: V10 Amy red x Lady (V10RxL), Diva x Pink peppermint (DxPP) and Pink peppermint x Diva (PPxD). Cytological studies were also performed prior to this investigation to determine the ploidy of some commercial cultivars, because differences in ploidy directly affect the production of seeds. The diploid 2n = 28 chromosome number has been previously reported (Ewart and Walker 1960, Bempong 1967). Preliminary studies showed four current commercial cultivars possessed the following chromosome numbers: Freedom red 2n = 28, Freedom white 2n = 28, V10 Amy red 2n = 28 (all diploid) and Supjibi 2n = 56 (tetraploid) (cytology protocol presented in Appendix 3).

Study of the pollination process was divided into two components i. - quantification of male and female fertility, as based on the ability of plants to set seed under an artificial short day environment and to produce plants from these seeds and ii. - determination of additional pollination parameters contributing to seed production such as the developmental stage of stigmas, pollination repetition requirement and pollen germination on stigmas. Additionally, seed germination *in vivo* and *in vitro* was briefly explored.

Materials and methods

Experimental set-up

Environmental conditions and parent plant descriptions

Plants from a range of cultivars and lines were grown (Table 4.1) in 150 or 200 mm pots as per standard commercial practices (NFP media, drip irrigation and the recommended rate of Osmocote®Plus 3 - 4 month slow release fertiliser was applied. Refer to Appendices 1 and 2).

Plants were grown in 'microclimate' growth rooms maintained at 21°C ± 1°C in a greenhouse (Figure 4.1.1 - Environment E1). Short photoperiod (10 h) for floral induction and continued cyathial production was provided. During the light phase of the photoperiod of 10 h, in addition to natural light, supplementary light was provided by Osram® Powerstar HQI-T 250W/D metal halide lamps at approximately one lamp per 1.5m^2 bench area. Light flux density was approximately 300 $\mu mol\ m^{-2}\ s^{-1}$ at approximate plant height measured at noon in winter. During the skotoperiod (dark phase), rooms were covered with a light impenetrable black cloth (Figure 4.1.1).

Plants were tested (refer to Experiment 3.1) for presence/absence of PnMV, either via the ELISA method or by visual observations by growing at 18 - 20°C for approximately 2 - 4 weeks and scoring for mottling. Plants that tested negative to PnMV were re-tested 2 - 3 times via ELISA over a period of at least twelve months. Two lines (V10RxL1 and V10RxL2) and one cultivar (Freedom white) were also tested for PoiBI via PCR however, most lines/cultivars were assessed visually for the presence of 'free-branching', which indicates infection with this micro-organism.

Figure 4.1.1. Environment E1 (utilised for short photoperiod growth). Top: inside microclimate room, bottom: external view

Table 4.1.1. Details of germplasm used and infection status

Plant code	Cultivar, seedling or line name	Pedigree (and breeder)	PoiBI	PnMV
DxPP1	Diva x Pink peppermint	Seedling grafted to obtain PnMV[1] (Bernuetz)	-	+
DxPP2	Diva x Pink peppermint	Seedling, grafted to obtain PoiBI[1] (Bernuetz)	+	+
DxPP3	Diva x Pink peppermint	Seedling, grafted to obtain PoiBI[1] (Bernuetz)	+	+
FR4	cv. Freedom red	Induced sport of a seedling[2] (Paul Ecke Ranch)	+	+
FR5	cv. Freedom red	Induced sport of a seedling[2] (Paul Ecke Ranch)	+	+
FW3	cv. Freedom white	Induced mutation of Freedom red[2] (Jacobsen)	+	+
FW4	cv. Freedom white	Induced mutation of Freedom red[2] (Jacobsen)	+	+
FW5	cv. Freedom white	Induced mutation of Freedom red[2] (Jacobsen)	+	+
FW6	cv. Freedom white	Induced mutation of Freedom red[2] (Jacobsen)	+*	+
HP2	cv. Hot pink	Mutant of Annette Hegg Dark red[3] (Hegg)	+	+
LD4	cv. Lemon drop	Seedling[3] (Paul Ecke Ranch)	+	+
PP4	cv. Pink peppermint	Seedling[2] (Fruehwirth)	+	+
PP5	cv. Pink peppermint	Seedling[2] (Fruehwirth)	+	+
PPxD2	Pink peppermint x Diva	Seedling, grafted to obtain PoiBI[1] (Bernuetz)	+	+
S2.1	S2.1	Induced mutant segregant from A.H. Diva[4] (Derera)	+	+
S2.3	S2.3	Induced mutant segregant from A.H. Diva[4] (Derera)	+	+
S4	S4	Induced mutant segregant from A.H. Diva[4] (Derera)	+	+
SR	cv. Single red	Unknown	n.t.	n.t.
SUP	cv. Supjibi	Possible tetraploid of V-17 Angelika[5] (Gross)	+	+
TW1	cv. Top white	Mutant of Annette Hegg [3] (Jacobsen)	+	+
TW2	cv. Top white	Mutant of Annette Hegg [3] (Jacobsen)	+	+
V10R1	cv. V10 Amy red	Seedling[2] (Gutbier)	+	+
V10R2	cv. V10 Amy red	Seedling[2] (Gutbier)	+	+
V10R3	cv. V10 Amy red	Seedling[2] (Gutbier)	+	+
V10R4	cv. V10 Amy red	Seedling[2] (Gutbier)	+	+
V10RxL1	V10 Amy red x Lady	Seedling grafted to obtain PnMV[1] (Bernuetz)	-**	+
V10RxL2	V10 Amy red x Lady	Seedling, grafted to obtain PoiBI[1] (Bernuetz)	+**	+
V10RxL3	V10 Amy red x Lady	Seedling, grafted to obtain PoiBI[1] (Bernuetz)	+	+
V10W2	cv. V10 white	Mutant of V10 pink[3] (Gutbier)	+	+
Lady	cv. Annette Hegg Lady	Mutant of Annette Hegg Dark red[3] (Hegg)	n.a.	n.a.
Diva	cv. Annette Hegg Diva	Mutant of Annette Hegg red[3] (Hegg)	n.a.	n.a.

Abbreviations: (+): infected, (-): not infected, (n.t.): not tested, (n.a.): not applicable, (*): PCR confirmation once, (**): PCR confirmation twice. [1] Genotype developed by the author, [2] U.S.A. Patent and Trademark Office, [3] Ecke *et al.* 1990, [4] Original genotype developed by Derera, [5] Starman and Abbitt 1997.

Crossing

Pollinations (self and cross) were randomly performed among plants during the course of cyathia production. Pollen was applied liberally to all areas of the stigma. Cyathia were individually tagged and tags were labelled with the cross number. Emasculation was not performed because i. it would result in destruction of the cyathial wall and subsequent cyathial abortion, ii. observation of hundreds of plants in the controlled environment rooms

(which were insect-free) revealed that self-pollinated seeds were never produced and iii. the structure of the cyathium limited self-pollination because anthers do not contact the stigma. The following data were recorded in a crossing book for each pollination: cross number, female parent, male parent, date, time, cyathia order, gland description (open/closed and colour), nectar (amount), stamens (number, dehiscence), stigma description and pollen appearance (old or fresh). Stigma descriptions were based on a scale from 0 to 1. Zero was equivalent to unopened, 1/8 open was equivalent to the tri-lobed stigmas just beginning to separate, ½ described stigmas that had reflexed approximately 90° and 1 was noted as fully open stigma lobes prior to the reflexion of the tips. Repeated pollinations were performed on some stigmas one or two days after the first pollination. The number of replicates was noted.

Post-pollination observations

Ovaries were observed approximately every second day after pollination. The date of ovary abortion (including diameter and contents of locules) and the date of seed harvest were recorded. Where seeds were collected, the date of harvest and number per ovary were also recorded. Seed quality was graded as per visual observation (1 = best, solid seed; 2 = average seed with some air pockets remained between the seed coat and endosperm and 3 = shrivelled).

Pollen germination assessment

Pollen germination was investigated on 159 stigmas from both self and cross pollinations between 1 and 4 weeks post-pollination. Pistils were collected in small glass vials and covered with 0.1% aniline blue stain dissolved in 0.05M Na_2HPO_4 that was adjusted to pH 11 (Ehrenfeld 1976, Dumas and Knox 1983). Approximately after one hour at 21°C, pistils were dissected and observed under the fluorescent compound microscope at x 125 magnification (Figure 4.1.6). The number of germinated pollen grains on a stigma was counted.

Sowing harvested seeds

Most seeds (84%) were sown 1 - 7 days after collection in the greenhouse. Seeds were placed into 70 ml plastic growth tubes containing NFP potting mix, at a depth of 10 - 20 mm. Temperature during germination and growth was maintained at 25°C ± 2°C, and long photoperiod conditions were provided with intermittent supplementary lights turned on initially for 10 h, then subsequently for 4 h from 10 pm. Supplementary lighting was provided by Osram® Powerstar HQI-T 250W/D metal halide lamps approximately at one lamp per 4 m^2

bench area. Light intensity was approximately 350 μmol m^{-2} s^{-1} measured 1 m directly below the lamps.

Some seeds (16% - all grade 2) were sown *in vitro*. Each seed was placed into a vial containing 5% NaOCL (Sodium hypochlorite) and a drop of Tween 20. Vials were closed with a lid and intermittently shaken for 10 minutes. Seeds were then removed, rinsed once in autoclaved distilled water and allowed to dry in a laminar flow cabinet. Once dry, seed coats were removed and the resulting embryo/endosperm complex was measured. These de-coated seeds were graded according to length, large = 4 - 6 mm, medium = 3 - 4 mm and small <3 mm. Measured de-coated seed were randomised and then placed firmly onto one of four possible media. Media types used were CH, MP, MS and SE (refer to Table 3.2.6). Vessels were then placed for incubation in the growth room (described in Experiment 3.2). After 21 days, each de-coated seed was visually scored for germination, swelling or no change. Seeds that germinated *in vitro* were deflasked inside the greenhouse and propagated in Jiffy® plugs at 23 ± 2°C. High humidity and light intensity of approximately 150 μmol m^{-2} s^{-1} were provided. After 3 - 4 weeks, plants were gradually acclimatised to the greenhouse environment. Once acclimatised, plants were treated in a similar manner to plants derived from greenhouse sown seeds.

Date, location and results of sowing (germination, non-germination, albino development, death post-germination) were recorded.

Seedling growth and characterisation

Subsequent to the emergence of the second set of leaves on each greenhouse sown seedling, planting was performed into 200 mm pots containing NFP potting mix and Osmocote®Plus 3 - 4 month slow release fertiliser applied as a top dressing at the recommended rate per pot. Plants were grown under conditions as described at sowing. Interpot distance was 350 mm as measured from the centre of the pots. Water was applied manually. Conventional pest and disease control measures were utilised when necessary. Approximately after 16 weeks from sowing, plants were visually characterised for height and abnormalities, if any.

Gibberellic acid application

The effect of gibberellic acid application on some plants was briefly investigated (Mynett and Wilkonska 1989, Bernuetz 1995 (unpublished)). Two vegetative plants of the cultivars Freedom red, Freedom white, Lemon drop, Hot pink and Pink peppermint received a foliar

spray application to completely cover leaves with 1000 mg/l GA_3. Control plants of each cultivar were also grown. Controls were sprayed with the ethanol/water solution without GA_3.

Results

General observations

Seeds were successfully produced under artificial short photoperiod conditions and approximately 59% of seeds developed into plants. Table 4.1.2 presents data on the number of pollinations performed through to the number of plants produced in total.

Twelve to sixteen weeks were required from the date of pollination to seed ripening, depending upon the female genotype. Production of seeds could be predicted by observing ovary swelling within one to three weeks post-pollination. Ovary swelling was correlated with seed production. This correlation was high because 87% of all swollen ovaries produced seed(s). The remaining 13% of swollen ovaries that did not produce seed(s) aborted. These swollen ovaries always contained at least one enlarged ovule, which appeared arrested prior to developing into a seed. Ovary swelling was used as an indicator of successful pollination and this development enabled early identification of compatible crosses.

The number of pollinations performed totalled 709. Of these, 24% produced seed(s), 63% aborted without swelling and 13% aborted with swelling (always containing at least one enlarged ovule). Enlarged ovules and shrivelled seed(s) were frequently found in remaining locule(s) of ovaries that produced less than three seeds. Figure 4.1.2 shows ovaries during seed development and the appearance of viable seeds. Figures 4.1.3 and 4.1.4 show the various ovary contents produced.

The efficiency of seed production calculated on a per ovule basis was 14.3%. From a total of 304 seeds produced, 39% were grade 1, 45% were grade 2 and 16% were grade 3. Seeds germinated within 2-4 weeks in the greenhouse and *in vitro*. In the greenhouse, grade 1 seeds were most fertile, resulting in 93% germination, grade 2 recorded 63% and grade 3 was 0%, indicating total infertility. However, 6.6% of grade 1 and 2 seeds that germinated died soon after germinating. These seedlings were frequently either albinos or chimeric. Two fully expanded leaves were produced on normal seedlings within 4 - 6 weeks of germination in the greenhouse. At this stage, sufficient root development had occurred on most plants to allow planting into 200 mm pots.

Figure 4.1.2. Ovaries during seed development (top) and ripe seeds (x 4 mag., bottom)

Table 4.1.2. Outcomes of pollinations conducted among poinsettia cultivars and lines listed in Table 4.1.1.

Parameter	Pollination results and efficiency
Number of ovaries pollinated	709
Number of ovules pollinated	2127
Number of seeds produced	304
Ovaries producing seeds	**24%** (167)†
Ovaries containing a single seed	10% (72)
Ovaries containing two seeds	8% (55)
Ovaries containing three seeds	6% (40)
Ovaries containing three shrivelled ovules (aborted)	63% (450)
Ovaries containing at least one enlarged ovule (aborted)	13% (92)
Ovules producing seeds	**14.3%** (304)
Grade 1 seeds produced	5.5% (118)
Grade 2 seeds produced	6.4% (136)
Grade 3 seeds produced	2.4% (50)
Single pollinations that produced seeds	22% (55/245)
Double pollinations that produced seeds	24% (105/429)
Triple pollinations that produced seeds	20% (7/35)
Mean seed set per ovary for effective pollinations	1.8 (304/167)
Seeds sown in greenhouse	84% (256/304)
Seeds sown *in vitro*	16% (48/304) (43 grade 2, 5 grade 3)
Seed germination	**64%** (196/304)
Grade 1 seeds germinated	93% (110/118)
Grade 2 seeds germinated	63% (86/136) (22/43 *in vitro*, 64/93 greenhouse)
Grade 3 seeds germinated	0% (0/50)
Rapid death post germination	6.6% (13/196)
Grade 1 seeds	1
Grade 2 seeds	12
Grade 3 seeds	0
Total plants produced per ovule	**8.6%** (183/2127)
Grade 1 seeds producing plants	92% (109/118)
Grade 2 seeds producing plants	54% (74/136)
Grade 3 seeds producing plants	0% (0/50)
Plants remaining *in vitro*, etc	4
Final plant number in the greenhouse	179
Normal plants	85% (153/179)
Weak plants‡	15% (26/179)
Grade 1 seeds producing weak plants	16
Grade 2 seeds producing weak plants	10

† Parentheses indicate actual number for each calculation. ‡ See following text and figure 4.1.5.

Figure 4.1.3. Ovary appearance (top) and range of contents (bottom) from unpollinated non-swollen, aborted ovaries through to pollinated swollen aborted ovaries

Figure 4.1.4. Range of contents from unpollinated non-swollen, aborted ovaries through to pollinated swollen aborted ovaries (Figure 4.4.3) longitudinally bisected to reveal ovule contents

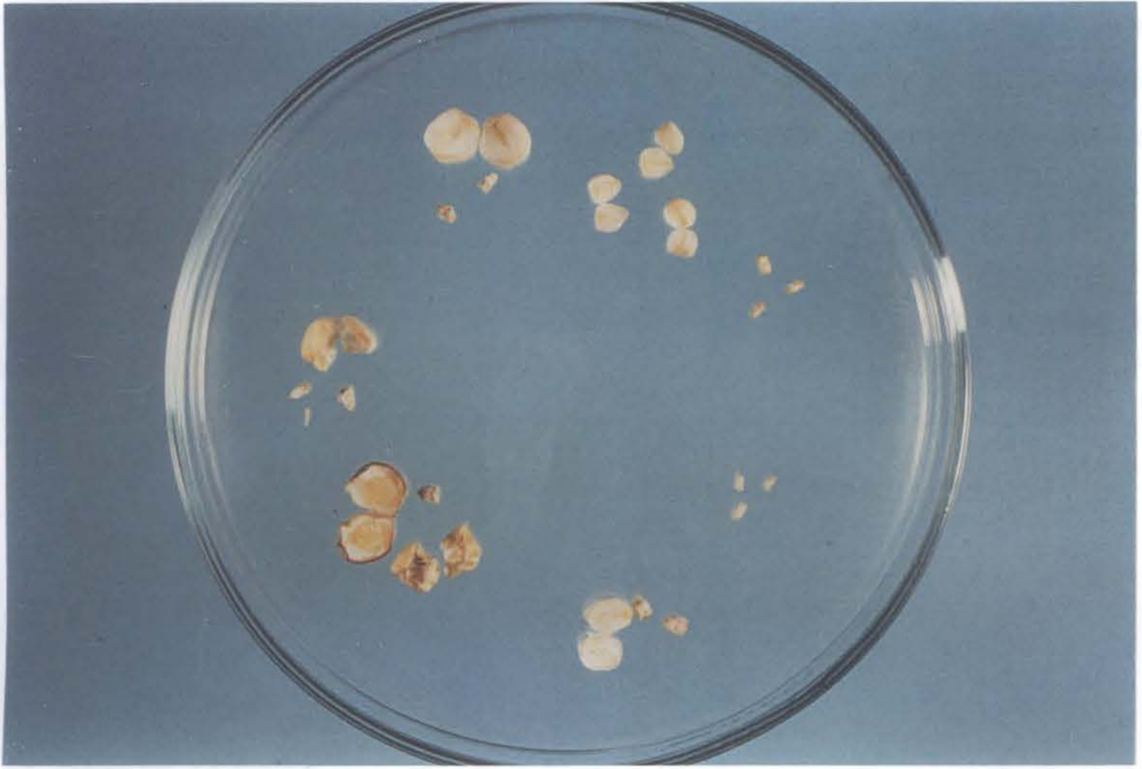


Figure 4.1.5. Weak plant phenotype (far right) shown in comparison to three normal seedlings (left)

Germination of grade 2 seeds sown *in vitro* (49%) was less than that recorded for the same seed grade sown in the greenhouse (69%). From the 43 seeds sown *in vitro*, 49% germinated (21/43), 32% became swollen (14/43) and 19% did not change (8/43). Large, de-coated seeds (4 - 6 mm) had a higher *in vitro* germination rate (67%) than smaller sizes. There appeared to be no effect of media on germination, because a similar number of seeds germinated on all media types (Table 4.1.3). A single seed developed a shoot derived from the endosperm tissue, and a germinated embryo. The plants derived from these two tissues are characterised in Experiment 7.3.

Table 4.1.3. Seed number, grade and germination results after 21 days from planting de-coated seeds *in vitro*

De-coated seed size	Media	Number of seeds sown	Number germinated	Total % germinated	Number swollen	Number unchanged
Large	CH	4	2		2	0
	MP	4	3	67%	1	0
	MS	6	3		3	0
	SE	4	4		0	0
Medium	CH	5	3		1	1
	MP	7	3	43%	2	2
	MS	5	2		2	1
	SE	4	1		2	1
Small	CH	1	0		0	1
	MP	0	0	0%	0	0
	MS	0	0		0	0
	SE	3	0		1	2
Total		43	21		14	8

Seedlings from *in vitro* culture were successfully acclimatised under the greenhouse environment.

The final number of plants in the greenhouse was 179 of which 15% were termed 'weak'. Weak plants were characterised by very short internodal spacing (<5 mm), chlorophyll deficiencies (mosaic leaves), slow growth and leaf abnormalities (refer to Figure 4.1.5). Weak plants resulted from both grade 1 and grade 2 seeds, indicating that abnormalities arose regardless of seed quality or germination potential. The remainder appeared morphologically normal. Within this 'normal' population, variation for height and internode length was

displayed. Parents generally considered as tall tended to produce tall progeny when selfed or intermated and likewise, short parents tended to produce short progeny.

Quantification of female and male fertility

Data for male and female fertility and seed produced on a per ovule basis is presented in Table 4.1.4. The five most fertile female parents determined based on seed production after random pollinations were (in descending order of fertility) V10RxL1, V10RxL2 and 3, DxPP1, FR and S4. The five most fertile male parents were FW, FR, V10RxL2 and 3, PP and V10RxL1.

Some genotypes possessed abnormalities, e.g. cv. Lemon drop appeared female sterile, PPxD never produced female cyathia, S2.3 and cv. Top white both had very low pollen production and DxPP appeared chimeric. Pollinations using cv. Supjibi as a female resulted in ovary swelling on three occasions (twice crossed with FR and once with V10R). Enlarged ovules were produced from the swollen pods that eventually aborted (refer to Table 4.1.5). The reciprocal cross never resulted in swelling.

Seeds were produced from plants infected with both PnMV and PoiBI. However, plants infected with PoiBI had reduced mean fertility based on seed production per ovule pollinated. i.e. DxPP1 (-p/+mv) = 27.1% vs. DxPP2+3 (+p/+mv) = 11.4%, V10RxL1 (-p/+mv) = 36% vs. V10RxL2+3 (+p/+mv) = 27.5%. All plants were infected with PnMV, indicating differences in seed production were not due to infection with this virus. Both V10RxL and DxPP infected with PoiBI produced a greater proportion of grade 1 + grade 2 seeds in comparison to their non-PoiBI infected counterparts, indicating that PoiBI infection may reduce seed number, but increase seed quality of the seeds obtained. i.e. the percentage of grade 1 + grade 2 seeds from DxPP1 (-p/+mv) was 61.5% vs. DxPP2+3 (+p/+mv) with 71.4% and V10RxL1 (-p/+mv) recorded 79.7% vs. V10RxL2+3 (+p/+mv) with 88.7%. Further studies with larger numbers of plants, crosses and cross combinations are required to determine if the effects of PoiBI on seed production are significant.

Table 4.1.4. Male and female fertility data and production efficiencies†

♂ ♀	DxPP1	DxPP2 DxPP3	FR4 FR5	FW (3,4,5,6)	HP2	LD4	PP4 PP5	PPxD2	S2.1 S2.3	S4	Single Red	SUPJ.1	TW1 TW2	V10R (1,2,3,4)	V10RxL1	V10RxL2 V10RxL3	V10W2	TOTAL	Efficiency seed/ovule plant/ovule
DxPP1	4,0,0		3,5,2	1,0,0		1,0,0				1,0,0				2,4,2	2,2,0	2,2,1		16,13,5	27.1% 10.4%
DxPP2 DxPP3	1,0,0	8,0,0	6,6,2	3,3,0		4,1,1	3,0,0		2,0,0					9,2,2		5,2,1		41,14,6	11.4% 4.9%
FR4		1,0,0	4,3,2			2,0,0								3,5,3				10,8,5	26.7% 16.7%
FW (3,4,5,6)	2,0,0	2,0,0	16,2,1	6,0,0	6,0,0	7,0,0	1,0,0			5,1,1	3,0,0	1,0,0		26,8,2	2,2,2	8,1,0		85,14,6	5.5% 2.3%
HP2		2,0,0	6,7,5	1,1,0	15,0,0	3,0,0	5,4,1			1,0,0		6,0,0		4,2,1		2,2,1		45,16,8	11.9% 5.9%
LD4			3,0,0			1,0,0					1,0,0	3,0,0		2,0,0				10,0,0	0% 0%
PP4 PP5		4,0,0	5,0,0	3,0,0	14,4,4		14,0,0	2,0,0		3,2,2			1,0,0	12,7,7		2,1,1		60,14,14	7.8% 7.8%
S2.3			5,0,0			2,3,3			5,0,0					3,1,1		5,6,2		20,10,6	16.7% 10%
S4			7,5,2			3,1,0	3,3,3			9,0,0				15,13,5				37,22,10	19.8% 9%
SUPJ.1			12,0,0									11,0,0		12,0,0				35,0,0	0% 0%
TW1 TW2	4,0,0	7,0,0	19,2,1	11,0,0	7,0,0	5,0,0				4,0,0	3,1,0	8,0,0	4,0,0	31,1,1		6,0,0		109,4,2	1.2% 0.6%
V10R (1,2,3,4)			12,9,3	6,6,5	1,0,0		4,0,0				9,5,0	3,0,0		36,19,6		8,3,0		79,42,14	17.7% 5.9%
V10RxL1	4,2,1	1,1,1	10,21,13	4,14,11		10,9,8	1,2,2			4,0,0		7,0,0		8,10,6	12,6,3		1,2,1	62,67,46	36% 24.7%
V10RxL2 V10RxL3		4,0,0	13,21,14	7,13,11	7,1,1	9,5,5	5,11,9	2,2,0		7,1,0		5,0,0	2,0,0	18,9,9	3,0,0	15,17,8		97,80,57	27.5% 19.6%
V10W2		1,0,0	2,0,0															3,0,0	0% 0%
TOTAL	15,2,1	30,1,1	123,81,45	42,37,27	50,5,5	47,19,17	36,20,15	4,2,0	7,0,0	34,4,3	16,6,0	44,0,0	7,0,0	181,81,45	19,10,5	53,34,14	1,2,1	709,304,179	14.3% 8.4%
Efficiency seed/ovule plant/ovule	4.4% 2.2%	1.1% 1.1%	21.9% 12.2%	29.4% 21.4%	3.3% 3.3%	13.5% 12.1%	18.5% 13.9%	16.7% 0%	0% 0%	3.9% 2.9%	12.5% 0%	0% 0%	0% 0%	14.9% 8.3%	17.5% 8.8%	21.4% 8.8%	66.7% 33.3%	14.3% 8.4%	

† The numbers in each cell of the table represent: the number of pollinations, seed set and the number of surviving plants.

Pollination factors contributing to seed production

The following determinations were made from 167 pollinations, which resulted in seed production.

Cyathia production. The cyathia order at which seed could be produced depended upon the position at which hermaphrodite cyathia were located. Male cyathia occupy the first and second order positions on most plants, as such the earliest hermaphrodite cyathium was either tertiary or quaternary.

Pollen. Pollinations conducted using both fresh (new stamens produced daily) and older pollen (>1 day old) resulted in seed production.

Pollination time. Pollinations were successful between the times of 8:00am through to 4:00pm.

Stigma developmental stage. Seeds were produced from stigmas of maturity from ¼ to ¾. Older pistils with fully reflexed stigmas subtending ovaries that were fully exerted from the cyathial cup were not effective for seed production.

Pollination repetition. Repetition of pollination did not result in significantly increased seed production (refer to Table 4.1.2).

Other parameters. Colour, openness and nectar content of the cyathial gland did not correlate with successful pollination. Presence of stamens did not influence pollination success, however the absence of stamens at pollination is desired to avoid any chance of self pollination.

Pollen germination

From a total of 159 stigmas investigated, 125 subtended an ovary that later aborted and 34 subtended an ovary that produced seed. Pollen was autofluorescent and pollen tubes were only visible upon staining with aniline blue stain (Figure 4.1.6). 151 stigmas possessed at least one germinated pollen grain on the stigma surface. Pollen germination occurred on both (a) stigmas subtending ovaries that produced seed, and (b) stigmas subtending ovaries that later aborted (Table 4.1.5). This indicated that self incompatibility (SI) mechanisms that prevent

pollen germination were not in operation at the stigma surface because both aborted and successful ovaries possessed germinated pollen at the stigma surface.

To determine if SI mechanisms were in operation inside the stigma and upper style, presence of pollen tubes within these tissues were observed. In most cases, pollen tube penetration of the stigma was difficult to distinguish from the auto-fluorescent internal structures of the stigma. However, of the 151 pistils with pollen germination present, penetration of the stigma and growth of the pollen tube down the style was unambiguously recorded in 18 (12%) (refer to Table 4.1.5). Out of these 18 pistils, 12 (67%) were from ovaries that produced seed(s). The other 6 (33%) of these pistils subtended ovaries that aborted. This showed that even when pollen tubes penetrate the stigma and the upper style, seed production may still not occur. Therefore, self incompatibility mechanisms are not likely to be operational inside the stigma or upper style. They may however, be present further down the style or at the ovule. If such mechanisms exist, they appear to be overcome at least to some extent as three genotypes produced self pollinated seeds, and backcross seeds were produced.

Figure 4.1.6. Fluorescent pollen and germinated pollen tubes stained with aniline blue

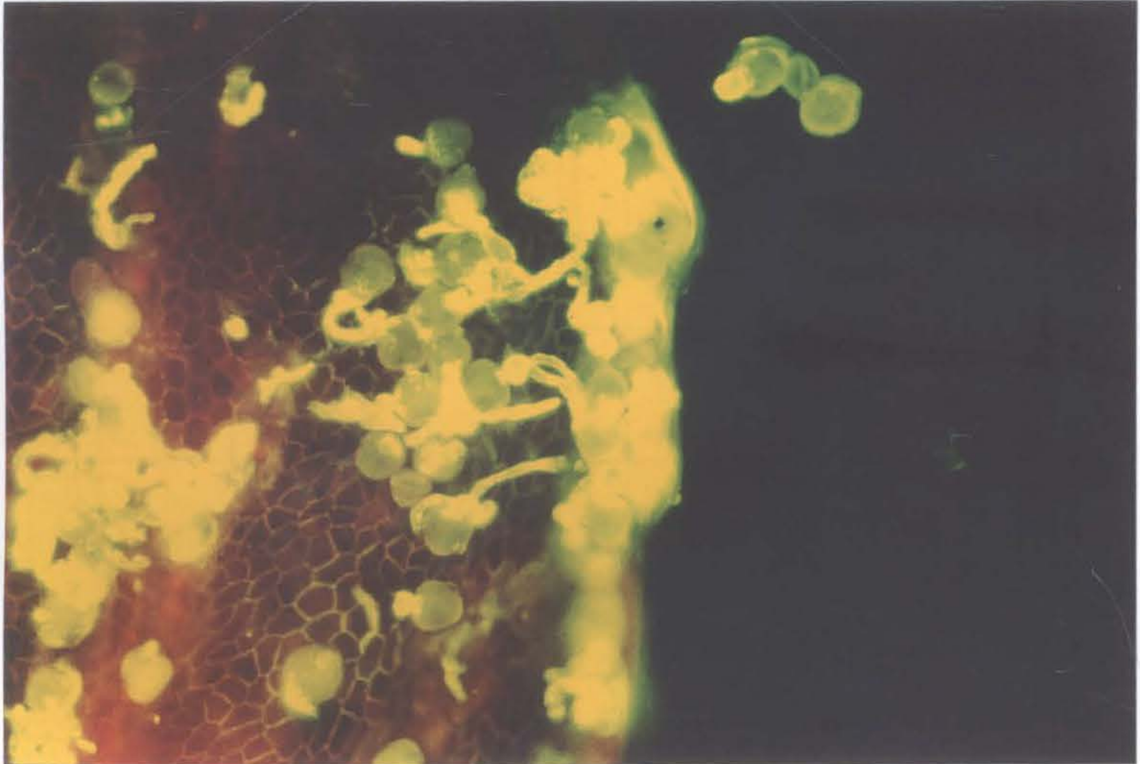


Table 4.1.5. Aniline blue stained pollen germination results†

♂ ♀	DxPP1	DxPP2 DxPP3	FR4 FR5	FW (3,4,5,6)	HP2	LD4	PP4 PP5	S4	Single Red	SUPJ.1	TW1 TW2	V10R (1,2,3,4)	V10RxL1	V10RxL2 V10RxL3	Total tested
DxPP1	16\1a*			16\1a				0\1a						12\1a	4
DxPP2 DxPP3	78\1a, 1p	15\5a, 1p		4\1a		70\1seed						193\3a		77\1a	12
FR4			29\1a 86\2seed, 1p			29\1seed, 1p						36\3a 120\1seed			8
FW (3,4,5,6)		64\1a	243\3a 160\1seed	63\1a	3\2a	267\1a		17\1a	55\1a			165\3a 24\1seed	60\2seed		17
HP2		8\2a		2\1seed, 1p	14\2a	3\1a				35\2a		482\1a			9
LD4			5\1a			10\1a				1\1a					3
PP4 PP5			0\1a	35\1a	6\1seed			37\2seed		0\1a	0\1a				7
S2.3			8\2a			53\1seed								43\1a	4
S4			4\1a				1\1a	9\3a				16\1a 29\1seed			7
SUPJ.1			308\2a, 1p (2x enlarged ovary)									220\1a, 1p (1x enlarged ovary)			3
TW1 TW2	11\1a		24\4a 106\1seed	13\2a	1\4a	15\3a	3\2a		60\1seed, 1p (grade 3)	5\2a	0\2a	21\3a 6\1seed, 1p		23\3a	29
V10R (1,2,3,4)			52\2a	3\1a		38\1a, 1p	91\1a		100\2a 144\1seed (grade 3)						8
V10RxL1	7\1seed		9\3a	121\1seed, 1p	0\2a	2\1a 71\2seed	3\1seed, 1p	4\4a		4\4a		140\2a	87\5a 174\1seed, 1p		27
V10RxL2 V10RxL3		2\1a	3\1a 86\3seed			14\3a 59\1seed, 1p		4\2a		9\1a, 1p		31\1a 154\2seed, 2p		79\3a 116\3seed, 1p	21
Total tested	4	9	28	9	11	17	5	13	5	11	3	24	8	12	159

† Abbreviations: Mean pollen germination number \ number of pistils observed for a = aborted ovary, seed = one or more seed produced from ovary. p = pollen tube penetration and growth down the style observed for the number of pollinations indicated.

Effects of gibberellic acid application

All plants that received foliar GA_3 application exhibited internode elongation and increased height greater than untreated controls within 1 - 2 weeks after treatment. This indicated that these genotypes were sensitive to GA_3.

Discussion

The main objectives of this study were to quantify female and male fertility and optimise conditions for seed production.

Pollination data indicated that single pollinations were as effective in producing seeds as repetitive pollinations. Thus, repetition was likely to be unnecessary and could be eliminated, which in turn leads to decreased cost of production. Pollinations were effective irrespective of time of day when they were conducted (on stigmas graded ¼ - ¾) indicating that pollen and stigmas remained functional during this period. Additionally, stigmas appeared to be receptive over a number of days (from stages ¼ to ¾). This long receptive period allows for flexibility during manual pollinations, thus improving efficiency.

Plants from two genotypes (V10RxL and DxPP) were used with and without PoiBI infection. PoiBI infected plants recorded lower seed/ovule efficiencies (approx. 10%) compared to uninfected plants, indicating non-infected plants may be used to marginally increase seed production efficiency. However, seed quality was not adversely affected by PoiBI infection in this study. Because pollinations were performed randomly and only one or two plants of each line were used, further investigations are required to conclusively elucidate the effects of PoiBI on seed production and quality.

Measurement of male and female fertility indicated that differences existed among genotypes and certain genotypes were more fertile than others, as shown by seed production. Compatible combinations should be targeted for future breeding studies to improve efficiency of production. Sowing seeds in the greenhouse achieved greater germination of grade 2 seeds than *in vitro*. Reduced *in vitro* germination may have been due to random differences in de-coated seed sizes sown in these two environments and small sample size. Sample size was limited by the availability of seeds.

The present study resulted in a mean seed set of 14% on a seed/ovule pollinated basis. The mean plant number produced per ovule was 8.6%. These low mean seed and plant production results clearly demonstrate the difficulty in producing seed as suggested by Sink (pers. comm. 1997) and mentioned by Hartley (1995) where an average of one seed was produced from 10 pollinations of diploids (3.3% seed/ovule efficiency). In contrast, Semeniuk and Stewart (1960) obtained a mean seed set value of 62% but they did not report plant production efficiency. Milbocker and Sink reported a 76% germination rate from self and cross pollinations of diploids however, they did not present seed production efficiencies. In this study 64% germination was recorded, which is similar to Milbocker and Sink. These differences are most likely due to genotypes and methodologies used.

For inheritance studies as well as breeding in general, a large number of seeds need to be produced. Genotypes with increased ability to produce seeds are necessary to improve efficiency. The majority of germplasm used in this study produced at least one seed, but overall seed production was low. Low seed production may be due to one or several combined factors such as self- incompatibility (SI), ploidy differences (Bempong 1967), mutational load and inbreeding depression (Sink pers. comm.1997).

SI is unlikely because several genotypes produced self-pollinated seeds and/or back cross seed (e.g. seed/ovule efficiencies for self pollinations of: V10RxL1 = 17%, V10RxL2 and V10RxL3 = 38%, V10R = 16% and FR = 25%). Sporophytic SI is generally characterised by the presence of trinucleate pollen and lack of pollen germination at the stigma surface in incompatible combinations. Corriveau and Coleman (1988) determined *E. pulcherrima* pollen was trinucleate. With sporophytic SI, penetration of the stigma by the pollen tube rarely occurs (Brewbaker 1967, Anderson *et al.* 1989). Sporophytic-SI is unlikely because pollen germination was frequently seen on and occasionally inside the styles of aborting ovaries from, self, cross and back-cross pollinations. Gametophytic self incompatibility is characterised by binucleate pollen and inhibition in the style or at the ovary (Brewbaker 1967, Anderson *et al.* 1989). Poinsettias could possess binucleate pollen as Sink (1963) demonstrated that eight commercial poinsettias possessed this type. Late acting SI is characterised by arrest of pollen tube growth just prior to fertilisation. Further studies are required to conclusively determine the nucleate number of poinsettia pollen from a range of genotypes.

It is also important to note that low seed set was not due to lack of pollen germination (although TW and S2.3 appeared almost completely male sterile). Pollen germination was noted on approximately 95% of stigmas investigated (one or more pollen grains germinated).

Differences in ploidy between parents can result in reduced seed production in poinsettia (Ewart and Walker 1960, Bempong 1967, Bempong and Sink 1968b, Milbocker and Sink 1969a and b). Of the cultivars investigated, only Supjibi (2n = 56) had a ploidy varying from 2n = 28. Supjibi as a female parent produced no seed, but three swollen ovaries were recorded. Pollinations using Supjibi as a male parent never resulted in seed production or ovary swelling. These results may be due to the differences in ploidy between parents. Embryo rescue or ovule culture may enable the production of triploid plants from these enlarged ovules.

Mutational load could have contributed to the reduced seed set. Mutational load is described as the induction of deleterious (often recessive) mutations produced in plants after treatment with mutagenic agents. Treatment of poinsettias with mutagenic agents (mutation breeding) has been practiced extensively (U.S.A. Patent and Trademark Office, Sander pers. comm. 1999, Rinehart pers. comm. 1997) but is not often reported in the literature. Induction of bract colour mutations (white, pink and marble types) can be accomplished by subjecting red bracted plants to mutagens (e.g. gamma and x-rays - Sander pers. comm. 1999, Rinehart pers. comm. 1997). Some of the accessions used in this study were developed through mutation breeding (i.e. FW, S2.1, S2.3, S2.4 and TW). Some were selected as variants from other cultivars. The method of induction of these 'sports' is often not specified, therefore they may be induced or naturally occurring.

Plants generated via mutation breeding are likely to possess mutations that could affect fertility, seed production and germination. If sexual hybridisation occurs, recessive mutations may be uncovered, thereby reducing seed production. These deleterious effects could mimic inbreeding depression (IBD).

Inbreeding depression can lead to reduced seed production. IBD has been observed as a major factor resulting in reduced seed production in poinsettias by Sink (pers. comm. 1997). IBD is often expressed when closely related genotypes are crossed in outbreeding species (Anderson *et al.* 1992, Willis 1999). Breeding records (Ecke *et al.* 1990, Table 4.1.1 and Appendix 5) and DNA marker experiments have shown that many commercial cultivars are closely related

(Ling *et al.* 1997, Starman and Abbitt 1997). According to the classification of Cruden 1977, poinsettias would be classed as xenogamous (outbreeding). Kobayashi (pers. comm. 2000) also suggests poinsettias are outbreeders. It could be assumed that if poinsettias are an outbreeding species, then IBD could be exhibited between commercial cultivars due to close genetic similarity.

Results from this study concur with of those presented by Sage *et al.* 1994, who suggests IBD is characterised by a continuation of failure to survive and reproduce during the life cycle. Evidence for IBD in this study was observed at several stages of the life cycle. i.e. 13% of ovaries that aborted contained partially developed seed or enlarged ovules, 41% of seed did not germinate, 8% of germinated seedlings died within a few weeks of germination and some were albinos. 15% of plants were characterised by slow growth, very short internodes and leaf deformations and these were termed 'weak'. The flowering ability and functionality of male and female reproductive structures were observed by placing these plants under a 10 h short photoperiod environment in a commercial nursery. The results of growth under this environment support the evidence presented thus far for presence if IBD. i.e. only 10/26 (38%) plants identified as weak actually reached anthesis, these plants did not produce hermaphrodite cyathia and anthers were scarce.

Therefore, for progress to be made towards producing shorter, ornamentally acceptable poinsettias, further studies need to be implemented to identify short growing cultivars/lines (not infected with PoiBI) that are highly fertile to increase efficiency and determine if IBD, mutational load or other factors are present in commercially grown and wild growing poinsettias. Difficulty lies in determining whether IBD and/or mutational load are acting because one may mimic the other. To determine the presence of IBD, pollination investigations of wild type poinsettias that have not been subjected to mutation breeding could be performed.

Experiment 4.2. Investigating wild-type poinsettia seedlings to ascertain the presence of inbreeding depression

Introduction

Many cultivated poinsettias have been subjected to mutation breeding. In Experiment 4.1, evidence for the presence of mutational load and/or inbreeding depression (IBD) was found for several cultivars/lines. However, the symptoms of mutational load are largely indistinguishable from IBD. Therefore, it is necessary to determine if IBD does exist in poinsettias by examining seedlings from plants not subjected to mutation breeding. If IBD exists, breeding via intraspecific hybridisation could be hampered, due to a reduced ability to produce inbred lines. Since the origins/pedigrees of many poinsettia cultivars are not known, use of a wild-type poinsettia could clarify if IBD is expressed in poinsettias.

Materials and methods

Twelve seeds produced by self pollination of a wild-type poinsettia (*Euphorbia pulcherrima*) collected in Mexico, were provided by C. Underhill (pers. comm. 1998). These seeds were derived via natural self pollination from an original plant collected from the wild in Mexico. This original plant was not subjected to any form of mutation breeding prior to collecting seeds. The plant produced large numbers of self pollinated seeds and was not in the vicinity of any other poinsettias. Seeds were characterised for quality in the same manner described in Experiment 4.1 and then sown in a greenhouse as per the procedure detailed in Experiment 4.1. The number of germinated seeds was recorded and plants were observed during vegetative growth. Plants were analysed for evidence of IBD, by observing characteristics such as albinism, deformities and reduced rate of growth, which may indicate expression of IBD. Following these observations, plants were placed under an artificial short photoperiod environment of 10 h duration and observed for reproductive growth and development. Floral abnormalities and sterility, if any, were noted as they could also indicate expression of IBD.

Results

Among the 12 seeds obtained, 10 were of grade two and 2 of grade three. Seven plants were derived from 10 grade two seeds. Grade three seeds did not germinate. Plants produced exhibited a variety of morphological abnormalities ranging from distorted and epinastic

leaves, thin stems and corky bark to lack of bract development and very late flowering (Table 4.2.1).

Table 4.2.1. Vegetative and reproductive characteristics of seven seedlings derived via self pollination of a wild-type poinsettia

Seedling accession	Vegetative Characteristics	Reproductive characteristics
97/176.1	Distorted leaves, nodules on stem	Normal, self-fertile
97/176.2	Normal	Normal, self-fertile
97/176.3	Normal	Normal, self-fertile
97/176.5	Deformed leaves, thin stem, corky bark	Very late flowering, fertility not determined
97/176.6	Leaves and bracts exhibit epinasty, thin stem	Normal, self-fertile
97/176.7	Normal	Few, partially coloured bracts at anthesis, low cyathia production, no seed produced
97/176.8	Normal	Normal, self-fertile

Discussion

Results from this investigation suggest IBD could have been expressed in the population of seedlings obtained. First, only 7 from 10 grade 2 seeds germinated. Second, a high level of deleterious mutations was observed for the seedlings that developed (four from seven plants exhibited deleterious abnormalities). Therefore, the poinsettia breeding system (when not influenced by induced mutation) could be subject to IBD. A larger number of seeds would have been desirable to provide a more robust study, however, these could not be obtained. The production of self-seeds from the wild-type parent and five of its progeny indicates a complete self-incompatibility system is unlikely in this wild-type poinsettia.

Presence of IBD and/or mutational load could have repercussions for breeding dwarf poinsettias, because short plant height may be associated with reduced vigour. Reduced vigour is undesirable for commercially produced poinsettias because the production period is lengthened. To avoid the effects of IBD, plants with diverse genetic backgrounds are necessary for use as parents. These parents can be expected to produce seeds from crosses and these seeds could be expected to eventuate into vigorous plants.

Experiment 4.3. Assessment of the intraspecific hybrid population for dwarf characteristics

Introduction

Breeding dwarf poinsettias via intraspecific hybridisation entails intermating parental genotypes for the production of seeds followed by growing and assessing these seedlings for characteristics such as height, internode length, response period, growth rate, etc. This study was undertaken to investigate a subset of plants selected from a population of plants derived from various cross combinations. The parental material consisted of various cultivars and lines presented in Table 4.1.1. The aim was to assess for parameters relevant to breeding poinsettias for short height, such as those listed above. Since the population was derived from parental material that were commercially available, this study could prove that there is scope for selection within the current commercial cultivar genepool for development of dwarf poinsettias that meet the market demand for height and if possible, ornamental merit.

Materials and methods

A population of 179 plants was developed from various cross and self pollinations of lines and cultivars in Experiment 4.1. This population contained a mixture of genotypes with differing heights. It was expected that short genotypes could be selected from this population, for use in further breeding studies to develop dwarf poinsettias. To breed poinsettias with reduced height and commercial acceptability, ornamental merit must also be considered. To expediate investigations, initial visual selections were made from the population whereby seedlings with undesirable features in the vegetative phase (such as slow growth, 'weak' phenotype, leaf and stem abnormalities, long internodes, etc.) were discarded. The remaining 'selection pool' consisted of seedlings of 'normal' appearance, but with short internode length.

From the 'selection pool', 14 seed derived plants (Table 4.3.1) were selected for characterisation and fertility testing, based upon visual appraisal of short internode length and vigorous growth. These 14 plants represented 7.8% of the population and were grown under the conditions described in Experiment 4.1 during the vegetative phase. Measurements were performed as described below to provide a basis from which further selections could be made.

Vegetative measurements

Measurements of the 14 seedlings were performed after approximately16 weeks from the date of planting in a controlled environment (Experiment 4.1). The measurements are described below.

Height. Calculated in millimetres from the first node to the stem apex.

Node number. Counted from the second node to the node subtending the uppermost leaf greater than 30 mm in length.

Mean internode length (H/N). Calculated as height (mm) divided by number of nodes. This measurement was used to determine compactness for the selection of compact, short plants.

Mean node production rate (T/N). This measurement was calculated as the duration of time in days from the date of planting to the date of scoring (approximately 16 weeks) divided by the number of nodes produced during that time. This value represents the mean time period required to produce one node and provides a measure of plant vigour.

Table 4.3.1. F_1 germplasm accession number and pedigree

Accession number	Female parent	Male parent
97/24.1	Freedom white	V10 Amy red
97/40.1	V10 Amy red	Self
97/43.1	V10RXL	Freedom red
97/54.1	V10 Amy red	Self
97/59.1	V10RXL	Freedom white
97/93.1	Freedom white	V10 Amy red
97/96.1	Freedom red	V10 Amy red
97/102.1	V10RXL	Freedom red
97/135.1	V10 Amy red	Freedom white
97/135.2	V10 Amy red	Freedom white
97/143.1	Freedom red	V10 Amy red
97/172.2	Freedom red	Self
97/199.1	V10 Amy red	Freedom white
97/201.1	Freedom marble	V10 Amy red

Reproductive growth environmental conditions

Subsequent to recording vegetative measurements, plants were transferred to the artificial short photoperiod environment designated as E1 (refer to Experiment 4.1).

Measurements conducted under short photoperiod

At anthesis, response period was recorded. This was measured as the time from the start of the short photoperiod phase to anthesis. Plants were observed 2 - 3 times per week for 10 weeks after placement in E1. A scale of 1 - 3 was used for categorising ornamental merit. The most ornamentally acceptable plants were scored as 1 (similar in appearance to current commercial cultivars), average plants as 2 and unacceptable plants as 3 (e.g. lack of reproductive structures, deformed bracts, protrusions from bracts). Bract colour and size, stem strength, leaf colour, cyathia number and overall appearance were combined in determining the final score.

Fertility testing performed under short photoperiod

Upon development of hermaphrodite cyathia, manual self pollinations were performed and seeds collected according to the procedure defined in Experiment 4.1. Seed production efficiencies were determined (using grade 1 and 2 seeds) as a percentage of the number of ovules pollinated.

Results

Four plants (97/24.1, 97/54.1, 97/96.1 and 97/172.2) were selected for further breeding studies according to the following protocol (refer to Table 4.3.2 for data).

i. - H/N. Three plants with the shortest H/N scores were selected (97/172.2, 97/54.1 and 97/96.1).

ii. - Pedigree. A plant derived via self pollination of V10 Amy red (97/54.1) and another derived via self pollination of Freedom red (97/172.2) was selected because they were developed via inbreeding and could be used for further studies.

iii. - Ornamental appeal. The most ornamentally desirable plants were selected (97/24.1, 97/54.1, 97/96.1 and 97/172.2).

iv. - Fertility. Two plants were selected with (partial) self fertility (97/24.1 and 97/54.1) to enable production of inbred (F_2) seedlings. 97/54.1 was chosen because second generation inbreds could be developed from this line.

The selection pool consisted of fourteen plants with response periods from approximately 7 to 8 weeks, mean internode lengths ranging from 12.3 - 18.9 mm and node production rates ranging from 2.6 - 5.3 days (Table 4.3.2). Some abnormalities were observed, such as elevated ridges either side of the main bract vein in some progeny with V10R as a parent. One line was sterile (lacked male and female appendages). Ornamental appeal was favoured over relatively high self-seed production for selection of four plants for further studies. Seed production from manual self pollination ranged from 0 - 50.9%. The number of pollinations performed was related to the number of cyathia formed during the crossing period. The line 97/172.2 was particularly slow at producing hermaphrodite cyathia, with only seven produced.

Table 4.3.2. Selection and fertility data based on seed development for fourteen seedlings

Accession number	Female parent	Male parent	Bract colour	Response period (days)	H/N (mm)	T/N (days)	Ornamental merit rating	Self- fertility %†
97/24.1	FW	V10R	white /cream	50	15.8	5.0	1	24.5(25/34)
97/40.1	V10R	Self	red	49	16.8	4.0	3	1.4(4/95)
97/43.1	V10RXL	FR	red	42	16.4	3.8	2	5.0(11/73)
97/54.1	V10R	Self	white /cream	57	12.3	4.1	1	9.6(13/45)
97/59.1	V10RXL	FW	red	51	18.0	4.4	3	9.3(5/18)
97/93.1	FW	V10R	red	56	17.4	4.8	3	8.7(6/23)
97/96.1	FR	V10R	red	50	12.8	3.1	1	1.6(1/21)
97/102.1	V10RXL	FR	red	50	18.7	3.9	2	10.0(6/20)
97/135.1	V10R	FW	white	56	17.6	2.6	2	50.9(29/19)
97/135.2	V10R	FW	white	57	18.9	3.2	2	28.6(12/14)
97/143.1	FR	V10R	red	52	15.0	3.7	2	18.2(6/11)
97/172.2	FR	Self	red	56	12.3	4.5	1	4.8(1/7)
97/199.1	V10R	FW	white	48	18.1	4.7	3	28.6(24/28)
97/201.1	FM	V10R	white/cream	55	14.4	5.3	2	sterile

† Seed/ovule production followed by number of seed produced/pollinations performed. Parent codes are described in Table 4.1.1.

Discussion

This experiment involved the selection of four short plants (for further studies) based upon measurable parameters within a seedling population developed through random crossing. These four seedlings were 97/24.1, 97/54.1, 97/96.1 and 97/172.2. Within the population, visual observations indicated most 'normal' short internode seedlings were the result of pollinations between what were considered short parents (e.g. V10 Amy red and the Freedom series). Therefore, future studies should aim to utilise short parents for breeding shorter poinsettias. The relationships between parameters measured in this experiment are important and need to be investigated further to determine if any adverse/advantageous correlations exist. Correlations may be used for breeding purposes as a means of indirect selection and provide an understanding of the problems which might be associated with breeding dwarf poinsettias (e.g. shorter height may be correlated with reduced stem diameter). Studies are also required to determine the merit of these selected seedlings in a commercial production environment, in comparison to current commercial cultivars.

Experiment 4.4. Comparative assessment of selected lines from the F_1 population with commercial cultivars and effects of PoiBI on height during commercial production

Introduction

Plant height in poinsettias is not only determined by genotype and growing environment, but also suggested to be due to the presence of phytoplasma (PoiBI). Commercial cultivars devoid of PoiBI have not been assessed for height in the published literature. Characterisation for height is required for further breeding studies aimed at producing dwarf poinsettias because selection of parents for short progeny production should be based upon genetic differences not influenced by PoiBI. In addition, it was also necessary to determine if four lines selected based upon short height in Experiment 4.3 were significantly shorter than their cultivar progenitors and other commercial cultivars considered short. To achieve these aims, the desired lines and cultivars were grown according to commercial production practices (similar to methodology described in Experiment 3.3).

The main aims of this experiment were therefore i. - to categorise cultivars and lines based on height, without the confounding effects of PoiBI, ii. - characterise cultivars and lines for traits relevant to future breeding experiments for production of genetically shorter poinsettias and iii. - determine the effects of PoiBI on plant height and morphology in the four selected lines to further estimate the effect of PoiBI on genotype, especially in shorter genotypes.

Materials and methods

Plant materials

The lines/cultivars used in this experiment are listed in Table 4.4.1. Cultivars devoid of PoiBI and PnMV were produced in Experiment 3.2. Two to three stockplants of each cultivar/line were established. Several cultivars with very low or no fertility were also included in this experiment as comparators because they were considered as short. However, these cultivars were not used in further breeding studies due to their inherent low or no fertility, which results in very low or no seed production (e.g. Pepride, Peterstar White and Supjibi). Determination of fertility was performed concurrently with this experiment and the results are presented in Chapter 5.

Table 4.4.1. Cultivar/line, pedigree, breeder, PnMV and PoiBI infection status and code

Code	Cultivar/line	Pedigree and breeder	PoiBI	PnMV†
24.1	97/24.1*	Freedom white x V10 Amy red[3] (Bernuetz)	-	-
54.1	97/54.1*	V10 Amy red x V10 Amy red[3] (Bernuetz)	-	-
96.1	97/96.1*	Freedom red x V10 Amy red[3] (Bernuetz)	-	-
172.2	97/172.2*	Freedom red x Freedom red[3] (Bernuetz)	-	-
24.1+	97/24.1+**	Freedom white x V10 Amy red[3] (Bernuetz)	+	-
54.1+	97/54.1+**	V10 Amy red x V10 Amy red[3] (Bernuetz)	+	-
96.1+	97/96.1+**	Freedom red x V10 Amy red[3] (Bernuetz)	+	-
172.2+	97/172.2+**	Freedom red x Freedom red[3] (Bernuetz)	+	-
DxPP	DxPP	Annette Hegg Diva x Pink peppermint[3] (Bernuetz)	-	-
V10RxL1	V10RxL1	V10 Amy red x Annette Hegg Lady[3] (Bernuetz)	-	+
FR	cv. Freedom red	Induced sport of a seedling[4] (Fruehwirth)	-	-
FW	cv. Freedom white	Induced mutation of Freedom red[4] (Jacobsen)	-	-
LD	cv. Lemon drop	Seedling[1] (Paul Ecke Ranch)	-	-
Pep	cv. Pepride	Induced mutation of Freedom Red[4] (Jacobsen)	-	+
PSW	cv. Peterstar white	Induced mutant of Peterstar red[4] (Jacobsen)	-	+
Sup	cv. Supjibi	Probably tetraploid of V-17 Angelika[2] (Gross)	-	-
V10R	cv. V10 Amy red	Seedling[4] (Gutbier)	-	-

[1] Ecke *et al.* (1990), [2] Starman and Abbitt (1997), [3] Produced by author, [4] U.S.A. Patent and trademark office. * Lines developed in Experiment 4.3. ** Approach grafting used to transfer PoiBI to cuttings of these lines from cv. V10 Amy red as described in Experiment 3.1. The PoiBI source was free of PnMV. † PnMV infection does to not alter plant height (refer to Experiments 3.3 and 3.4).

Experimental protocol

This experiment was performed concurrently with Experiment 3.3, during late summer to early winter. Protocols from stockplant management to final scoring are identical to those in Experiment 3.3 (Table 4.4.2). These included, harvesting of uniform 60mm long cuttings from stockplants maintained under long photoperiod conditions. Cuttings were propagated in a commercial nursery and were planted into NFP potting mix in 150 mm pots. Plants were maintained under long photoperiod conditions for approximately 5 - 6 weeks, during which time, apical decapitation was practiced to leave 6 nodes on each plant. Plants were then transferred to a short photoperiod area (10 h) and allowed to flower. Measurements such as height, node number, branch number and stem diameter were recorded at each critical production stage. The differences between this experiment and Experiment 3.3 were plant materials, the date of final scoring (one day earlier) and data analyses procedures, which are noted below.

Table 4.4.2. Developmental stages when measurements were taken and mean minimum and maximum temperatures

Stages of plant development	Stage	Day	Temperature (°C) Min.	Temperature (°C) Max.
Start of propagation	0	0		
Planting cuttings with roots	1	25	16	36
Pre-apical decapitation to 6 nodes	2	43	18	29
Post-apical decapitation to 6 nodes	3	43	18	29
Start of short photoperiod	4	65	18	29
Final scoring (close to anthesis)	5	120	18	29

Data analyses

Data analyses were performed with the SuperANOVA software package version III (ABACUS Concepts Inc., Berkley, CA, U.S.A.). General linear models (GLM) using Type I sums of squares were performed with Tukey pairwise comparisons to determine significant differences between treatment means for the whole data set. Pairwise comparisons were only conducted at stage 5 (final scoring) because this stage concludes the production cycle and plants would be sold at this stage under commercial conditions. Therefore, this stage was regarded as most important. Graphs display standard errors for this stage alone. A subset of the experiment data (PoiBI infected and non-infected selections) was also analysed separately to determine the morphological effects of phytoplasma infection.

Results

The number of uniform cuttings available from stockplants varied between cultivars/lines. In particular, cultivars/lines not infected with phytoplasma produced fewer cuttings within the available time frame than those infected with phytoplasma. Therefore, uneven replication resulted among cultivars/lines (Table 4.4.3). Cuttings that were taken grew uniformly (Figure 4.4.1) and at potting 95% had greater than 5 roots extending from the propagation plug. Photographs taken during throughout the experiment are shown in Figures 4.4.1 and 4.4.2.

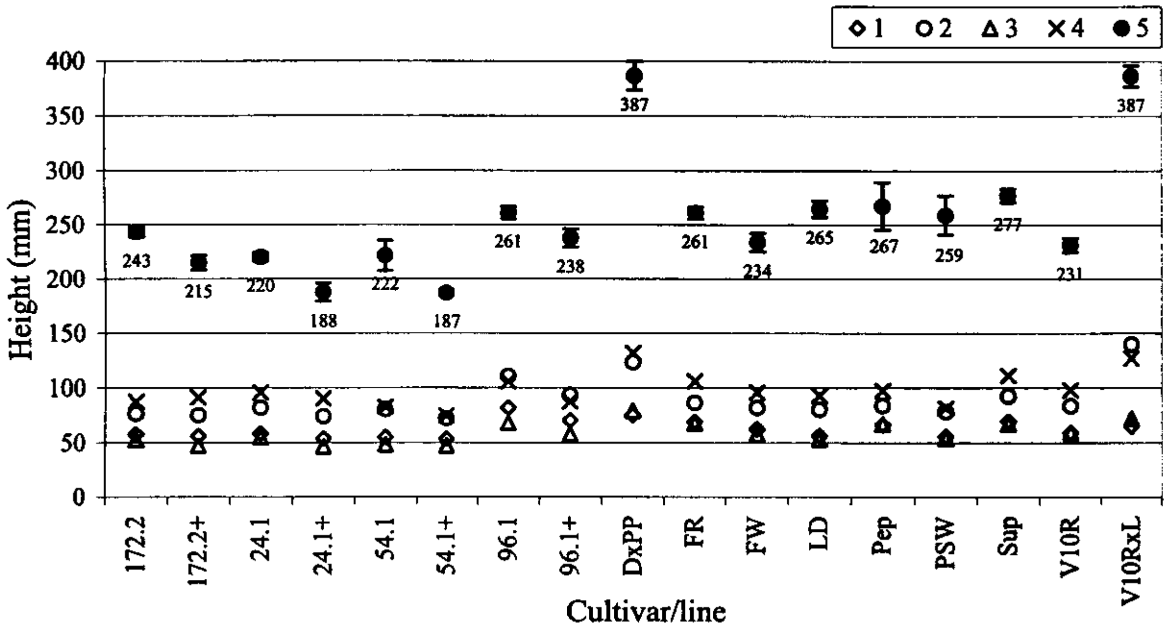
Figure 4.4.1. Experimental layout and morphology of plants (top) during propagation and (bottom) one week after planting. (Note Experiment 3.3 in foreground)

Figure 4.4.2. Experimental layout and morphology of plants (top) at approximately four weeks under short photoperiod environment and (bottom) at final scoring (stage 5)

Height

Means for cultivars/lines remained in a similar order (smallest to largest) over the entire duration of the experiment (Figure 4.4.3 and Table 4.4.3). Mean height ranged from approximately 55 - 82 mm at stage 1, 72 - 140 mm at stage 2, 46 - 79 mm at stage 3, 75 - 132 mm at stage 4, and 187 - 387 mm at stage 5.

Figure 4.4.3. Mean height (mm) for cultivars and lines at stages 1 - 5 (with SE at stage 5)



At each production stage, significant differences were found for height among cultivars/lines (Table 4.4.3). At stage 5, all commercial cultivars and the four selected lines from Experiment 4.3 were within or below the commercially acceptable height range (225 – 300 mm) as measured from the top of the propagation plug. When measured from the pot rim, mean height was reduced further, between 3 – 8 mm. This result showed that when growing poinsettias during a cooler period of the year, growth regulator usage is not required and all cultivars and the four selected lines could be grown successfully within commercially accepted standards.

Pairwise comparisons at stage 5 demonstrated that the three shortest cultivars/lines were lines infected with phytoplasma (24.1+, 54.1+ and 172.2+). Among those lines without phytoplasma, both 24.1 and 54.1 were again the shortest. These two lines were significantly shorter than the cultivars Lemon drop, Pepride and Supjibi, but not significantly shorter than their parents (i.e. FW and V10R for 24.1 and V10R for 54.1). All cultivars and selected lines were significantly shorter than DxPP and V10RxL. Both recorded the same upper maximum height of 387 mm.

Table 4.4.3. Mean heights (mm) and standard errors for cultivars and lines at stages 1 – 5 and pairwise comparisons at stage 5

Stage		1		2		3		4		5		5†		5
Cultivar/line	n	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Pairwise comparisons‡
54.1+	9	53.3	1.2	72.1	2.2	47.2	1.2	74.6	8.5	187	3.1	179	3.7	
24.1+	10	53.6	2.4	73.8	3.9	46.5	1.9	90.1	3.7	188	8.3	180	8.0	
172.2+	7	56.0	1.6	75.0	2.9	47.3	1.5	91.3	4.0	215	6.7	209	6.6	
24.1	18	58.3	1.0	81.8	1.4	54.8	1.0	95.8	3.6	220	4.2	215	4.2	
54.1	4	55.0	4.9	80.2	4.3	48.2	2.2	82.5	0.9	221	14	218	16	
V10R	8	59.1	1.8	83.5	3.7	59.3	1.9	98.1	3.2	231	6.5	227	6.1	
FW	12	62.2	2.0	81.8	2.0	57.7	1.4	95.7	3.1	234	8.5	227	8.5	
96.1+	9	70.3	2.0	93.3	3.3	57.6	1.3	87.6	4.5	238	8.5	231	7.5	
172.2	14	57.6	1.2	76.4	1.4	52.6	1.0	87.4	2.3	243	4.5	235	5.0	
PSW	4	55.7	1.2	78.2	2.3	53.7	1.5	81.5	2.1	259	18	252	17	
96.1	13	82.0	3.1	110	3.0	68.1	2.4	105	3.5	261	5.8	253	6.1	
FR	15	68.9	1.8	86.4	1.7	67.7	1.8	106	2.3	261	5.6	254	5.8	
LD	8	56.2	0.9	80.1	1.9	53.1	2.6	93.0	4.6	265	7.8	259	7.9	
Pep	3	66.3	1.8	83. 7	1.7	67.0	2.6	97.3	10	267	22	262	22	
Sup	11	69.5	2.5	92.4	2.8	67.3	2.5	111	3.2	277	6.2	269	6.7	
DxPP	7	75.1	6.7	123	7.2	79.1	4.6	132	7.7	387	13	381	12	
V10RxL	3	65.0	3.2	140	4.2	72.0	2.9	127	8.0	387	9.7	381	12	

Source	Mean square					
	1	2	3	4	5	5†
Cultivar/line	692.4***	2095***	751.2***	1547***	19471***	19533***
Residual	54.35	79.13	38.51	176.8	517.0	521.0

‡ Means with common line are not significantly different. †Mean height measured from pot rim. *, **, ***: $p<0.05, 0.01, 0.001$ respectively.

Effect of phytoplasma

The main effect of phytoplasma was to reduce height. This effect was shown to be significant at all five stages (Table 4.4.4, Table 4.4.5). On an individual genotype basis, only six significant differences were recorded across all five stages and none at final scoring according to pairwise comparisons (Table 4.4.3). These analyses are not presented because there were no significant effects at stage 5 (final scoring), which is the crucial stage. Increased replication could lead to observation of significant differences in these individual genotypes. A genotype x phytoplasma interaction was observed at stage 2, but not at any other stage (Table 4.4.4).

Table 4.4.4. ANOVA for height as influenced by phytoplasma and genotype

Stage		1	2	3	4	5
Source	df			Mean square		
Genotype	3	2425***	3852***	994.7***	1331***	11481***
Phytoplasma	1	568.4***	1535***	978.6***	955.6*	16366***
Genotype*Phytoplasma	3	106.2^{ns}	210.6*	62.47^{ns}	401.2^{ns}	107.6^{ns}
Residual	76	44.37	72.55	27.42	204.0	409.3

*, **, ***: p<0.05, 0.01, 0.001 respectively. ns: not significant.

Table 4.4.5. Means and standard errors for effect of phytoplasma on height (mm) across genotypes

Stage		1		2		3		4		5	
	n	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
- Phytoplasma	49	64.1a	1.8	87.7a	2.3	57.2a	1.2	94.9a	2.0	237.6a	3.6
+ Phytoplasma	35	58.3 b	1.5	78.6 b	2.2	49.7 b	1.1	85.7 b	2.9	205.7 b	5.0

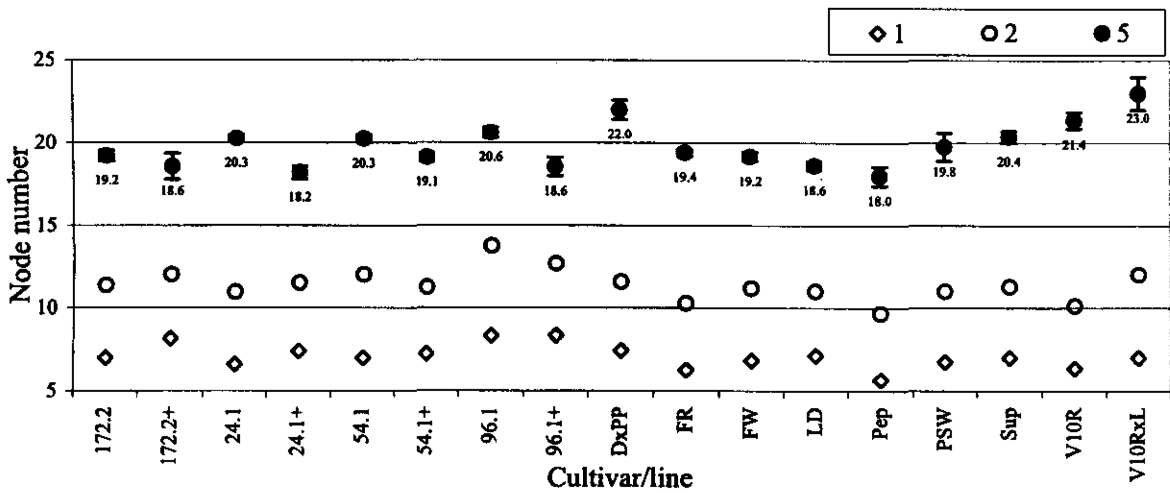
Means followed by the same letter are not significantly different.

Impact of node number on height and rate of growth

Node number contributes to plant height. A moderate correlation was found between node number and height (r = 0.436). Mean node numbers ranged from approximately 6 - 8 at stage 1, 10 - 14 at stage 2 and 18 - 23 at stage 5 (Figure 4.4.4). At each stage, significant differences were found for node number among all cultivars/lines (Tables 4.4.6 and 4.4.7).

Pairwise comparisons demonstrated that significant differences were not present between commercial cultivars and the selected four seed lines at stage 5. The two cultivars/lines with the highest mean node numbers were also the tallest (lines DxPP and V10RxL).

Figure 4.4.4. Mean node numbers and standard errors for cultivars and lines at stages 1, 2 and 5



Stages 3&4 not presented as all plants were apically decapitated to 6 nodes.

Table 4.4.6. Mean node numbers and their standard errors for cultivar/line at stages 1, 2 and 5 with pairwise comparisons at stage 5

Stage		1		2		5‡		5‡
Cultivar/line	n	Mean	SE	Mean	SE	Mean	SE	Pairwise comparisons
Pep	3	5.67	0.33	9.67	0.33	18.0	0.58	
24.1+	10	7.40	0.45	11.5	0.31	18.2	0.39	
96.1+	9	8.33	0.33	12.7	0.47	18.6	0.56	
172.2+	7	8.14	0.26	12.0	0.44	18.6	0.78	
LD	8	7.12	0.35	11.0	0.38	18.6	0.26	
54.1+	9	7.22	0.36	11.2	0.53	19.1	0.26	
FW	12	6.83	0.27	11.2	0.30	19.2	0.27	
172.2	14	7.00	0.26	11.4	0.32	19.2	0.32	
FR	15	6.27	0.18	10.3	0.15	19.4	0.21	
PSW	4	6.75	0.25	11.0	0.00	19.8	0.85	
54.1	4	7.00	0.58	12.0	0.58	20.3	0.25	
24.1	18	6.61	0.24	10.9	0.21	20.3	0.23	
Sup	11	7.00	0.33	11.3	0.20	20.4	0.36	
96.1	13	8.31	0.21	13.8	0.20	20.6	0.31	
V10R	8	6.37	0.26	10.1	1.3	21.4	0.50	
DxPP	7	7.43	0.20	11.6	0.37	22.0	0.58	
V10RxL	3	7.00	0.00	12.0	0.00	23.0	1.0	
Source				Mean square				
Cultivar/line		4.300***		8.786***		11.71***		
Residual		0.8865		1.660		1.439		

† Stages 3&4 not presented as all plants were apically decapitated to 6 nodes. ‡ Total nodes measured on primary stem, top branch and to last bract >30 mm in a single line. These nodes were counted because they contribute to plant height.

Effect of phytoplasma

The main effect of phytoplasma was to significantly reduce mean node number at stages 1 and 5 (Table 4.4.7). Therefore, phytoplasma could reduce growth rate. The difference was smaller at stage 1 (0.54 nodes) as compared to stage 5 (1.5). At stage 5, a difference of 1.5 nodes is commercially significant (Table 4.4.7) because this can increase the production period by approximately one week (if node production rate is approximately 1 node per 5 days).

A genotype interaction effect was noted at stages 1 and 2 (but not stage 5) and a genotype x phytoplasma interaction effect was noted at stage 2, (but not in stages 1 and 5) (Table 4.4.8).

Table 4.4.7. Effect of phytoplasma on mean node numbers with standard errors at stages 1, 2 and 5

Stage		1		2		5	
	n	Mean	SE	Mean	SE	Mean	SE
- Phytoplasma	49	7.20a	0.16	11.9a	0.21	20.1a	0.17
+ Phytoplasma	35	7.74 b	0.20	11.9a	0.23	18.6 b	0.24
Mean difference		0.54		0		1.5	

Means followed by the same letter are not significantly different.

Table 4.4.8. ANOVA for node number as influenced by phytoplasma and genotype

		Mean square		
Stage		1	2	5
Source	df			
Genotype	3	8.825***	21.41***	2.250^{ns}
Phytoplasma	1	6.489*	0.0676^{ns}	48.38***
Genotype*Phytoplasma	3	1.249^{ns}	3.847*	2.484^{ns}
Residual	76	1.051	1.250	1.529

*, **, ***: $p<0.05$, 0.01, 0.001 respectively. ns: not significant. At stages 3&4 all plants possess *6 nodes* due to decapitation.

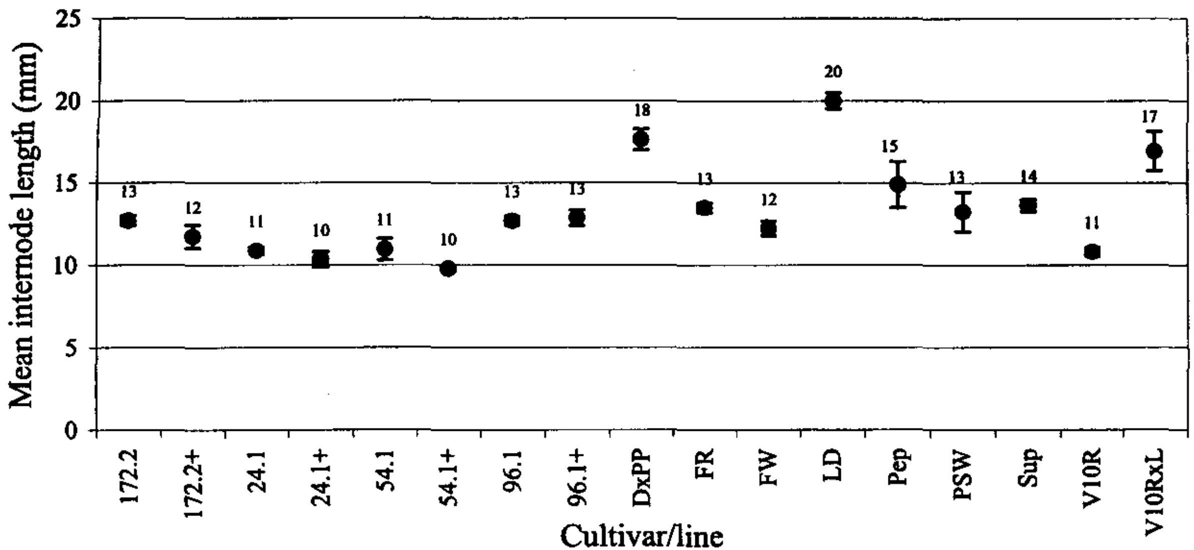
Mean internode length (H/N) assessment and its implications at stage 5

H/N means ranged from approximately 10 - 18 mm. Pairwise comparisons showed the tallest cultivars/lines (lines DxPP and V10RxL) had significantly greater mean internode lengths compared to all others. Increased height in these lines was attributed to an increase in H/N and node number.

Among non-PoiBI infected cultivars/lines, the three with the shortest H/N means were also the shortest for height (i.e. V10R, 24.1 and 54.1). A very high correlation between H/N and height (r = 0.902) for non-PoiBI infected cultivars/lines suggests that H/N is the primary factor contributing to height within the context of this experiment, and node number per se, although possessing a moderate correlation (r = 0.367) is of less influence.

Analysis of mean internode length at stage 5 effectively highlighted cultivars/lines with reduced node numbers (Figure 4.4.5 and Table 4.4.9). Lemon drop for example, had a high mean internode length, indicating fewer, more widely spaced nodes compared to other plants of similar height (height data presented in Figure 4.4.3 and Table 4.4.3).

Figure 4.4.5. Mean internode length (mm) and standard errors for cultivars and lines at stage 5 (with SE at stage 5)



Effect of phytoplasma

Phytoplasma did not strongly influence H/N (P = 0.053, means and standard errors are presented in Table 4.4.9). Therefore, reduction in height in phytoplasma infected lines can be primarily attributed to a reduction in node number. A reduction in H/N can also be expected to contribute, as three from four lines had a reduced mean H/N, although the overall combined effect was not significant. This result may be attributed to different effects of phytoplasma on different genotypes, insufficient replication and/or small effect in these genetically short genotypes.

Table 4.4.9. H/N means (mm) and standard errors for cultivar/line at stage 5 with pairwise comparisons

Line	n	Mean	SE	Pairwise comparisons
54.1+	9	9.79	0.13	
24.1+	10	10.3	0.47	
V10R	8	10.8	0.26	
24.1	18	10.9	0.23	
54.1	4	10.9	0.66	
172.2+	7	11.7	0.71	
FW	12	12.2	0.45	
96.1	13	12.7	0.27	
172.2	14	12.7	0.30	
96.1+	9	12.9	0.48	
PSW	4	13.2	1.2	
FR	15	13.5	0.30	
Sup	11	13.6	0.37	
LD	8	14.2	0.48	
Pep	3	14.9	1.4	
V10RxL	3	16.9	1.2	
DxPP	7	17.6	0.64	

Source	Mean square
Line	32.35***
Residual	1.737

‡ Means with common line are not significantly different.

*, **, ***: $p < 0.05, 0.01, 0.001$ respectively.

Stem diameter

Stem diameter ranged from approximately 4.1 - 7.4 mm at stage 1, 4.8 - 7.6 mm at stage 2, 5.6 - 9.3 mm at stage 4 and 7.1 - 11 mm at stage 5. Increase in stem diameter appeared linear regardless of cultivar/line, and was dependent upon initial size (Figure 4.4.6, Table 4.4.10).

Pairwise comparisons at stage 5 (Table 4.4.10) showed the tetraploid Supjibi had significantly greater stem diameter than any other cultivar/line. Most cultivars/lines were not significantly different, however Freedom white was notable for a large mean stem diameter.

Figure 4.4.6. Mean stem diameter (mm) and standard errors for cultivars and lines at stages 1, 2, 4 and 5

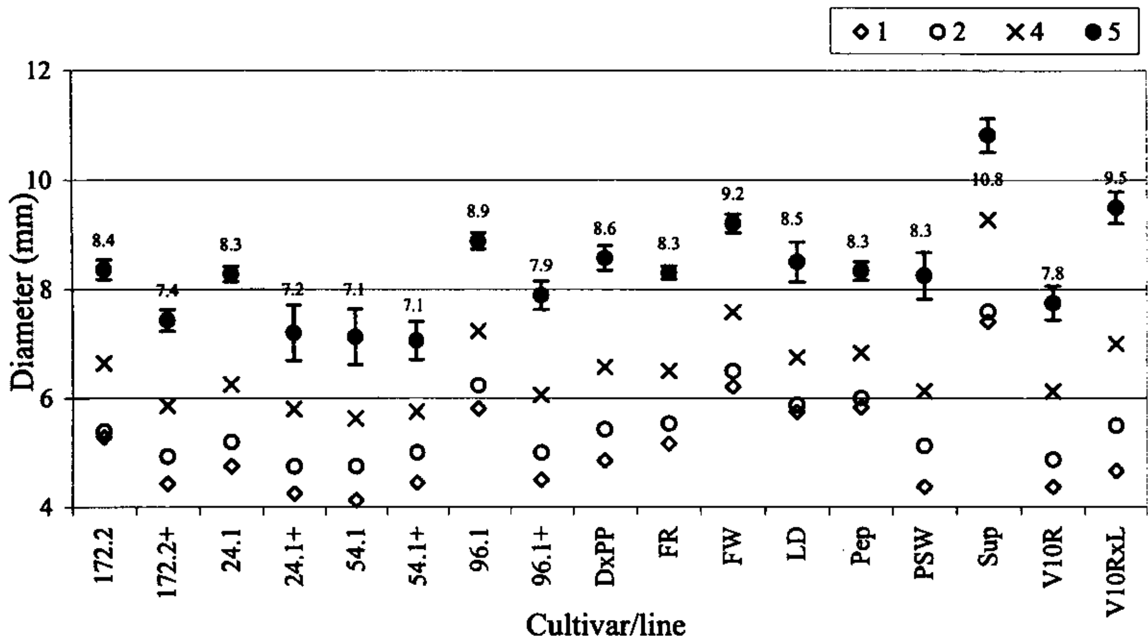


Table 4.4.10. Mean stem diameters (mm) and standard errors for cultivar/line at stages 1, 2, 4 and 5 with pairwise comparisons at stage 5

Stage		1		2		4		5		5
Cultivar/line	n	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Pairwise comparisons‡
54.1+	9	4.44	0.27	5.00	0.31	5.75	0.31	7.06	0.35	
54.1	4	4.12	0.43	4.75	0.32	5.62	0.47	7.12	0.51	
24.1+	10	4.25	0.28	4.75	0.21	5.80	0.31	7.20	0.51	
172.2+	7	4.43	0.23	4.93	0.23	5.86	0.34	7.43	0.20	
V10R	8	4.37	0.26	4.87	0.23	6.12	0.31	7.75	0.31	
96.1+	9	4.50	0.17	5.00	0.19	6.06	0.35	7.89	0.26	
PSW	4	4.37	0.37	5.12	0.51	6.12	0.43	8.25	0.43	
24.1	18	4.75	0.15	5.19	0.14	6.25	0.15	8.28	0.14	
FR	15	5.17	0.10	5.53	0.12	6.50	0.15	8.30	0.12	
Pep	3	5.83	0.17	6.00	0.29	6.83	0.44	8.33	0.17	
172.2	14	5.29	0.18	5.39	0.18	6.64	0.24	8.36	0.18	
LD	8	5.75	0.31	5.87	0.28	6.75	0.35	8.50	0.37	
DxPP	7	4.86	0.18	5.43	0.20	6.57	0.20	8.57	0.23	
96.1	13	5.81	0.17	6.23	0.16	7.23	0.18	8.88	0.15	
FW	12	6.21	0.29	6.50	0.21	7.58	0.24	9.21	0.17	
V10RxL	3	4.67	0.17	5.50	0.29	7.00	0.00	9.50	0.29	
Sup	11	7.41	0.18	7.59	0.20	9.27	0.31	10.8	0.31	
Source										
Cultivar/line		7.201***		5.514***		7.715***		8.071***		
Residual		0.4799		0.4337		0.6820		0.6733		

‡ Means with common line are not significantly different. Stage 3 not included because there was no change from stage 2.

*, **, ***: p <0.05, 0.01, 0.001 respectively.

Effect of phytoplasma

Phytoplasma and genotype both significantly influenced stem diameter at all stages (Table 4.4.12). Phytoplasma significantly reduced stem diameter (Table 4.4.11) as demonstrated in Experiments 3.3, 3.4 and 3.5. Significant differences within individual genotype means were not observed (except for 96.1 at stages 1 and 2 - data analyses not presented), although means for phytoplasma infected lines were consistently less than non-infected lines of the same genotype at stage 5 (Table 4.4.10). Lack of significant effects between phytoplasma infected and non-infected plants of the same genotype may have been due to insufficient replication to observe a possibly small difference.

Table 4.4.11. Effect of phytoplasma on mean stem diameter (mm) with standard errors at stages 1, 2, 4 and 5

Stage		1		2		4		5	
	n	Mean	SE	Mean	SE	Mean	SE	Mean	SE
- Phytoplasma	49	5.13a	0.12	5.49a	0.11	6.57a	0.12	8.37a	0.11
+ Phytoplasma	35	4.40 b	0.12	4.91 b	0.12	5.87 b	0.16	7.39 b	0.19
Mean difference		0.73		0.58		0.7		0.98	

Means followed by the same letter are not significantly different. Stage 3 is identical to stage 4.

Table 4.4.12. ANOVA for stem diameter as influenced by phytoplasma and genotype at stages 1, 2, 4 and 5

		Mean square			
Stage		1	2	4	5
Source	df				
Genotype	3	3.278***	2.563**	3.338**	5.437***
Phytoplasma	1	9.199***	6.167***	7.998**	14.62***
Genotype*Phytoplasma	3	1.738*	1.446*	1.198^{ns}	0.7174^{ns}
Residual	76	0.4803	0.4349	0.7114	0.7568

*, **, ***: $p<0.05$, 0.01, 0.001 respectively. ns: not significant. Stage 3 is identical to stage 4.

Branch number

Branches were produced after apical decapitation on all plants. Mean branch number ranged from approximately 1 - 4.8 at stage 4 and 2.8 – 5.9 at stage 5 (Figure 4.4.7).

Figure 4.4.7. Mean branch number and standard errors for cultivars and lines at stages 4 and 5

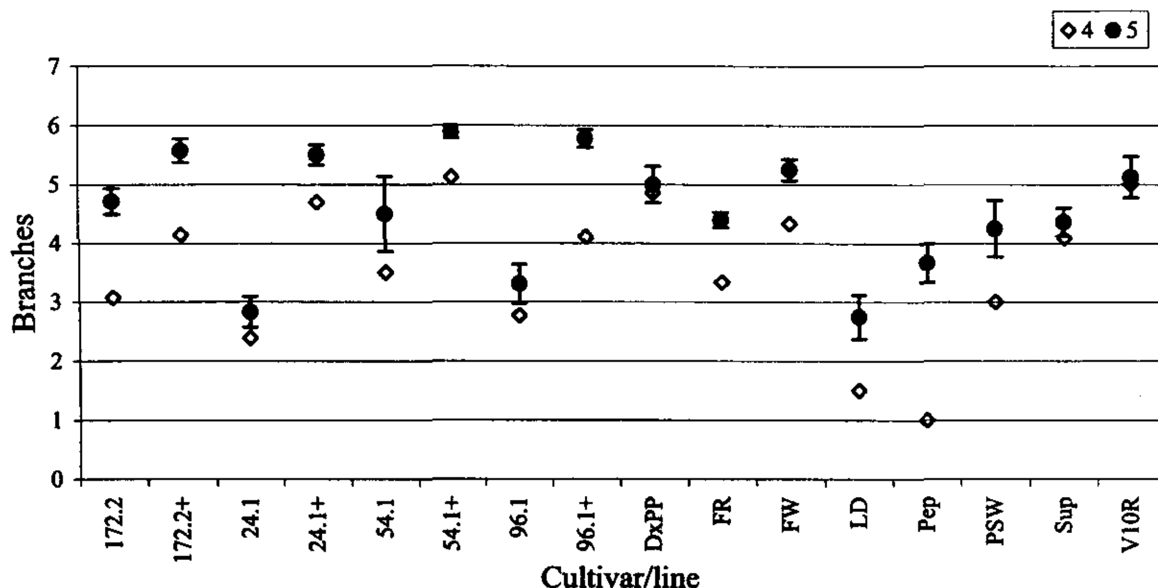


Table 4.4.12. Mean branch numbers and standard errors for cultivars and lines at stages 4 and 5 with pairwise comparisons at stage 5

Stage		4		5		5
Cultivar/line	n	Mean	SE	Mean	SE	Pairwise comparisons‡
LD	8	1.50	0.53	2.75	0.37	
24.1	18	2.39	0.26	2.83	0.26	
96.1	13	2.77	0.28	3.31	0.33	
Pep	3	1.00	0.58	3.67	0.33	
PSW	4	3.00	0.82	4.25	0.48	
Sup	11	4.09	0.25	4.37	0.24	
FR	15	3.33	0.16	4.40	0.13	
54.1	4	3.50	0.87	4.50	0.64	
172.2	14	3.07	0.30	4.71	0.22	
DxPP	7	4.86	0.34	5.00	0.31	
V10R	8	5.00	0.33	5.12	0.35	
FW	12	4.33	0.26	5.25	0.18	
V10RxL	3	6.00	0.00	5.33	0.33	
24.1+	10	4.70	0.33	5.50	0.17	
172.2+	7	4.14	0.96	5.57	0.20	
96.1+	9	4.11	0.65	5.78	0.15	
54.1+	9	5.12	0.35	5.89	0.11	

Source	Mean square	
Cultivar/line	11.72***	10.01***
Residual	1.486	0.6858

‡ Means with common line are not significantly different.

*, **, ***: $p < 0.05, 0.01, 0.001$ respectively. No branches were present prior to stage 4.

Lines with the highest branch numbers were infected with phytoplasma. The highest branch producing cultivars (devoid of phytoplasma) were V10R and FW.

Effect of phytoplasma

The main effect of phytoplasma was to increase branch number by approximately two branches at stages 4 and 5 (Table 4.4.13). Branch number was also influenced by genotype and a genotype x phytoplasma interaction (Table 4.4.14). Of the four lines, only 172.2 infected with phytoplasma was not significantly different to 172.2 without phytoplasma for branch number at stage 5. Data analyses for the four lines are not presented because they do not provide further information other than already shown in Table 4.4.12.

Table 4.4.13. Effect of phytoplasma on mean branch number with standard errors at stages 4 and 5

Stage		4		5	
	n	Mean	SE	Mean	SE
- Phytoplasma	49	2.77a	0.17	3.63a	1.9
+ Phytoplasma	35	4.54 b	0.28	5.69 b	0.08
Mean difference		1.77		2.06	

Means followed by the same letter are not significantly different.

Table 4.4.14. ANOVA for branch number as influenced by phytoplasma and genotype at stages 4 and 5

		Mean square	
Stage		4	5
Source	df		
Genotype	3	6.427*	10.76***
Phytoplasma	1	51.58***	75.77***
Genotype*Phytoplasma	3	1.626^{ns}	3.720**
Residual	76	1.937	0.7603

*, **, ***: p<0.05, 0.01, 0.001 respectively. ns: not significant.

Branches were not present at stage 3.

Top branch measurements at stage 5

Mean top branch length ranged from 94.2 – 326 mm (Table 4.4.15). Among the four selected lines without phytoplasma, 24.1 had the lowest top branch length and 96.1 had the highest.

Table 4.4.15. Means and standard errors for length, node, leaf and transitional-bract number, bract width and bract number on the top branch for cultivars and lines at stage 5

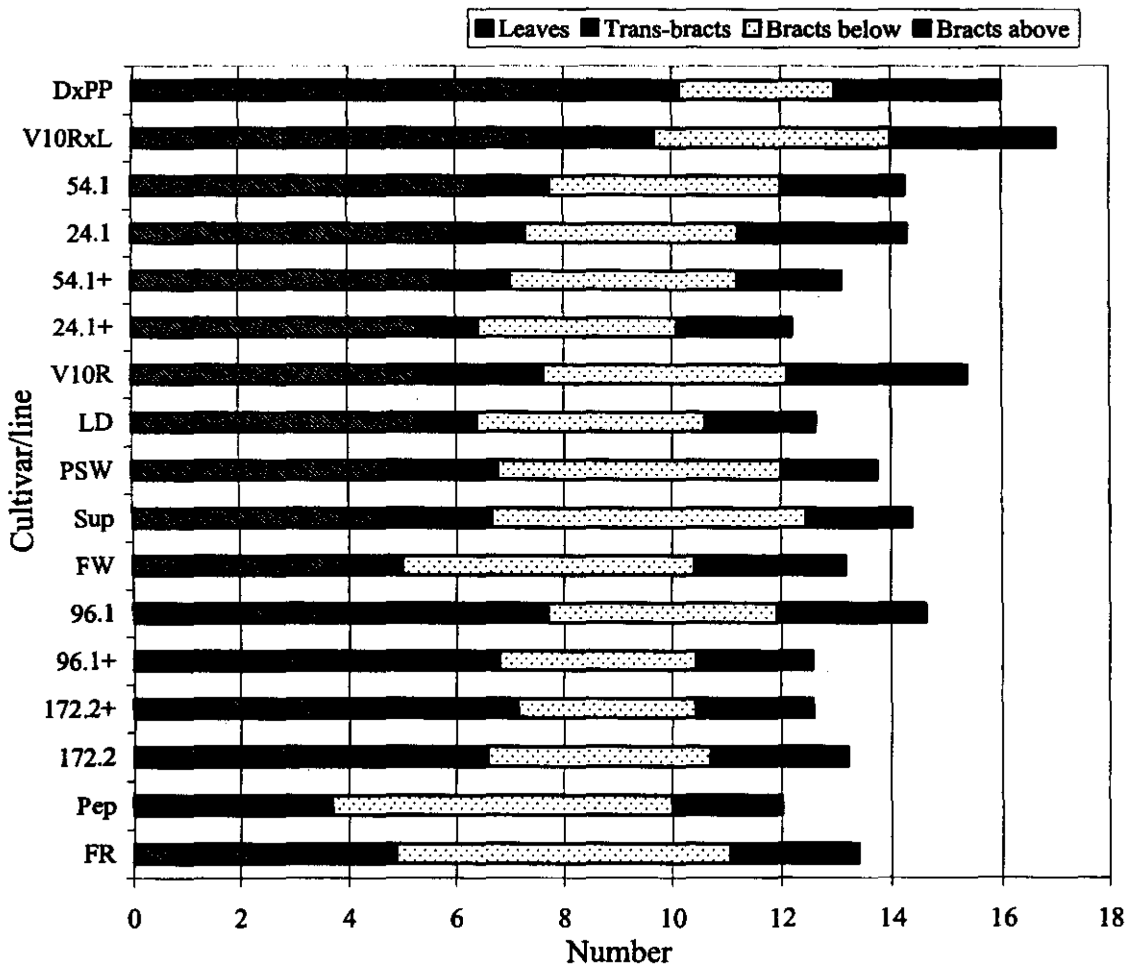
		Length		Node number		Leaf number		Trans-bracts		Bracts below†		Bracts above‡		Largest bract width	
Cultivar/line	n	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
172.2	14	142	5.9	10.7	0.32	3.14	0.18	3.43	0.17	4.14	0.27	2.50	0.14	110	3.3
172.2+	7	136	6.3	10.4	0.75	3.43	0.30	3.71	0.18	3.29	0.47	2.14	0.14	113	5.7
24.1	18	116	3.2	11.2	0.27	5.89	0.21	1.39	0.12	3.94	0.23	3.06	0.19	126	2.8
24.1+	10	109	5.5	10.1	0.35	5.30	0.21	1.10	0.10	3.70	0.21	2.10	0.10	121	4.0
54.1	4	136	13	12.0	0.00	6.25	0.25	1.50	0.29	4.25	0.25	2.25	0.25	82.2	2.8
54.1+	9	94.2	2.1	11.2	0.22	5.56	0.18	1.44	0.18	4.22	0.28	1.89	0.11	92.6	2.9
96.1	13	143	5.0	11.9	0.29	3.77	0.20	3.92	0.14	4.23	0.23	2.69	0.13	128	5.6
96.1+	9	136	4.3	10.4	0.58	3.56	0.38	3.22	0.32	3.67	0.37	2.11	0.11	128	3.3
DxPP	7	294	9.3	13.0	0.43	8.00	0.49	2.14	0.34	2.86	0.14	3.00	0.31	67.0	2.9
FR	15	153	3.8	11.1	0.18	2.27	0.12	2.60	0.16	6.20	0.14	2.33	0.13	136	3.0
FW	12	135	7.2	10.4	0.31	4.00	0.17	1.00	0.00	5.42	0.29	2.75	0.13	128	4.0
LD	8	176	14	10.6	0.18	5.25	0.16	1.12	0.12	4.25	0.16	2.00	0.19	91.1	4.4
Pep	3	129	6.3	10.0	0.58	2.67	0.33	1.00	0.00	6.33	0.33	2.00	0.00	116	7.4
PSW	4	151	15	12.0	0.82	4.75	0.48	2.00	0.00	5.25	0.48	1.75	0.25	84.2	1.1
Sup	11	170	5.0	12.4	0.37	4.45	0.25	2.18	0.12	5.82	0.26	1.91	0.91	116	3.6
V10R	8	151	6.4	12.1	0.44	5.25	0.31	2.37	0.26	4.50	0.38	3.25	0.16	89.5	3.3
V10RxL	3	326	6.9	14.0	1.0	7.67	0.33	2.00	0.56	4.33	0.33	3.00	0.00	92.0	5.3
Source		Mean square													
Cultivar/line		20187***		7.567***		20.31***		9.373***		8.535***		1.884***		3400***	
Residual		397.6		1.367		0.5910		0.3230		0.7696		0.2626		156.8	

† Bracts below primary cyathium. ‡ Bracts above primary cyathium. *, **, ***: $p<0.05$, 0.01, 0.001 respectively. ns: not significant.

Among the cultivars assessed, Freedom white had the shortest top branch length and Lemon drop had the longest. The two lines V10RxL and DxPP recorded the longest top branch lengths from all cultivars/lines.

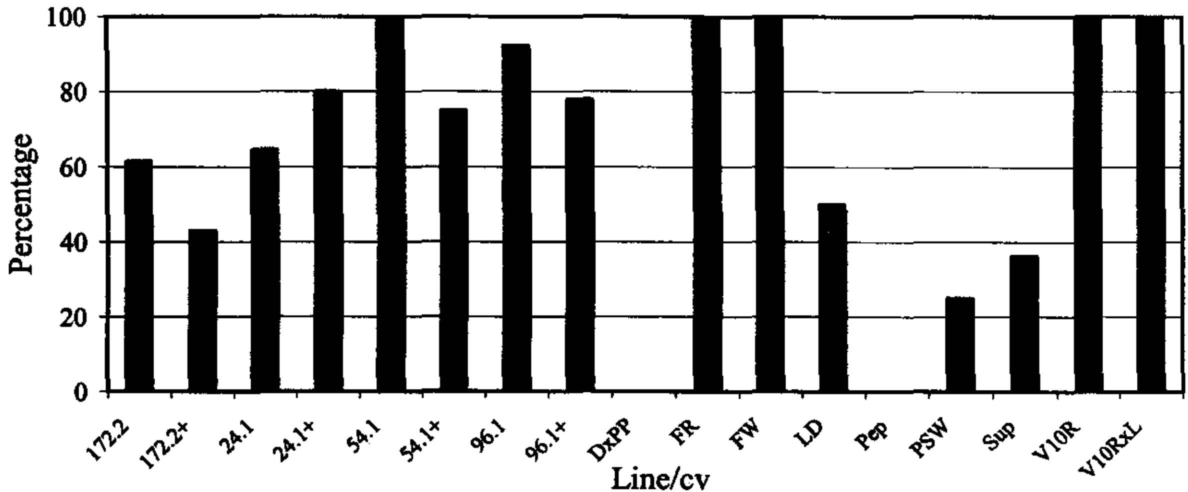
On the top branch, Freedom red and Pepride demonstrated the lowest mean leaf numbers (Figure 4.4.8, Table 4.4.15). Pepride had the lowest transitional bract count of the red bracted cultivars/lines, while lines 172.2 and 96.1 had the highest. Bract width contributes to ornamental appeal, and significant differences were found for this character. Freedom red and white, 24.1 and 96.1 possessed the largest bract widths.

Figure 4.4.8. Number of leaves, transitional bracts, bracts below the primary cyathium and bracts above the primary cyathium on the top branch according to cultivar/line at stage 5



Anthesis was observed for a proportion of plants of each cultivar/line (except DxPP and Pepride, which had a small sample size). All plants of cultivars Freedom red, Freedom white, V10 Amy red, line V10RxL and selected line 54.1 reached anthesis (Figure 4.4.9).

Figure 4.4.9. Percentage of plants of each cultivar/line that reached anthesis at stage 5



Correlations and their implications for breeding

Correlations were performed between the characters measured at stage 5 for all non-PoiBI infected plants to determine positive or negative relationships that could impact upon breeding (Table 4.4.16). Numerous significant positive and negative correlations were observed.

Plant height was highly correlated with the length of the top shoot (r = 0.921). In addition, plant height was highly correlated with H/N (r = 0.902) although a moderate correlation was found with total node number (r = 0.367) and node number on the top shoot (r = 0.418). These correlations emphasise the fact that plant height is determined by the number of nodes in a vertical line to the top of the plant and their internodal distance. Selection based upon the length of the top shoot can be expected to concurrently select for plant height and internode length as these factors are strongly correlated.

A low negative correlation was observed between T/N of the top shoot and length of the top shoot (r = -0.372). This negative correlation could be considered as unusual, as an increase in node number could be considered to result in increased height. However, this population of cultivars and lines was primarily chosen for short height and vigorous growth and this correlation reflects the selection of plant material.

Stem diameter was not highly correlated with any parameters, although a low correlation was observed with height (r = 0.281) and H/N (r = 0.259). The low correlation between stem diameter and height indicated that short plants with large stem diameter could be selected from this population.

Total node number was highly correlated with node number on the top shoot (r = 0.887). This was expected as all plants were decapitated to six nodes. Node number on the top shoot was also moderately correlated with the length of the top shoot (r = 0.448) and number of leaves on the top shoot (r = 0.546). Total nodes had a low correlation with height (r = 0.364), but moderate correlation with length of the top shoot (r = 0.428), leaves on the top shoot (r = 0.610) and bracts above the primary cyathium (r = 0.406). Length of the top shoot had good correlation with H/N (r = 0.783).

A low negative correlation was found between leaves on the top shoot and transitional bracts on the top shoot (-0.398). A similar correlation was found between leaves on the top shoot and bracts on the top shoot below the primary cyathium (-0.445). These two negative correlations provide evidence that a reduced number of leaves on the top shoot results in a subsequent increase in transitional bract number and bract number below the primary cyathium.

Bract number on the top shoot below the primary cyathium was moderately negatively correlated with bracts above the primary cyathium on the top shoot (r = -0.432).

Increased leaf number on the top shoot appeared to result in reduced largest bract diameter, which is not desirable (r = -0.514). By selecting for low leaf number breeders may be able to concurrently make increases in largest bract diameter in this population of lines and cultivars. The length of the top branch was moderately negatively correlated with largest bract diameter (r = -0.438), indicating plants with shorter top branch length also had larger bracts.

Branch number was not correlated with height when assessing the whole data set or when assessing only non-PoiBI infected data.

Table 4.4.16. Correlations between parameters measured at stage 5 for all non-PoiBI infected cultivars and lines

	Anthesis	Stem diameter (mm)	Branch number	Height (mm)	Length (mm) of top shoot to base of primary cyathium	Leaves on top shoot	Transitional bracts on top shoot	Bracts on top shoot below primary cyathium	Nodes on top shoot	Bracts on top shoot above primary cyathium	Total nodes‡	Largest bract width (mm)	T/N of top shoot (days)
Stem diameter (mm)	0.144^{ns}												
Branch number	-0.049^{ns}	-0.201*											
Height (mm)	0.220*	0.281**	0.173^{ns}										
Length (mm) of top shoot to base of primary cyathium	0.201*	0.202*	0.237**	0.921***									
Leaves on top shoot	0.244**	0.002^{ns}	-0.083^{ns}	0.390***	0.476***								
Transitional bracts on top shoot	-0.098^{ns}	-0.006^{ns}	0.078^{ns}	0.064^{ns}	0.025^{ns}	-0.398***							
Bracts on top shoot below primary cyathium	-0.191*	0.232*	0.141^{ns}	-0.141^{ns}	-0.194*	-0.445***	-0.174^{ns}						
Nodes on top shoot below primary cyathium	0.057^{ns}	0.212*	0.086^{ns}	0.418***	0.448***	0.546***	0.134^{ns}	0.214*					
Bracts above primary cyathium on top shoot	-0.119	-0.215*	0.076^{ns}	-0.034^{ns}	0.040^{ns}	0.237**	0.044^{ns}	-0.432***	-0.061^{ns}				
Total nodes‡	-0.003^{ns}	0.095^{ns}	0.043^{ns}	0.367***	0.428***	0.610***	0.143^{ns}	0.004^{ns}	0.887***	0.406***			
Largest bract width (mm)	-0.276**	0.100^{ns}	-0.275**	-0.341***	-0.438***	-0.514***	0.110^{ns}	0.262**	-0.326***	0.112^{ns}	-0.247**		
T/N (days) of top shoot	0.027^{ns}	-0.092^{ns}	-0.012^{ns}	-0.318***	-0.372***	-0.589***	-0.144^{ns}	0.017	-0.883***	-0.395***	-0.991***	0.206*	
H/N (mm)	0.232*	0.259**	0.154^{ns}	0.902***	0.783***	0.125^{ns}	0.004^{ns}	-0.130^{ns}	0.043^{ns}	-0.227*	0.065^{ns}	-0.224**	0.113^{ns}

‡ Total nodes measured on primary stem, top branch and to last bract >30 mm in a single line. These nodes were counted because they contribute to plant height. *, **, ***: $p<0.05$, 0.01, 0.001 respectively. ns: not significant.

Characterisation and selection of cultivars and lines for future breeding studies

For breeding purposes, vigorous short growing plants with good ornamental merit are desirable. Considering these parameters, the lines DxPP and V10RxL can be discounted due to their tall height and undesirable ornamental characteristics (such as small bract size and poor bract colour). The cultivar Supjibi can also be discounted due to its chromosome complement of 2n = 56. Producing seeds by crossing between 2n = 56 and 2n = 28 plants has been shown to be difficult (Experiment 4.1) and probably requires embryo rescue therefore, the use of this cultivar in further studies is limited. Thus, these two lines and one cultivar are not included in the following characterisation/selection process.

The seven shortest cultivars/lines were 24.1, 54.1, V10 Amy red, Freedom white, 172.2, 96.1 and then Freedom red. These cultivars/lines would be suitable for further breeding studies to produce dwarf poinsettias.

High node number coupled with short height is desirable for the production of vigorous dwarf poinsettias. The following cultivars/lines recorded a high node number and short height: V10 Amy red, 96.1, 24.1, 54.1 and Freedom red. Increased node number indicates increased rate of growth, which in turn could lead to increased vigour. The upper limit for node production is reached at approximately 25°C mean daily temperature when one node is produced every four to five days (Tayama et al. 1990). Therefore, under ideal conditions, a difference of one node can increase the production cycle by approximately 5 days if all other factors remain constant. The cultivars Pepride and Lemon drop had low mean node number at stage 5 concurring with the generally accepted view that they have a slow rate of growth (Rinehart pers. comm. 1997). Mean internode length was lowest on V10 Amy red, 24.1, 54.1 FW and 96.1.

Large stem diameter is important to reduce branch breakage and enable production of strong plants. Stem diameter was greatest for Freedom white, 96.1, Lemon Drop and 172.2. Branch number is important for the production of multi-branched plants after apical decapitation. Cultivars V10 Amy red and Freedom white may be suitable for further breeding studies to produce plants that possess high branch number without phytoplasma infection as these two cultivars had the highest branch numbers.

Low leaf number on the top shoot indicates a rapid response to the short photoperiod, which thereby results in rapid development of the first transitional bract. A rapid response is desirable for producing cultivars with a short response period (period from start of short

photoperiod to date of sale), thus reducing the growing cycle and cost of production. Early responding cultivars included Freedom red and Pepride (an induced mutation of Freedom red).

Low transitional bract number is desirable because a high transitional bract number could be visible on plants ready for sale in some circumstances, and detracts from ornamental appeal. The lowest transitional bract numbers were observed for cultivars Pepride and Freedom red. White bracted cultivars/lines possessed low transitional bract numbers in comparison to red bracted plants because anthocyanin is not produced in white bracted plants and influences transitional bract production.

A high bract number is also desirable for ornamental appeal. Cultivars/lines with high bract numbers below the primary cyathium had low leaf and transitional bract numbers. These genotypes are desirable for further breeding studies (i.e. cultivars Pepride and Freedom red). Bract number above the primary cyathium was greatest for cv. V10 Amy red and line 24.1.

Freedom red and white, 24.1 and 96.1 possessed the largest bract widths. indicating these cultivars/lines are suitable for breeding for increased bract width.

Poinsettias are often sold when anthesis has been reached, however, date of sale also depends upon sufficient bract coverage. Therefore, plants that reach anthesis earliest with sufficient bract coverage are suitable for further breeding in the context of reducing the production period. Three cultivars (Freedom red, Freedom white, V10 Amy red) and one line (54.1) had 100% of plants at anthesis at stage 5.

Discussion

The main aims of this experiment were to i. - characterise cultivars and lines for height, ii. - to determine the effects of PoiBI on height and morphology in four selected lines with and without PoiBI and iii. - characterise cultivars and lines for potential use in future breeding studies aimed at the production of short, vigorous poinsettias.

The primary observation from classifying cultivars/lines depending on height was that significant height differences were minimal at stage 5. Only two seed derived lines (neither commercial cultivars nor selected short lines) were significantly taller than the rest. This

indicated one or more genes for short plant height were present in the cultivars/lines tested. The two tall lines were derived via crossing with at least one parent that is generally denoted as tall. The crossed parents were cv. Diva (tall) x cv. Pink peppermint (tall) and cv. V10 Amy red (short) x cv. Annette Hegg Lady (tall). These results reflect the general observation made in Experiment 4.2 that tall x tall pollinations generally produced tall progeny and short x short pollinations generally produce short progeny. The time of year may have influenced the results of this experiment substantially. It is known that cv. Freedom red can be grown to different heights at different times of the year (Experiment 3.4). Therefore, it can be presumed that other cultivars could likewise grow to different heights, depending upon season. It would therefore, be desirable to conduct this experiment several times during the year to ascertain the effects of changes in seasons, daylength, temperature, etc. on plant height and morphology. Such a study was beyond the scope of this project, but could be performed in future investigations.

The four selected lines (devoid of PoiBI) were not significantly shorter than their progenitors, although 24.1 and 54.1 recorded the lowest mean heights amongst non-PoiBI infected lines. Increased replication could result in clear significant differences between these seedlings and their parents, however it is likely that the difference would be small as observation of their means (i.e. ~10 mm) indicate.

Incorporation of phytoplasma into the selected four short lines consistently resulted in a decrease in mean height and stem diameter at stage 5. The three shortest lines were infected with PoiBI, although they were not significantly shorter than their non-infected counterparts. This indicates that a reduced effect of phytoplasma occurred in these lines, which are genotypically short. Low replication of some cultivars/lines may have also contributed to reduced precision.

PoiBI infected lines responded in a similar manner to PoiBI infected Freedom red in Experiment 3.4. In addition to height and stem diameter reductions, node number was reduced at stage 5, and branch number was increased for infected plants. Mean top branch length was also reduced. Time to anthesis was slightly reduced on phytoplasma infected lines, but a genotype interaction was evident.

Analysis of correlations between parameters measured at stage 5 revealed selection of short, vigorous (increase node production) and large stem diameter plants could be possible as no strong negative correlations were found among any of these parameters.

For future breeding studies, several cultivars/lines can be utilised due to their reduced height (24.1, 54.1, V10R, FW, FR, etc), increased node numbers (V10R, 96.1), reduced mean internode length (V10R, 24.1, 54.1, FW, etc), increased stem diameter (FW, 96.1) and increased branch number (FW, V10R, 172.2, etc). Additionally, plants that responded rapidly to the short photoperiod (FR, Pep, 96.1, etc) may be useful for creating lines with reduced response period. However, transitional bract number should be considered, as higher numbers are ornamentally undesirable. Reduced transitional bract number shows a low negative correlation with increased leaf number on the top shoot ($r = -0.398$) in this population. Time to anthesis is also important, providing bract development is acceptable. Increased bract number is related to decreased leaf and transitional bract number on the top shoot. Therefore, earlier responding cultivars/lines can be expected to produce more bracts and less leaves.

The results from this preliminary study indicated advances towards producing shorter poinsettias through intraspecific hybridisation may be made if i. - some genes of small effect can be combined and ii. - population sizes can be increased to observe these effects. Among the commercial cultivars, V10 Amy red, Freedom white and Freedom white appear to be suitable candidates for future studies to breed ornamentally acceptable, shorter, vigorous poinsettias. The four selected lines are also acceptable (for height and ornamental appeal). Further studies on fertility of potential breeding candidates needs to be undertaken to determine the efficiency of seed production prior to conduction breeding experiments for the production of dwarf, ornamentally desirable poinsettias.

In conclusion, it appears that genetic diversity for plant height during commercial production is limited within the range of commercial cultivars utilised in this study. Major genes for short height appear to be present in these cultivars, as seedlings derived from tall parents (DxPP and V10RxL) were significantly taller than the current commercial cultivars. Introduction of novel genes outside of the commercial population may result in reduced height, or further intensive breeding efforts using current short cultivars may produce the same result. However, the effects of IBD may reduce vigour.

Chapter 5. Development of dwarf, commercially desirable poinsettias by targeted intraspecific hybridisation

Introduction

Producing shorter poinsettias compared to current commercially available cultivars via intraspecific hybridisation is limited by several factors such as availability of dwarfing genes, mutational load (due to accumulation of deleterious genes), heterozygosity leading to segregation and limitations on selfing due to inbreeding depression. Characterisation of seven available current commercial cultivars devoid of phytoplasma, during commercial production showed that differences in height were not significant, indicating limited genetic variability for this character in the cultivar genepool.

Intraspecific hybridisation for the production of dwarf poinsettias involves intermating the shortest, most ornamentally appealing and fertile parents and selecting the most suitable progeny for further development. Therefore, prior to any breeding effort, suitability of desirable short lines and cultivars as parents should be ascertained. A key consideration is the fertility of the female parent. In addition, interference from genetic factors causing mutational load and IBD need to be preferably eliminated. Due to the close relationship among poinsettia cultivars, selfing or intermating could lead to IBD. In addition, as several cultivars have been subjected to mutation breeding, mutational load could be a significant factor. Therefore, it was hypothesised that the best method to ascertain female fertility would be to conduct crosses to a male wild genotype that would be free of or have a minimal number of deleterious genes.

Following selection of suitable fertile female parents, hybridisation needs to be performed and desirable plants isolated. A large seed derived population is desirable for optimum selection efficiency and to develop a commercially acceptable product.

To select for superior progeny in a seed derived population, parental checks need to be utilised as a basis for selection. This poses a problem in poinsettias as all cultivars are vegetatively propagated. Therefore, two options were envisaged for obtaining controls, i. – develop controls from vegetative cuttings and grow them to match a similar stage of development as the seed derived population and ii. – utilise plants from self pollinated seeds of the parents as controls. Both methods have implications in regard to their use. For example,

vegetatively derived controls exhibit different physiological status to seed derived plants, and their growth needs to be estimated. On the other hand, controls derived from self pollinated seeds may exhibit the deleterious effects of mutational load/IBD, resulting in poor quality controls. Therefore, in this experiment, both vegetatively derived controls (VDC) and seed derived controls (SDC) were used to assess the seed derived population (SDP) obtained from various crosses.

Considering heat tolerance is a desirable attribute in Australia, the experiment was conducted predominantly during summer and selections were made after eight weeks under short photoperiod conditions.

Following selection of dwarf, ornamentally desirable lines from the seed derived population, grafting is required to infect each selected line with phytoplasma. A replicated vegetative cutting experiment as per commercial production then needs to be performed using the phytoplasma infected lines during summer in Australia to determine which lines, if any, are shorter than current short cultivars.

Part I. Assess female fertility of desirable dwarf germplasm

Part II. Produce seeds via intermating selected dwarf lines and cultivars

Part III. Grow plants from seeds and select shorter, ornamentally desirable, vigorous plants in comparison to seed derived and vegetatively derived controls

Part IV. Infect selections with phytoplasma and trial phytoplasma infected selections with cultivar controls under commercial production conditions during summer in Australia

Part I. Crossing with a male wild type poinsettia for evaluation of female fertility of selected parents

Materials and methods

Parents assessed for fertility were selected based upon demonstrated short height and ornamental appeal in a commercial production experiment (Experiment 4.4). Parents are listed in Table 5.1.

Two plants of each commercial cultivar and four selected lines (97/24.1, 97/54.1, 97/96.1 and 97/172.2 developed in Experiment 4.3) all without PoiBI and PnMV, were grown according to standard production practices (Experiment 3.1). One wild poinsettia line, devoid of PoiBI and PnMV was also grown (97/176.2WT) and used as a male parent. This line was derived via seed from a line collected in the wild in Mexico.

Table 5.1. Germplasm assessed for female fertility

Female cultivar/line	Pedigree	Breeder
97/24.1	Freedom white x V10 Amy red	Bernuetz
97/54.1	V10 Amy red x V10 Amy red	Bernuetz
97/96.1	Freedom red x V10 Amy red	Bernuetz
97/172.2	Freedom red x Freedom red	Bernuetz
cv. Freedom red	Induced sport of a seedling[3]	Fruehwirth
cv. Freedom white	Induced mutation of Freedom red[3]	Jacobsen
cv. Lemon drop	Seedling[1]	Paul Ecke Ranch
cv. Pepride	Induced mutation of Freedom red[3]	Jacobsen
cv. Peterstar white	Induced mutation of Peterstar[3]	Jacobsen
cv. Supjibi	Probably a tetraploid of Angelika[2]	Gross
cv. V10R	Seedling[3]	Gutbier

Male cultivar/line	Pedigree	Breeder
97/176.2WT	wild type x self	Underhill

[1] Ecke *et al.* (1990), [2] Starman and Abbitt (1997), [3] U.S.A. Patent and trademark office.

Plants were placed inside environment E1 (as in Experiment 4.1) to induce flowering. Pollinations were performed using cultivars and lines as female parents and 97/176.2WT as the male parent. Self pollinations of 97/176.2WT were also performed.

Pollination methodology and seed collection were conducted as per procedures stated in Experiment 4.1. Seed production efficiencies (using grade 1 and 2 seeds only) were determined as a percentage of the number of ovules pollinated. Fertility was assessed based on seed production efficiencies.

Results

A total of 124 crosses were performed. Among cultivars, fertility ranged from 0 – 88.9% (Table 5.2). It was observed that cultivars with low or no seed production ability had been derived via mutation breeding methods (e.g. Peterstar white and Pepride). This suggested that their inability to produce seed could be a result of infertility due to their mode of development. However, cv. Freedom white, a cultivar bred via mutation breeding, was relatively fertile with 44% seed production efficiency. Supjibi, a tetraploid (2n = 56) could be expected to produce triploid offspring. However, no seeds were produced. Cultivar Lemon drop also produced no seeds.

Table 5.2. Seed production efficiencies from crosses conducted with a wild poinsettia line (97/176.2WT) used as a male parent

Female parent	Number of pollinations	Seeds produced	Seed production efficiency† (%)
97/24.1	5	14	93.3
97/54.1	5	15	100
97/96.1	8	18	75.0
97/172.2	6	8	44.4
cv. Freedom red	15	28	62.2
cv. Freedom white	3	4	44.4
cv. Lemon drop	7	0	0
cv. Pepride	21	1	1.58
cv. Peterstar white	16	0	0
cv. Supjibi	16	0	0
cv. V10 Amy red	24	64	88.9
97/176.2WT (self)	7	17	81.0

† Determined by dividing seeds produced by number of ovules pollinated.

Among the four selected lines, fertility ranged from 44.4 – 100%. These lines were derived from crosses conducted between V10 Amy red, Freedom red and Freedom white (Table 5.1).

The cultivar parents of these lines also exhibited good fertility (44.4 – 88.9%). Self pollination of 97/176.2WT (a parent derived from a self-pollinated seed) resulted in 81% seed production efficiency, showing two generations of selfing can result in high seed production efficiency. Selection of parents for producing dwarf poinsettias is dealt with in Part II.

Part II. Seed production from selected parents

Materials and methods

Plant materials and environmental conditions

Cultivars and lines (Table 5.3) were selected from those shown in Table 5.1 based upon fertility results from crossing with a wild poinsettia (from Part I). Plants with red bracts were considered as most desirable for selection because this colour has the largest market share. Cultivar Freedom white, with white bract colour was not chosen, although it had good fertility of 44.4%.

Cultivars/lines listed in Table 5.3 were utilised as parents for conducting diallel crosses (Tables 5.4 and 5.5) to develop a seed derived population from which dwarf, ornamentally desirable plants could be selected. The four parental lines (24.1, 54.1, 96.1, 172.2) did not carry either PnMV or PoiBI. However, they could have been infected with PnCV. Both cultivars Freedom red and V10 Amy red were infected with PnMV, PoiBI and PnCV due to their vegetative propagation from infected parents.

Table 5.3. Parental germplasm used for conducting diallel crosses

			Infection status			
Code	Line or cultivar	Pedigree	PoiBI	PnMV	PnCV	Breeder
24.1	97/24.1	Freedom white x V10 Amy red	-	-	n.t.	Bernuetz
54.1	97/54.1	V10 Amy red x V10 Amy red	-	-	n.t.	Bernuetz
96.1	97/96.1	Freedom red x V10 Amy red	-	-	n.t.	Bernuetz
172.2	97/172.2	Freedom red x Freedom red	-	-	n.t.	Bernuetz
FR	cv. Freedom red	Induced sport of a seedling[1]	+	+	+	Fruehwirth
V10R	cv. V10 Amy red	Seedling[1]	+	+	+	Gutbier

[1] United States Patent and Trademark Office. Note: - : not infected, + : infected, n.t. : not tested.

Four to ten plants of each genotype were grown from cuttings according to standard practices described in Experiment 3.1. Plants were allowed to flower and pollinations were performed in environment E1 (Experiment 4.1) as described next.

Diallel crosses among i. - four select lines and ii. - two cultivars for production of seed derived population and seed derived controls

Two separate diallel crossing experiments were performed (Tables 5.4 and 5.5). The first set involved only the four selected lines. The second set consisted of two cultivars. Crosses among selected lines and cultivars were not performed due to genetic similarity between the cultivars and select four lines. i.e. all select lines were derived from various cross or self combinations of cv. Freedom red, cv. V10 Amy red or cv. Freedom white , which is a mutant of cv. Freedom red. As many crosses as possible were conducted within each diallel to produce the largest number of seeds possible from each cross combination. Pollinations were performed (Table 5.3) as per methodology detailed in Experiment 4.1. As seeds ripened, they were collected in glassine bags and individually labelled prior to being placed inside a seed storage room maintained at 18°C and 30% relative humidity until sowing. Poor quality seeds (grade 3) were discarded. The number of seeds produced from each parental combination and the length of each seed (mm) was recorded prior to sowing.

Table 5.4. Diallel crossing design for four lines

♂ ♀	97/24.1	97/54.1	97/96.1	97/172.2
97/24.1	self	X	X	X
97/54.1	X	self	X	X
97/96.1	X	X	self	X
97/172.2	X	X	X	self

X: cross performed. Seeds derived via self pollination were used as controls in Part III.

Table 5.5. Diallel crossing design for two cultivars

♂ ♀	Freedom red	V10 Amy red
Freedom red	self	X
V10 Amy red	X	self

X: cross performed. Seeds derived via self pollination were used as controls in Part III.

Seed germination

All seeds were sown on the same day into 400 ml pots filled with NFP potting mix. Each pot was labelled with the code of the seed planted in it and the pots were placed inside a glasshouse maintained under long photoperiod conditions provided by >2 µmol m^{-2} s^{-1} incandescent light for four hours starting at 10 pm. Germination date was recorded two to three times per week and pots were manually watered as required.

Results

A total of 4257 ovules were pollinated during the two diallel cross combinations conducted, to produce 576 seeds resulting in a 13.5% seed/ovule efficiency (Table 5.6). This is similar to that obtained in Experiment 4.1 (14%). The efficiency of seed production from the four line diallel (23.1%, refer to Table 5.7) was twice as great compared to the efficiency recorded from the two cultivar diallel (10.5%, refer to Table 5.8). Only 1020 ovules were pollinated for the four line diallel to produce 236 seeds, whereas 3237 ovules were pollinated in the two cultivar diallel to produce 340 seeds.

Table 5.6. Total number of pollinations conducted, seed and plant production efficiencies for diallel crosses of four lines and two cultivars

				Efficiencies of production	
Pollinations	Ovules pollinated	Seeds produced	Plants produced	Seeds/ovule pollinated (%)	Plants/ovule pollinated (%)
1419	4257	576	310	13.5	7.3

When seeds were planted, germination efficiency was always less than 100% for each cross combination (except for 172.2 x 172.2 which only produced one seed, which germinated). A total of 310 seeds germinated (54% germination rate) and produced plants (Table 5.6). The resulting plant production efficiency per ovule pollinated for all crosses was 7.3% and is similar to that obtained in Experiment 4.1 (8.6%).

Seed production was highly dependent upon the cross performed (Table 5.7). Lines 24.1 and 54.1 were generally more successful as female parents as compared to 96.1 and 172.2. The line 24.1 had the greatest seed production. When line 24.1 was used as a female parent in crosses with lines 54.1, 96.1 and 172.2 as male parents, high seed production efficiencies were recorded, indicating that these lines were fertile when used as male parents (Table 5.7). When lines 172.2 and 96.1 were used as female parents, seed production was rarely observed,

except when crossed with 24.1. Self pollinations of 96.1 and 172.2 were also rarely successful. This indicated the presence of reciprocal differences among lines. Reciprocal differences were also present in the two cultivar diallel (V10R x FR vs. FR x V10R). The highest seed production efficiency in the two cultivar diallel was from the cross FR x V10R (20.5%).

Table 5.7. Number of pollinations conducted, seed and plant production efficiencies for diallel crosses conducted among four lines

Parents						Efficiencies of production	
Female	Male	Pollinations	Ovules pollinated	Seeds produced	Plants produced	Seeds/ovule pollinated (%)	Plants/ovule pollinated (%)
24.1	**24.1**	**34**	**102**	**25**	**10**	**24.5**	**12.7**
24.1	54.1	15	45	22	15	48.9	33.3
24.1	96.1	18	54	46	37	85.2	72.2
24.1	172.2	14	42	32	17	76.2	42.9
54.1	24.1	28	84	25	18	29.8	21.4
54.1	**54.1**	**45**	**135**	**13**	**2**	**9.6**	**3.7**
54.1	96.1	33	99	13	9	13.1	9.1
54.1	172.2	34	102	16	10	15.7	10.8
96.1	24.1	20	60	37	13	61.7	23.3
96.1	54.1	24	72	0	0	0	0
96.1	**96.1**	**21**	**63**	**1**	**0**	**1.6**	**0**
96.1	172.2	25	75	0	0	0	0
172.2	24.1	6	18	3	2	16.7	11.1
172.2	54.1	8	24	2	0	8.3	0
172.2	96.1	8	24	0	0	0	0
172.2	**172.2**	**7**	**21**	**1**	**1**	**4.8**	**4.8**
Total		340	1020	236	134	23.1%	13.1%

Plants produced via self pollination, for use as seed derived controls in Part III, are indicated in bold.

Table 5.8. Number of pollinations conducted, seed and plant production efficiencies for diallel crosses conducted between two cultivars

Parents		Pollinations	Ovules pollinated	Seeds produced	Plants produced	Efficiencies of production	
Female	Male					Seeds/ovule pollinated (%)	Plants/ovule pollinated (%)
FR	**FR**	**170**	**510**	**66**	**41**	**12.9**	**8.0**
FR	V10R	112	336	69	51	20.5	15.8
V10R	FR	415	1245	81	46	6.5	3.9
V10R	**V10R**	**382**	**1146**	**124**	**38**	**10.8**	**3.3**
Total		1079	3237	340	176	10.5%	5.4%

Plants produced via self pollination, for use as seed derived controls in Part III, are indicated in bold.

Part III. Characterisation and selection of seed derived plants

Materials and methods

Three distinct populations were used to enable selection of dwarf, ornamentally desirable plants. a) The seed derived population (SDP) comprised all plants derived from reciprocal crosses between lines and cultivars produced in Part II. b) The seed derived controls (SDCs) were all plants derived from self pollinations of lines and cultivars produced in Part II. c) Vegetatively derived controls (VDCs) were vegetative cuttings of lines and cultivars used in Part II (refer to Table 5.10). The SDP, SDCs and VDCs were not infected with PoiBI or PnMV. All plants were placed in a completely randomised order in the experimental area.

a) Seed derived population (SDP)

Approximately 8 weeks from the date of sowing (Table 5.9), most seedlings had developed 3-4 true leaves. All seedlings were then transplanted into 200 mm pots containing NFP potting mix in an environment maintained under long photoperiod as described previously. Pots were randomised and placed at an the interpot distance of 350 mm, as measured from the centre of pots. Osmocote Plus® 3-4 month slow release fertiliser was applied to all plants at 18g/pot, 28 days after planting. Water was applied manually.

Following the first scoring, all plants were decapitated to remove approximately 20 mm of the apical meristem. Decapitation promotes uniformity of growth and thereby minimises differences in growth among and between seed derived and vegetatively derived plants. During normal commercial production, after apical decapitation, plants are grown for two weeks under long photoperiod conditions and then placed into a short photoperiod

environment. However, in this experiment, to gain a better understanding of mean internode length and premature floral initiation (PFI), plants were grown for eleven weeks prior to being moved to an artificial short photoperiod area of 10 h (commencing at 7 am) to induce flowering. Interpot spacing was maintained at 350 mm between the centres of pots. Drip irrigation was used and a general liquid fertiliser was applied as required. Plants were scored for the second time after eight weeks under short photoperiod conditions.

Table 5.9. Procedures conducted with number of days at each step from sowing and temperature conditions for the SDP

Procedure	Duration (days)	Mean daily temperature min. - max. (°C)
Sowing	0	
Planting	59	16 – 25
First scoring followed by apical decapitation	137	17 – 29
Transferral to short days	214	17 – 39
Second scoring (8 weeks under short photoperiod)	270	18 – 30

b) Seed derived controls (SDCs)

Among the two diallels conducted, progeny derived via self pollination were used as seed derived controls (SDC). These seeds were treated identically to the SDP.

c) Vegetatively derived controls (VDC)

Genotypes used as vegetative derived controls (VDC) are listed in Table 5.10.

Table 5.10. Cultivar/line, pedigree and number of plants grown for VDCs

Code	Line or cultivar	Pedigree	Number of plants
24.1	97/24.1	Freedom white x V10 Amy red[1]	13
54.1	97/54.1	V10 Amy red x V10 Amy red[1]	5
96.1	97/96.1	Freedom red x V10 Amy red[1]	13
172.2	97/172.2	Freedom red x Freedom red[1]	13
FR	cv. Freedom red	Induced 'sport' of a seedling[2]	13
V10R	cv. V10 Amy red	Seedling[2]	13

[1] Produced by author, [2] United States Patent and Trademark Office.

Stockplant production and vegetative propagation was performed as described in Experiment 3.3. Approximately 28 days after the start of propagation, uniform acclimatised cuttings from each cultivar/line were potted into 200 mm pots filled with NFP mix. Pots were randomly

placed at an interpot distance of 350 mm as measured from the centre of pots. To ensure that VDCs were at a similar stage of growth as the SDP at planting, standardisation was performed by decapitation (which re-instates juvenility) to 6 nodes after approximately 5 weeks from the date of planting (Table 5.11). Subsequently, after 4 weeks, all branches except the top branch were removed from VDCs, to match/equalise the mean level of development of the SDP and SDCs. From this stage, all plants were treated identically.

Table 5.11. Procedures conducted with days from propagation and temperature conditions for VDCs

Procedure	Duration (days)	Mean daily temperature min. – max. (°C)
Start of propagation	0	
Planting	25	20 – 27
Standardise (decapitate to 6 nodes)	60	17 – 33
Standardise (1 top branch only)*	91	16 – 30
First scoring followed by apical decapitation	169	17 – 29
Transferral to short days	246	17 – 39
Second scoring (8 weeks under short photoperiod)	302	18 – 30

*Date of standardising to one branch (day 91) is the same as date of planting seed derived plants (day 59) in Table 5.9. All procedures from this point onward were identical for VDCs and seed derived plants.

Data collection for SDP, VDCs and SDCs

Scoring was performed on two occasions, i. - during vegetative growth and ii. - after eight weeks under a short photoperiod environment (Tables 5.9 and 5.11).

First scoring

All plants including controls were scored (after 137 days from sowing seeds) for the following parameters.

- number of nodes from the first node to the uppermost node subtending the most apical leaf greater than 30 mm in length
- height from the surface of the potting media to the tip of the plant (mm)
- stem diameter measured at the approximate mid point of the stem (mm)
- H/N (height per node - mm) or mean internode length was derived by dividing height by the node number
- T/N (time per node - days) or mean node production rate was derived by dividing time (days) from sowing by the node number

Second scoring

This scoring was performed 133 days after the first scoring (Table 5.9). The number of branches greater than 100 mm in length was recorded for each plant. In addition, the following parameters were measured on the top branch. The top branch of plants was considered suitable as representative of the whole plant because previous experiments had shown this to be a consistent feature of cultivars and lines tested (Experiment 3.3, 3.4 and 4.4).

- anthesis (1 = yes, 2 = no) for at least one cyathium
- number of nodes from the junction of the top branch with the main stem below the primary cyathium
- height from the junction of the top branch with the main stem to below the primary cyathium
- leaf number
- number of transitional bracts (less than 90% coloured)
- number of bracts below the primary cyathium. The three bracts emerging beneath the primary cyathium were treated as one because they emanated from one nodal position
- number of bracts (greater than 30 mm) after the primary cyathium along one cyathial branch
- stem diameter at the approximately midpoint of the stem (mm)
- H/N (mm) was calculated as height of the top branch divided by the number of nodes along its length (i.e. addition of leaves, transitional bracts and bracts below the primary cyathium)
- T/N (days) was calculated as time in days from apical decapitation to second scoring divided by the total node number developed after apical decapitation along the top branch (i.e. addition of leaves, transitional bracts and bracts below and above the primary cyathium).

Additionally, bract colour (red/white/yellow), abnormalities (e.g. bract protrusions, green bracts, 'weak' plants, etc) and presence of premature floral initiation were recorded. Plants that exhibit premature floral initiation at a low node number are not commercially useful at present.

Correlations

Correlations were performed between and amongst first and second scoring parameters to determine if a linear relationship existed over time and between characters and to identify methods to improve selection efficiency.

Selections

After the second scoring, selections were conducted based upon SDC and VDC mean height and node numbers. Ornamental appeal was assessed and determined on a scale of 1 – 3, where 1 = excellent (better than cv. Freedom red), 2 = average (similar to cv. Freedom red) and 3 = poor (worse than cv. Freedom red). Factors such as bract colour, transitional bract number and lack of abnormalities were independently considered and used for culling to derive final selections.

Data analyses

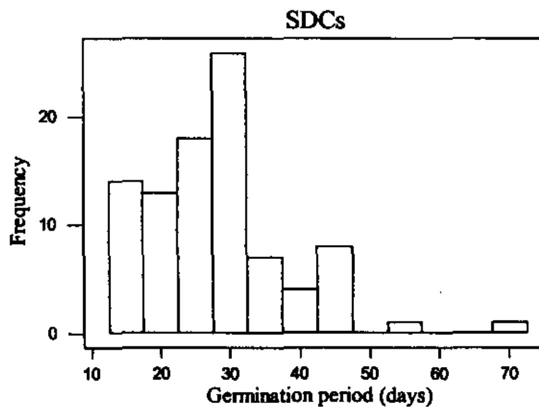
Data analyses were performed using the MINITAB® statistical software package (MINITAB® for windows release 12.22, Minitab Inc. 1998). The experiment was performed as a completely randomised design. Analysis of segregation of genes for height was conducted by developing histograms of self-pollinated seed derived plants from cultivars Freedom red and V10 Amy red.

Results

a) Analyses at first scoring for the SDCs and VDCs

Germination period for the SDCs ranged from 17 – 70 days (Figure 5.1). Germination of VDCs is not applicable.

Figure 5.1. Histogram for germination period of the SDCs



At first scoring, mean height for SDCs (163 mm) was much lower than VDCs (227 mm). The mean node number (14.9) and stem diameter (4.98 mm) for SDCs were also lower than for VDCs (21.0 and 8.84 mm respectively). Mean H/N for SDCs (10.9 mm) was similar to VDCs

(10.8 mm). T/N was slightly lower for SDCs (9.73 days) compared to VDCs (10.1 days). The maximum value for height for SDCs (289 mm) was slightly lower than VDCs (306 mm).

These results showed that there were differences between SDCs and VDCs for most parameters at first scoring.

Table 5.12. Means, standard errors and maximum and minimum values for parameters measured at first scoring for the SDCs and VDCs

Seed derived controls (SDCs)	n	Mean	SE	Min.	Max.
Height (mm)	92	163	5.5	55.0	289
Node number	92	14.9	0.36	7.0	22.0
Stem diameter (mm)	92	4.98	0.20	1.5	9.0
H/N (mm)	92	10.9	0.25	5.0	18.1
T/N (days)	92	9.73	0.26	6.23	19.6
Vegetatively derived controls (VDCs)					
Height (mm)	70	227	4.0	151	306
Node number	70	21.0	0.26	15.0	26.0
Stem diameter (mm)	70	8.84	0.16	5.5	11.0
H/N (mm)	70	10.8	0.15	7.55	13.7
T/N (days)	70	10.1	0.22	7.0	19.0

b) Analyses at second scoring for the SDCs and VDCs

At the second scoring (Table 5.13) mean height for SDCs (599 mm) was lower than VDCs (675 mm). Mean node number for the SDCs (27.3) and VDCs (28.8) was comparable. A difference was observed for mean stem diameter between SDCs (9.24 mm) and VDCs (11.1 mm). Mean H/N was slightly lower for SDCs (23.2 mm) compared to VDCs (24.8 mm). Mean T/N for SDCs (5.08 days) was slightly slower than VDCs (4.81 days). In addition to the above primary parameters, six other traits were measured at second scoring. Mean branch number for SDCs (4.39) was lower than VDCs (5.16). Mean leaf number for SDCs (19.8) was slightly lower than VDCs (20.6). SDCs had a lower mean transitional bract number (3.67) compared to VDCs (4.13). Mean bract number below the primary cyathium was similar for SDCs (2.11) and VDCs (2.26). Mean bract number above the primary cyathium was 1.68 for the SDCs and 1.88 for VDCs. Premature floral initiation (PFI) was noted for 6 SDC plants and 32 VDC plants (Table 5.14). Within the SDCs, 10 plants were recorded with an ornamental score of 1, 17 with a score of 2 and 65 with a score of 3. Within the VDCs, 57 plants obtained an ornamental score of 2 and 13 plants obtained a score of 3 (Table 5.15). All score 3 plants were cv. V10 Amy red. No plants were recorded as score 1 in the VDCs.

The above parameters measured showed that VDCs had greater stem diameter, faster production of nodes, increased branch number, more bracts and a majority plants with an ornamental score of 2. The SDCs were however, shorter in height and H/N, fewer plants exhibited PFI, transitional bract number was lower and 10 plants obtained an ornamental score of 1.

It was determined that both VDCs and SDCs could be used as controls to select ornamentally excellent, dwarf plants from the SDP. However, due to their mode of production, pooling of data would not be appropriate. Therefore, for each parameter selected, the most appropriate control was used (either SDCs or VDCs).

Table 5.13. Means, standard errors and maximum and minimum values for parameters measured at second scoring for the SDCs and VDCs

Seed derived controls	n	Mean	SE	Min.	Max.
Height (mm)	88	599	22	51.0	960
Node number	88	27.3	0.49	15.0	37.0
Stem diameter (mm)	88	9.24	0.28	2.0	15.0
Anthesis	88	1.31	0.05	1.0	2.0
Branch number	88	4.39	0.20	1.0	11.0
Leaf number	88	19.8	0.43	13.0	30.0
Transitional bracts	88	3.67	0.18	0.0	9.0
Bracts below primary cyathium	88	2.11	0.22	0.0	7.0
Bracts above primary cyathium	88	1.68	0.08	0.0	3.0
H/N (mm)	88	23.2	0.80	3.2	45.7
T/N (days)	88	5.08	0.10	3.6	8.9
Vegetatively derived controls					
Height (mm)	70	675	23	270	1010
Node number	70	28.8	0.56	17.0	36.0
Stem diameter (mm)	70	11.1	0.17	8.0	14.5
Anthesis	70	1.27	0.05	1.0	2.0
Branch number	70	5.16	0.20	2.0	10.0
Leaf number	70	20.6	0.31	14.0	28.0
Transitional bracts	70	4.13	0.26	0.0	8.0
Bracts below primary cyathium	70	2.26	0.22	0.0	6.0
Bracts above primary cyathium	70	1.88	0.10	0.0	4.0
H/N (mm)	70	24.8	0.65	13.5	42.6
T/N (days)	70	4.81	0.12	3.7	7.9

† Four SDC plants died between first and second scoring.

Table 5.14. Plants displaying premature floral initiation at second scoring

Population	Plant code	Number of plants displaying premature floral initiation (total plants)	Percentage of plants displaying premature floral initiation
SDP	24 x 54	1 (15)	6.6
	24 x 96	2 (37)	5.4
	24 x 172	1 (17)	5.9
	54 x 24	1 (18)	5.3
	54 x 96	1 (9)	11.1
	54 x 172	2 (10)	20
	FR x V10R	5 (51)	9.8
	V10R x FR	9 (46)	19.6
SDCs	24 x 24	1 (10)	10
	FR x FR	1 (41)	2.4
	V10R x V10R	4 (38)	10.5
VDCs	24	13 (13)	100
	54	3 (5)	60
	96	1 (13)	7.7
	172	2 (13)	15.4
	FR	6 (13)	46.1
	V10R	6 (13)	46.1

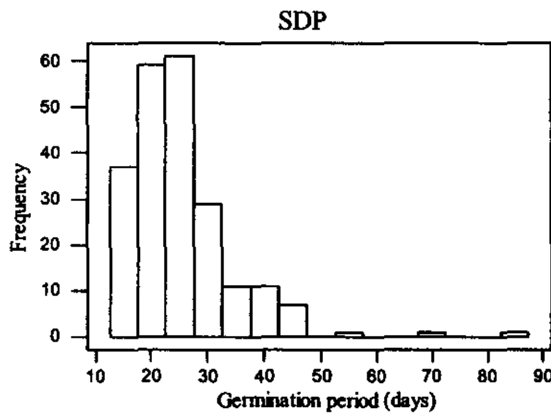
Table 5.15. Ornamental appeal results for SDCs and VDCs

	Number of plants	
Ornamental score	SDCs	VDCs
1	10	0
2	15	57
3	60	13

c) Comparative assessment of the SDP and controls at first scoring

The germination period for the SDP ranged from 17 - 85 days (Figure 5.2). At first scoring, mean height was closest between the SDP (215 mm) and VDCs (227 mm, Tables 5.12 and 5.16). H/N for the SDP (12.2 mm) was greater than both SDCs (10.9 mm) and VDCs (10.1 mm). Mean node number for the SDP (17.5) was between the means recorded for SDCs (14.9) and VDCs (21.0). Stem diameter of the SDP (6.92 mm) was also between SDCs (4.98 mm) and VDCs (8.84 mm). T/N for the SDP (8.28 days) was lower than both SDCs (9.73 days) and VDCs (10.1 days).

Figure 5.2. Histogram for germination period of the SDP



d) Comparative assessment of the SDP and controls at second scoring

At second scoring, mean height for the SDP (687 mm, Table 5.17) and VDCs (675 mm, Table 5.13) was very similar, however, SDCs had much lower mean height (599 mm). This showed that selfing resulted in shorter plants. Therefore, SDCs could be used as controls to select for plants with short height. Mean node numbers of VDCs (28.8) and the SDP (30.3) were closest. Therefore, VDCs could be used as controls to select for high node number. The SDP had the fastest node production rate (4.54 days) followed by VDCs (4.81 days) and SDCs (5.08 days). Mean branch number was lowest for the SDP (4.23) followed by SDCs (4.39) and VDCs (5.16). Lower branch number is not a desired feature. Mean leaf number was highest for the SDP (22.7) compared to SDCs (19.8) and VDCs (20.6). Lower transitional bract number (which is a desirable feature) was observed for the SDP (3.79) and SDCs (3.67) as compared to VDCs (4.13). An undesirable feature of the SDP was the lowest value for bract number below the primary cyathium (1.83) compared to SDCs (2.11) and VDCs (2.26). However, for bracts above the primary cyathium, the SDP had the highest mean (2.0) compared to the SDCs (1.68) and VDCs (1.88). Assessment of ornamental appeal showed 25 plants within the SDP achieved a score of 1 (better than cv. Freedom red), 32 were score 2 and 159 were score 3. Score 1 plants accounted for 11.6% of the SDP, which is very similar to SDCs with 11.4% of the population recording a score of 1. No VDCs recorded an ornamental score of 1.

The data indicated that primary selections could be made for height by utilising the mean height recorded for SDCs and for node number by utilising the mean node number recorded for VDCs.

Table 5.16. Means, standard errors and maximum and minimum values for parameters measured at first scoring for the SDP

Seed derived population	n	Mean	SE	Min.	Max.
Height (mm)	218	215	4.6	55.0	406
Node number	218	17.5	0.24	6.0	26.0
Stem diameter (mm)	218	6.92	0.14	1.50	11.5
H/N (mm)	218	12.2	0.17	5.0	19.4
T/N (days)	218	8.28	0.16	5.27	22.8

Table 5.17. Means, standard errors and maximum and minimum values for parameters measured at second scoring for the SDP

Seed derived population	n†	Mean	SE	Min.	Max.
Height (mm)	216	687	13	60.0	1095
Node number	216	30.3	0.30	16.0	41.0
Stem diameter (mm)	216	10.3	0.16	2.0	15.0
Anthesis	216	1.25	0.03	1.0	2.0
Branch number	216	4.23	0.10	1.0	10.0
Leaf number	216	22.7	0.27	12.0	32.0
Transitional bracts	216	3.79	0.18	0.0	9.0
Bracts below primary cyathium	216	1.83	0.12	0.0	8.0
Bracts above primary cyathium	216	2.00	0.05	0.0	3.0
H/N (mm)	216	24.2	0.40	3.80	39.2
T/N (days)	216	4.54	0.05	3.30	8.40

† Two seed derived plants died between first and second scoring.

Correlations among and between parameters measured at first and second scoring for the SDP

Correlations for various parameters measured for the SDP at first and second scoring are presented in Table 5.18. Many significant positive correlations were observed.

Within the first scoring, good correlations (r >0.70) were found between height and stem diameter (r = 0.817), height and node number (r = 0.783), height and H/N (r = 0.805) and stem diameter and node number (r = 0.775). Within the second scoring, a high correlation was observed between height and H/N (r = 0.859). A similar high correlation between these two parameters has been observed in a previous experiment (Table 4.4.16). A good correlation was also observed between height and stem diameter (r = 0.702).

Table 5.18. Correlation table for linear relationship between SDP parameters measured at (1) first scoring and (2) second scoring

	Seed length	Germination period	Height (1)	Node no. (1)	Stem diam. (1)	H/N (1)	Height (2)	Node no. (2)	Stem diam. (2)	H/N (2)
Germination period	ns									
Height (1)	0.226***	-0.324***								
Node number (1)	0.174 *	-0.364***	0.783***							
Stem diameter. (1)	ns	-0.272***	0.817***	0.775***						
H/N (1)	0.180**	-0.163*	0.805***	0.283***	0.548***					
Height (2)	ns	ns	0.600***	0.439***	0.608***	0.547***				
Node number (2)	ns	ns	0.516***	0.608***	0.546***	0.248***	0.625***			
Stem diameter (2)	ns	ns	0.550***	0.453***	0.657***	0.455***	0.702***	0.638***		
H/N (2)	ns	ns	0.437***	0.199**	0.428***	0.520***	0.859***	0.170***	0.509***	
Branch number (2)	ns	ns	0.295***	0.250***	0.218**	0.210**	ns	ns	ns	ns

*, **, ***: significant correlation at $p<0.05$, 0.01, 0.001 respectively. ns: not significant. Height, stem diameter, H/N and seed length are measured in millimetres. Germination period is measured in days.

At second scoring, all correlations, except between height and H/N were less than the corresponding ones at first scoring.

Correlations between the same first and second scoring parameters were generally moderate. Stem diameter had the highest correlation (r = 0.657) followed by node number (0.608), height (r = 0.600) and then H/N (r = 0.520). Correlation for height was moderate between first and second scoring, even though at first scoring non-uniformity resulted from differences in date of germination. These correlations could be used to reduce the population after first scoring in breeding experiments aimed at producing poinsettias of reduced height. Thus, efficiency of production could be improved and cost of production reduced.

H/N could not be as reliably predicted at second scoring from the first scoring data, due to moderate correlation. The best correlations were with H/N (r = 0.520), height (r = 0.437) and stem diameter (r = 0.428). These correlations could also be utilised in breeding programs to select for mean internode length with moderate efficiency only.

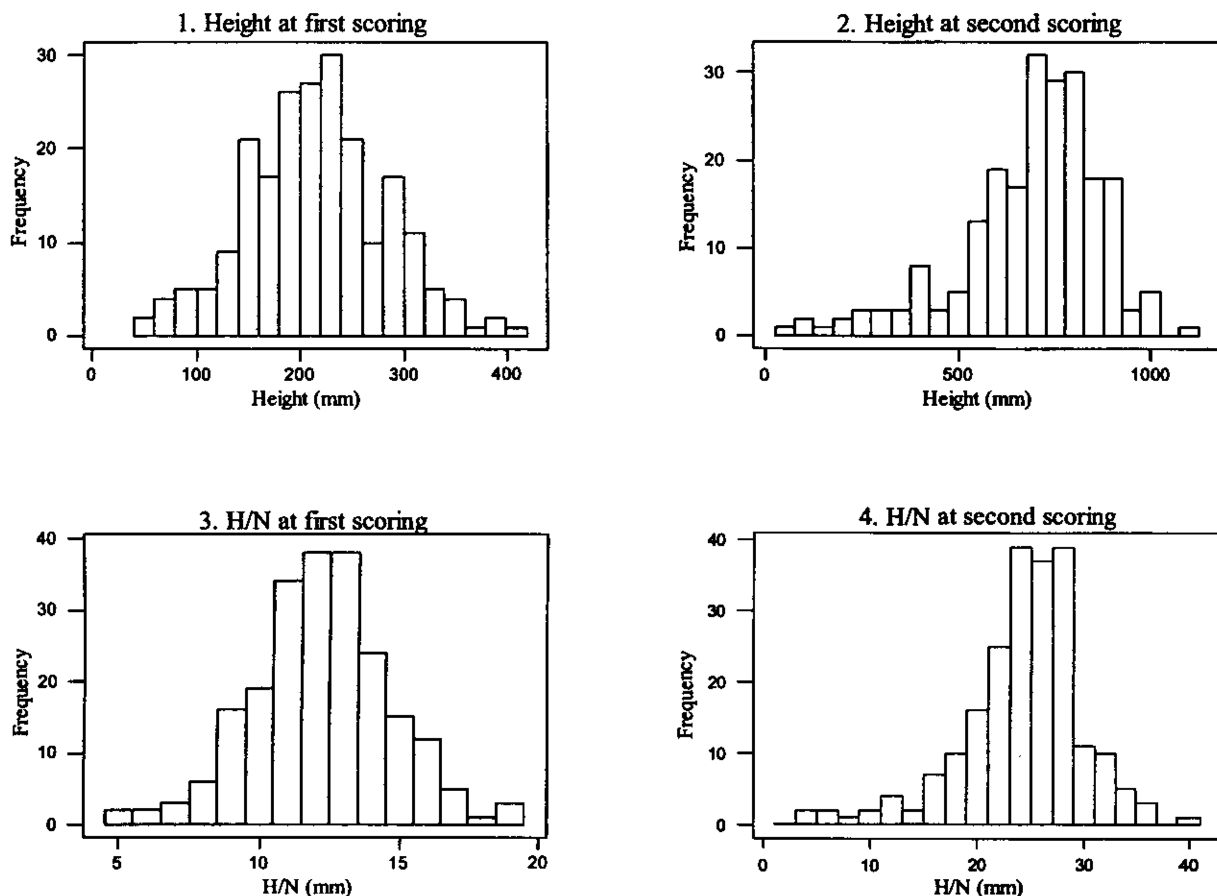
Branch number had a low correlation with several parameters, as did seed length. A low negative correlation was found for germination period and height (r = -0.364) and germination period and node number (r = -0.324) at first scoring. These correlations became non-significant at second scoring, probably due to apical decapitation.

Histograms for height and H/N of the SDP

Histograms showing the values for plant height and H/N within the SDP are presented in Figure 5.4. At first scoring height and H/N were both normally distributed. However, at second scoring, both height and H/N became skewed. This skewed effect was likely due, in part, to the presence of 'weak' plants with short internodes in the lowest area of the histograms. Since second scoring was conducted after apical decapitation, which confers uniformity, height at second scoring could be considered more accurate than at first scoring.

The predominantly normal distributions were likely due to a combination of factors including segregation of minor genes for height, heterosis, mutational load, and environmental differences. However, environmental variability was minimised by growing all plants in a uniform, greenhouse environment and providing adequate spacing to avoid competition effects.

Figure 5.4. Histograms for height (mm) and H/N (mm) at first and second scoring for the SDP



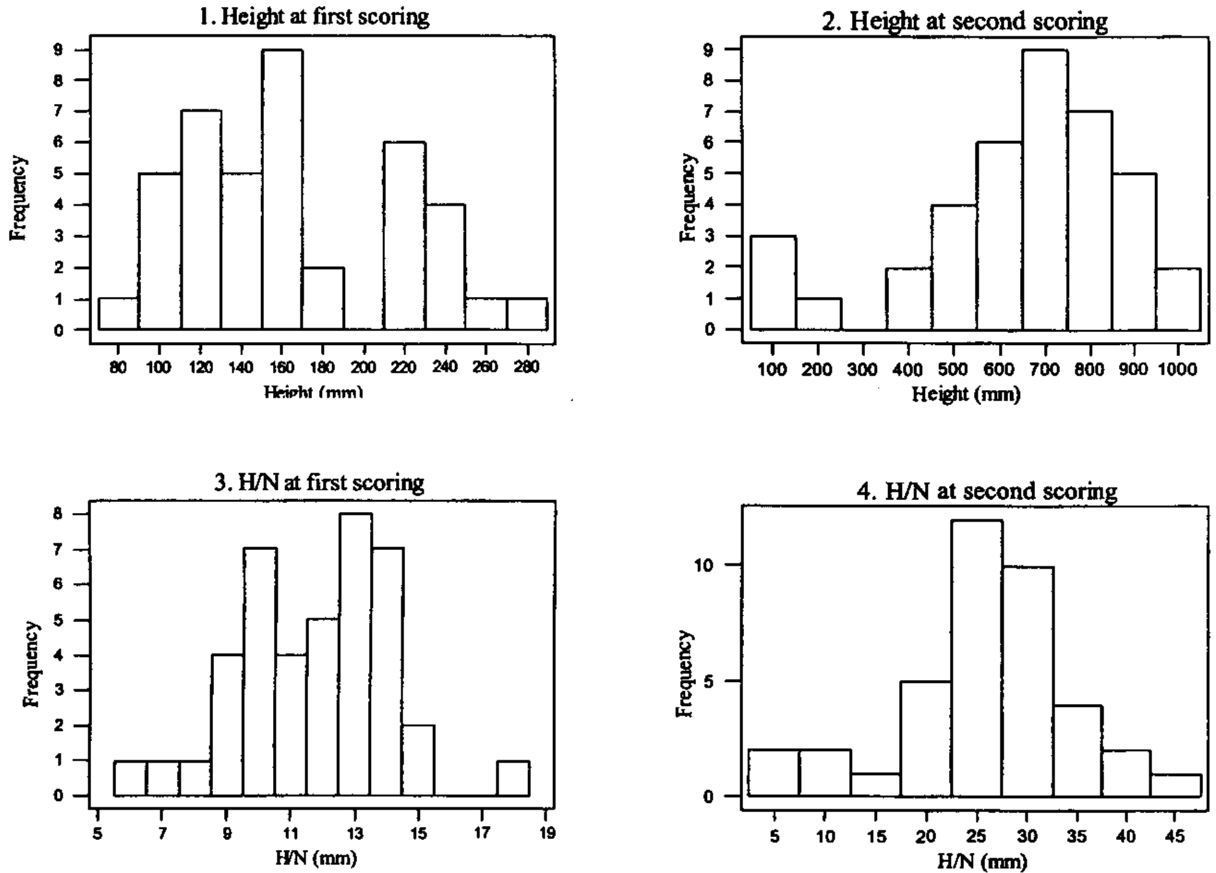
Analysis of the SDC populations for evidence of segregation

A large population of self-pollinated seed derived controls were available for analysis of segregation. Both V10 Amy red (38 plants) and Freedom red (41 plants) could be analysed.

a) Freedom red

Freedom red selfed progeny displayed a non-normal height distribution at first scoring (Figure 5.5). This may have been due to differences in the date of germination of seeds and seedling vigour, which increased the spread of data (which was from 80 – 280 mm). This histogram was normal at the second scoring, except for a few very short plants at the far left of the diagram. The spread of plants was from 51 mm – 960 mm, indicating several genes of minor effect could be involved in plant height in this population.

Figure 5.5. Histograms for height (mm) and H/N (mm) at first and second scoring for the cv. Freedom red self-pollinated seed derived control population

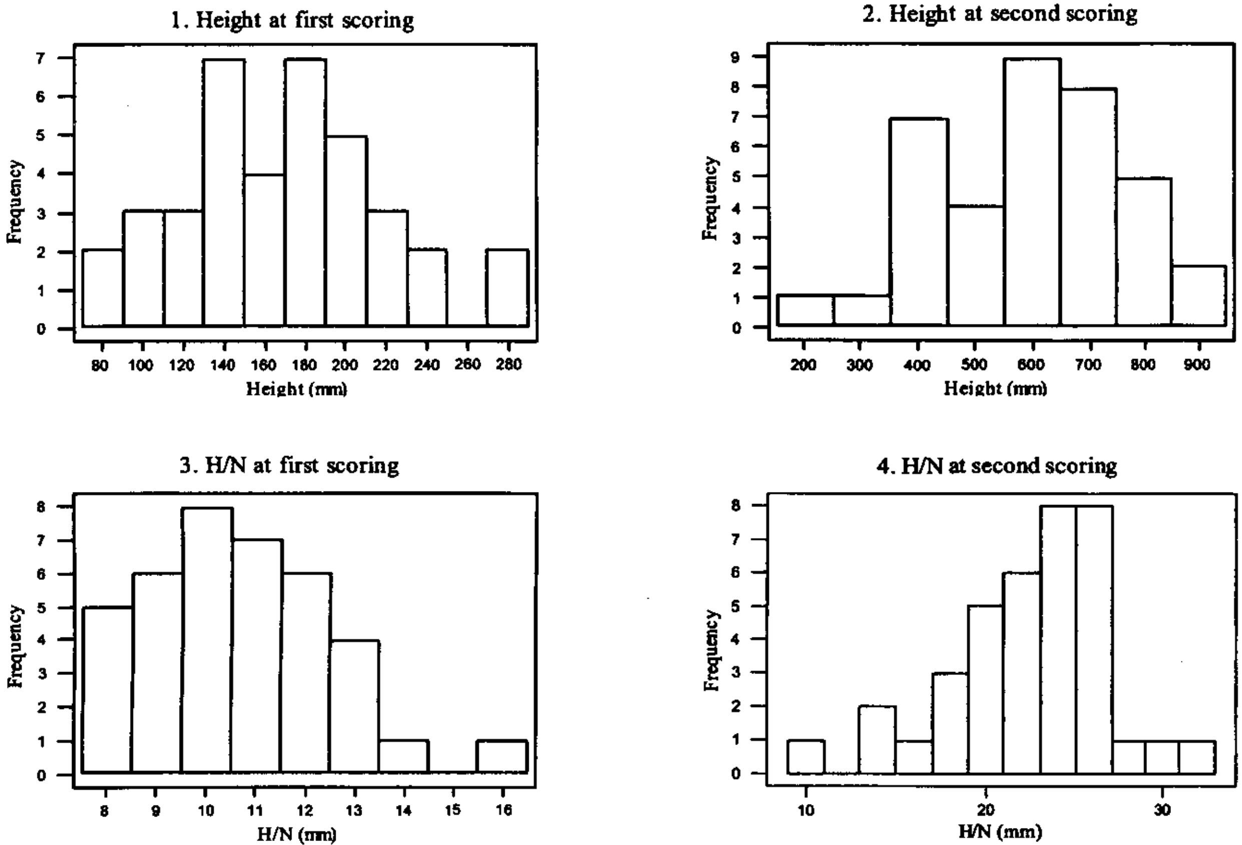


b) V10 Amy red

Height at first scoring for the cv. V10 Amy red selfed progeny population appeared normally distributed, with a spread of heights from approximately 82 - 289 mm (Figure 5.6). This large spread of plant heights was also observed at the second scoring (210 - 920 mm). This population also appeared to be influenced by several genes of minor effect on height. Differences in date of germination could be considered negligible at the second scoring due to decapitation.

H/N however, showed a different pattern. At first scoring, the population exhibited normality, except for one plant to the far right and a missing left tail. This changed dramatically at the second scoring with a skewed diagram, where approximately 50% of the population were found at 25 mm. Three plants were above this point, and a skewed spread found to the left. The spread may be due to plants bearing short internodes. A larger population may be required to adequately investigate segregation of genes for height and H/N.

Figure 5.6. Histograms for height (mm) and H/N (mm) at first and second scoring for the V10 Amy red self-pollinated seed derived control population



Selection for shorter, vigorous and ornamentally acceptable plants from the SDP

Plants were selected first, based upon node number at second scoring being greater than or equal to the mean node number for VDCs, 28.8 (Table 5.13). VDCs had a higher mean node number than the SDCs. High node number is desirable for selection of vigorous plants. Therefore, selected plants would be average or above average for node production rate. Plants were then selected for height. Plants were chosen if they recorded a height at second scoring less than or equal to the mean height for SDCs, 599 mm (Table 5.13). SDCs had the lowest mean node number.

A total of 157 plants had a node number equal to or greater than 28.8. (Table 5.19), and within these selected plants, 17 had a height equal to or lower than 599 mm. The selection intensity was therefore 7.8% (17/216). From these 17 plants, two were deemed ornamentally excellent and the remaining 15 were considered poor (e.g. score 3, deformed leaves, thin stems, poor bract colour). The final selection intensity, when considering only excellent plants was 0.9% (2/216).

Table 5.19. Ornamental score and pedigrees for plants selected from the SDP with node number ⩾28.8 and height ⩽599 mm

	1		2		3	
Ornamental appeal score	Excellent – better than cv. Freedom red	No. of plants	Good – similar to cv. Freedom red	No. of plants	Poor – worse than cv. Freedom red	No. of plants
Pedigrees	24.1 x 96.1	1	-	-	24.1 x 54.1	3
	24.1 x 172.2	1	-	-	24.1 x 96.1	6
					54.1 x 24.1	2
					54.1 x 96.1	3
					FR x V10R	1
Total plants		2		0		15

- : none recorded.

Five SDCs (from 88) and five VDCs (from 70) were also within the height and node selection criteria, indicating selection intensity was high. All five SDCs were classed as ornamentally poor (score 3). Among the five VDC plants within selection criteria two were V10 Amy red (ornamental score 3), two were line 54.1 (ornamental score 2) and one was line 172.2 (ornamental score 2).

Apart from the two ornamentally excellent plants selected in Table 5.19, 19 ornamentally excellent plants that did not meet both of the abovementioned criteria for height and node number were also selected from the SDP for further testing. These selected lines could be used for further breeding and selection to introgress desirable traits such as bract colour, cyathia number, earliness to flower, etc. Their pedigrees were primarily either FR x V10R or V10R x FR. Therefore, 9.7% of plants from the SDP (21/216) were deemed ornamentally excellent. From these 19 plants, 16 met either the node (⩾28.8) or height (⩽599 mm) criteria. Furthermore, five ornamentally good plants were also selected, but these did not meet the node and height criteria. Three of these had pedigree V10R x FR and the other two were 24 x 96 and 24 x 54. Six ornamentally excellent plants were also selected from the SDCs. These were all of the pedigree FR x FR and did not meet height and node criteria. Figure 5.3 shows ornamentally excellent and good selections and control plants after roguing was performed.

Figure 5.3. Randomised selections from the SDP with VDC and SDC plants

Part IV. Preliminary testing of ornamentally excellent selections under commercial conditions

Materials and methods

i. – Infection of selected lines with PoiBI

A total of 32 selections were used in this experiment (Table 5.20). The two ornamentally excellent selections from table 5.18 were used, in addition, 19 ornamentally excellent selections and 5 ornamentally good selections (as described earlier in Part III) from the SDP were chosen. Six ornamentally excellent selections from the SDCs were also used.

Table 5.20. Cultivar/line code, pedigree and ornamental appeal score for germplasm as recorded at completion of Part III

Cultivar/line	Pedigree	Ornamental appeal score
98-158.3	24 x 96	1
98-345	V10R x FR	1
98-350	V10R x FR	1
98-407.2	V10R x FR	1
98-435	V10R x FR	2
98-474.2	FR x FR	1
98-562.1	FR x V10R	1
98-574	FR x V10R	1
98-589	FR x V10R	1
98-621.2	FR x FR	1
98-630.2	V10R x FR	1
98-664	V10R x FR	2
98-682.1	FR x FR	1
98-694	V10R x FR	1
98-705.1	FR x V10R	2
99-6	V10R x FR	1
99-113.3	FR x V10R	1
99-133.2	FR x V10R	1
99-155.2	FR x V10R	1
99-185.1	24 x 96	1
99-200.1	24 x 96	1
99-230.1	24 x 172	1
99-240	FR x V10R	1
99-242	FR x V10R	1
99-253.1	FR x V10R	1
99-255.2	FR x V10R	1
99-269.2	FR x V10R	1
99-290	FR x FR	1
99-296	24 x 54	2
99-317.1	24 x 96	2
99-324	FR x FR	1
99-325.2	FR x FR	1
Freedom red	Induced sport of a seedling[1]	2
V10 Amy red	Seedling[1]	3

[1] United States Patent and Trademark Office. Bold indicates four ornamentally excellent selected lines meeting height and node criteria in Part III.

After completion of experimentation in Part III, each individual selected plant was pruned to remove stem tissue until only leaves were present on branches. Pruned plants were placed under a long photoperiod environment (>2 μmol m^{-2} s^{-1} incandescent light for four hours starting at 10 pm, in addition to natural photoperiod light) at 25°C ± 2°C to enable rapid development of vegetative shoots. When at least three vegetative shoots emerged and were greater than 60 mm long on all plants, they were removed as cuttings. Cuttings were propagated as per standard practices (Experiment 3.1). Cuttings of cv. V10 Amy red infected with PoiBI but not PnMV (+p/-mv) were also propagated to enable grafting and transmission of PoiBI.

Following development of roots, one cutting of each selection was planted close to one cutting of cv. V10 Amy red (+p/-mv) in a 200 mm diameter pot filled with NFP potting mix. After growing for approximately 4 weeks, each selection was approach grafted (Experiment 3.1) to the cv. V10 Amy red plant growing in the same pot. Grafted plants were allowed to grow until more than five branches were present on each grafted selection. A general liquid fertiliser was applied as required.

Stockplants of cv. Freedom red and V10 Amy red (+p/-mv) were also grown under the same conditions as grafted plants and used as cultivar controls. Cultivar V10 Amy red was used as a control because it recorded the lowest mean height of all cultivars in Experiment 4.4 and was a parent for many lines. Cultivar Freedom red was used as a control because it is the current leading cultivar, has desirable ornamental appeal and was a parent for many lines.

ii. – Evaluation of selected lines under commercial production conditions

The experiment was conducted during spring - summer in Australia (Table 5.21). When five or more uniform cuttings were available on each grafted plant and controls, they were removed with a knife and propagated according to standard procedures. Following propagation, four cuttings with roots from each line were planted into 150 mm diameter pots filled with NFP potting mix and allowed to grow under a long photoperiod environment for four weeks. After two weeks under long photoperiod conditions, apical decapitation was performed to remove approximately 20 mm of the shoot apex, which included young expanding leaves. In addition, Nutricote® 3-4 month slow release fertiliser was applied at approximately 8g/pot (Appendix 1) to the top of the potting mix in each pot at this date. Two weeks later, plants were allowed to flower by inducing an artificial short photoperiod of 10 h from 7 am. After 8 weeks of short photoperiod growth, the experiment was complete and each

plant was measured for height from the top of the potting mix to the top of the plant. Photographs were taken at completion of the experiment.

Table 5.21. Mean daily minimum and maximum temperatures recorded

Steps	Duration (days)	Mean daily temperature min. – max. (°C)
Start of propagation		
Planting	31	16 – 32
Apical decapitation	45	20 – 38
Transferral to short photoperiod	60	20 – 38
Anthesis/date of sale	116	20 – 32

Data analyses

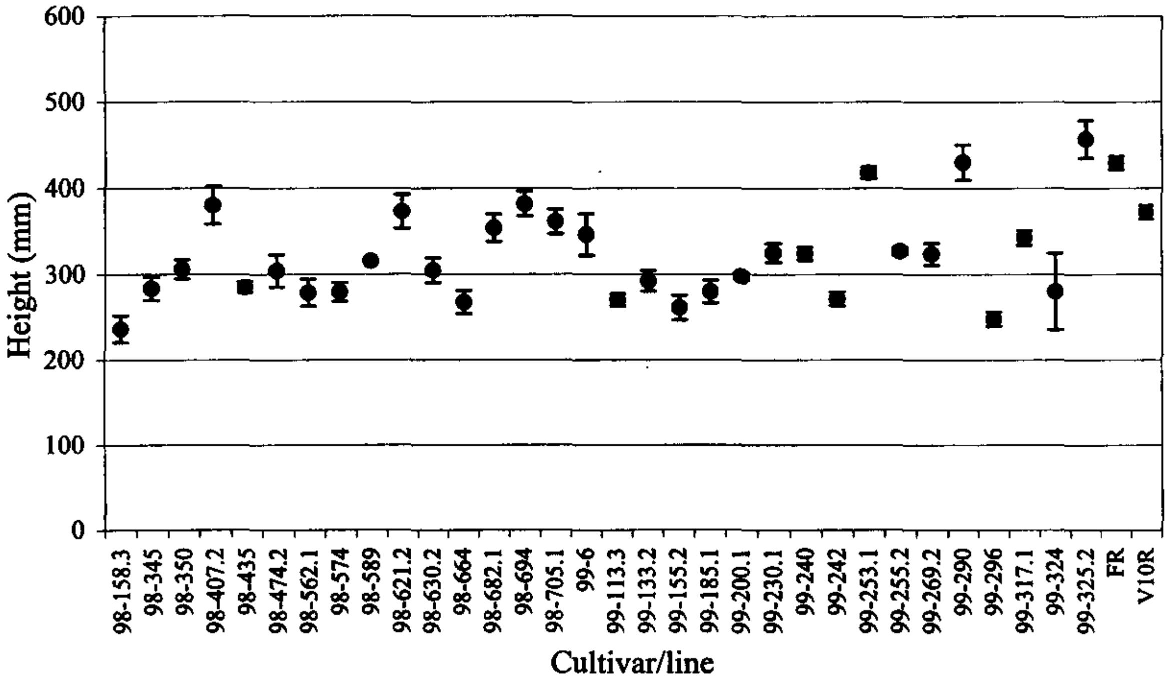
Data was analysed using the MINITAB® statistical software package (MINITAB® for windows release 12.22, Minitab Inc. 1998). The effects of genotype (line/cv.) on height were analysed by conducting Analysis of Variance (AVOVA). Dunnett pairwise comparisons with controls (cv. Freedom red and cv. V10 Amy red) were performed to determine significant differences between means of selected lines and control cultivars only, at a 95% confidence interval. Tukey pairwise comparisons were not performed because comparison with controls was not an available function (only complete pairwise comparisons).

Results

Grafted plants displayed PoiBI infection symptoms when cuttings were taken for the experiment. Cuttings of each line produced roots within 3 – 4 weeks from the start of propagation.

Mean height at the completion of the experiment is plotted for each selected line in Figure 5.5. The results of data analyses (Table 5.22) showed that 16 out of 32 lines were significantly shorter ($P < 0.05$) than both cv. Freedom red and cv. V10 Amy red. The two ornamentally excellent lines that met selection criteria for height (< 599 mm) and nodes (>28.8) in Part III were 98-158.3 and 99-230.1. Line 98-158.3 recorded the lowest mean height of all lines and the cultivar controls. Node number was not recorded, so the level of selection intensity was reduced compared to selections made in Part III where both height and node number were used for selection.

Figure 5.5. Mean height and standard errors for 32 lines and two cultivars at completion of testing



Further replicated commercial production trials, under various environments for the selected lines and cultivar controls, are required to ascertain if a reduction in height has occurred under a wide range of conditions. However, the results from this preliminary experiment strongly indicate that progress towards producing dwarf ornamentally desirable poinsettias has been made (Figure 5.5, Table 5.22). Figures 5.6 and 5.7 show two desirable selections from this experiment in comparison to cv. Freedom red.

Table 5.22. Means, standard errors and pairwise comparisons of ornamentally excellent selected lines for height with cultivar Freedom red and V10 Amy red controls

				Pairwise comparisons	
Cultivar/line	Mean height	n	SE	cv. Freedom red	cv. V10 Amy red
98-158.3	236.5	4	16	***	***
98-345	283.5	4	14	***	**
98-350	306.0	4	11	***	ns
98-407.2	380.3	4	22	ns	ns
98-435	284.8	4	6.3	***	**
98-474.2	304.0	4	19	***	*
98-562.1	278.8	4	16	***	**
98-574	279.5	4	11	***	**
98-589	315.3	3	0.3	***	ns
98-621.2	372.8	4	20	ns	ns
98-630.2	304.7	3	14	***	ns
98-664	267.8	4	13	***	***
98-682.1	353.8	4	16	*	ns
98-694	381.8	4	14	ns	ns
98-705.1	361.0	4	14	*	ns
99-6	346.3	4	24	**	ns
99-113.3	270.5	4	7.2	***	***
99-133.2	292.5	4	12	***	**
99-155.2	261.3	3	14	***	***
99-185.1	280.0	4	13	***	**
99-200.1	297.5	4	3.9	***	*
99-230.1	325.0	4	11	***	ns
99-240	324.0	4	7.6	***	ns
99-242	271.8	4	8.1	***	***
99-253.1	417.8	4	6.8	ns	ns
99-255.2	326.8	4	4.2	***	ns
99-269.2	323.8	4	13	***	ns
99-290	429.8	4	21	ns	ns
99-296	248.0	4	8.2	***	***
99-317.1	342.3	4	8.6	**	ns
99-324	280.3	4	44	***	**
99-325.2	456.8	4	22	ns	**
Freedom red	429.0	4	7.9	n.a.	ns
V10 Amy red	372.3	4	8.1	ns	n.a.

Source	Mean square
Cultivar/line	10908***
Residual	627

Bold indicates four ornamentally excellent selected lines meeting height and node criteria in Part III. *, **, ***: $p < 0.05, 0.01, 0.001$ respectively. ns = not significant, n.a. = not applicable.

Figure 5.6. Comparison of selected line 98-562.1 with control cv. Freedom red at completion of experiment

Figure 5.7. Comparison of selected line 99-155.2 with control cv. Freedom red at completion of experiment

Discussion

The key objectives of this chapter were to i. - test the female fertility of potential short parents, ii. - conduct crosses and produce seeds and plants from the selected fertile, short parents, iii. - characterise the resulting population with the aim of selecting dwarf poinsettias with ornamental merit and then iv. - test these selections (with PoiBI infection) for height and ornamental merit under commercial conditions during summer in Australia.

Fertility testing of potential parents showed that seed production was seldom 100% (except for 97/54.1) and may be attributed to genetic variation, mutational load, IBD partial female fertility, environmental variability or combinations of two or more of these factors. Some evidence for mutational load was demonstrated from the results of cultivar fertility testing. Several cultivars tested were developed as the result of mutation breeding and were apparently female sterile, indicating sterility could have resulted due to their method of development. Pepride, for example, is an induced mutation of Freedom red and has much lower female fertility. Cultivars showing complete female sterility were not suitable for further intraspecific breeding investigations because of their lack of efficient seed production. As stated previously, IBD may mimic mutational load effects, so this factor could be presumed to be influencing results here as well. Partial female fertility/sterility could also be proposed as an explanation for less than 100% seed set when crosses were performed with wild-type poinsettia pollen.

The reciprocal differences in seed production for 24.1 x 96.1 and 24.1 x 172.2 may be due to cytoplasmic effects or partial female fertility, resulting in decreased ovules available for fertilisation. Results from crossing these lines with a wild-type male parent support this hypothesis because only 44.4 and 75% seed production efficiency was recorded for lines 172.2 and 96.1 respectively.

Production of seeds and plants from selected fertile, short parents indicated seed production efficiency could be affected by another factor not previously recognised. This factor was postulated to be the cytoplasm because differences in seed production efficiency were noted between reciprocal crosses. For example, in the Freedom red x V10 Amy red reciprocal cross, 20.5% (Freedom red x V10 Amy red) and 6.5% (V10 Amy red x Freedom red) seed production efficiencies were recorded and 1581 ovules were pollinated. This result may also

be attributed to partial female fertility. Thus, another factor may need to be considered when performing intraspecific breeding studies.

From the seeds produced via reciprocal cross pollinations (SDP) two ornamentally desirable dwarf poinsettias could be selected within specified node number and height criteria. In addition, 19 other ornamentally excellent plants were also selected from the SDP, and 6 SDC plants were selected due to an excellent ornamental score.

Within the SDP, correlations were investigated to determine an effective method for culling the seed derived population at an early age and thereby increasing efficiency. In the SDP, correlations between height and H/N were moderate between first and second scoring. Therefore, in future studies, discarding the tallest plants early in the selection process could improve efficiency through reduced use of space and reduction in population size combined with retaining the most dwarf plants via single plant selection. This in turn could reduce the cost of cultivar development.

Another commercial application from this study was the use of parental vegetative (cutting) controls to aid in selection. Timing the cuttings to be at a similar stage of growth to seedlings was achieved, but was highly dependent on some factors that cannot be easily estimated such as temperature and germination period of seedlings. None the less, this method of control proved beneficial. Breeders could select single progeny with greater confidence based upon evidence of parents grown within the same seedling population.

Apical decapitation is suggested as another method to assist in commercial breeding projects. This practice is recommended because it tends to encourage uniformity, induce juvenility and reduce the effects of differences in seed germination date between vegetatively derived controls and seed derived plants. Ideally, all plants need to be scored at the same time interval from germination. This was, however, technically difficult because germination is not applicable for VDCs and even scoring by this method among seed derived plants would result in high labour under commercial application. Therefore, it was hypothesised that decapitation would allow re-instatement of uniformity among all plant types and because the period prior to decapitation was short in relation to the period post-decapitation, the effect of differences in date of seed germination could be negligible. Measurements could then be made on the new growth. In addition, plants were developed which can immediately be assessed for branching potential. Another consideration is that increased branch number results in increased

'inflorescence' number. This increase provides an improved representation of the bract display, which can sometimes appear distorted or malformed on single inflorescence plants. A larger number of inflorescences overcomes this problem, and can provide insurance against accidental stem breakages.

In this study, the length of the growing period was much greater than the conventional 16 week commercial production period. This allowed plants to reach a greater height, provide a better estimate of their growth rate, and mean internode length during summer. In addition, the extended period from decapitation to second scoring could have negated the effects of differences in date of seed germination, because the time to first scoring was small in relation to the time from decapitation to second scoring. Premature floral initiation could also be ascertained because of the high node numbers reached. This trait is undesirable and selections with this trait should be avoided. In the line 97/24.1, all 13 plants exhibited premature floral initiation. This phenomenon can be suppressed by exogenous application of gibberellic acid whereby the ontogenetic age of the meristem is inhibited (Evans *et al.* 1992b). This could suggest that very dwarf plants (such as line 97/24.1) have an increased likelihood to exhibit premature floral initiation at a reduced node number, possibly due to differences in GA, which is commonly associated with internode elongation in poinsettias. This phenomenon has been experienced with the short growing cv. Pepride at a commercial nursery in Australia (Swarbrick pers. comm. 2000). This cultivar apparently reaches the critical long day node number for floral initiation much earlier than other common cultivars (including its progenitor, cv. Freedom red). However, studies by Evans *et al.* 1992b showed application of GA inhibitors did not affect premature floral initiation. Further studies are required to ascertain the effects of GA on premature floral initiation, and if a correlation exists between short plant height and propensity to prematurely initiate floral structures.

Segregation for height and H/N was investigated and proposed to be under the control of several genes of minor effect in cultivars Freedom red and V10 Amy red. Further studies utilising a larger population of seedlings with accurate pedigree information are required.

Introduction of phytoplasma into 32 selected lines and testing of the now phytoplasma infected lines showed that 16 from 32 were significantly shorter than both Freedom red and V10 Amy red controls. Therefore, this experiment was successful in the production of ornamentally excellent, dwarf poinsettias that could be produced within the standard production period and according to standard production practices. However, further trials

under different environments (sites and temperature, etc.) are required to verify the worth of these selections to the commercial poinsettia industry. In addition, the selections also need to achieve desired height during growth through winter in Australia to be immediately commercially applicable.

Future intraspecific breeding studies should consider the impact of grafting all seedlings at an early stage and performing selections based upon PoiBI-infected phenotype, rather than performing selections on single, uninfected plants. In addition, replication at this stage, by harvesting cuttings of each seedling, would provide a more robust method of selection. In addition, any selections would be immediately available for stock multiplication and further trials, increasing efficiency of cultivar production.

In conclusion, poinsettias can be produced via intraspecific hybridisation, which are shorter than cultivars Freedom Red and V10 Amy red and are also ornamentally desirable. An alternative option to develop dwarf poinsettias would be to conduct interspecific and/or intergeneric crosses, which could lead to introgression of novel dwarfing genes. The next chapter investigates this option.

Chapter 6. Interspecific hybridisation for the introgression of novel genes for height

Introduction

The cultivated poinsettias have been developed from a single species, *Euphorbia pulcherrima* (Ling *et al.* 1997). Previous investigations have indicated genes for reduced plant height are already present within several current commercial cultivars (refer to Experiment 4.4). Introgression of genes for short height from unrelated *Euphorbia* species, through interspecific hybridisation and selection for poinsettia-like plants with a further reduction in height compared to current commercial cultivars, could lead to development of novel varieties. Following F_1 production, back crossing to the desired parent can be performed to obtain plants closely resembling parental poinsettia genotypes, but with a reduced height.

The production of interspecific hybrids requires an understanding of the crossability and compatibility of parental germplasm. Once combinations that produce true interspecific hybrids are identified, further investigations could be performed to increase F_1 seed/embryo production efficiency and the number of plants regenerated for selection purposes. Interspecific hybrid embryos often fail to develop into normal seeds (with endosperm and testa) therefore, putative hybrid embryos need to be rescued at an early stage of development and cultured *in vitro*. Ovary swelling in intraspecific pollinations is highly correlated with seed development (refer to Experiment 4.1) and increased ovule size (greater than non-pollinated controls) could indicate successful fertilisation. These indicators were used in this study. In many plant species, efficiency of regeneration of plants from cultured embryos increases with time up to a certain stage after pollination and increased stage of development of the embryo (Pickersgill 1993) Therefore, embryos should be removed at the latest possible stage to ensure best regeneration efficiency.

Commercially developed protocols for induction of growth and flowering of species related to *E. pulcherrima* are, at best, limited. Current information suggests that the tuberous Mexican species *E. colorata, E. restiacea, E. radians, E. hormorrhiza* and *E. strigosa* flower in response to short photoperiod (Mayfield pers. comm. 1997) and *E. cornastra* is day neutral and will flower in both long and short days (Le Duc and Albrecht 1996).

Materials and methods

Germplasm

Parental germplasm used in this experiment are detailed in Table 6.1. Four species required quarantine containment in Australia (refer to Table 6.1 and below). Chromosome numbers have been established in earlier studies by various authors for some species used in this experiment, however, additional chromosome counts (8) performed by the author have also been shown in Table 6.1 (methodology presented in Appendix 3).

Growth conditions

Plants used for pollinations were grown in two insect-free environments, namely E1 or E2.

Environment 1 (E1) was the standard environment used for intraspecific hybridisation (detailed in Experiment 4.1). Temperature was maintained at 21°C ± 1°C, and 10 h photoperiod was provided in 'microclimate' rooms contained within a greenhouse. Supplementary light of approximately 300 μmol m^{-2} s^{-1} was provided. Plants of all poinsettias and *Euphorbia* species were grown here, except for those species that required quarantine containment (see below).

Environment 2 (E2) was the quarantine greenhouse at the Plant Breeding Institute, Cobbitty. Temperature was maintained between 21°C and 24°C, natural daylength and light were provided and plants were positioned in a northerly aspect to ensure good light intensity (Figure 6.2.). The species *E. colorata*, *E. hormorrhiza*, *E. radians.1* and *E. radians.2* were grown in E2. The poinsettia cultivars/lines 97/24.1, 97/176.2, 97/176.3, cv. Freedom red, cv. Hot pink, cv. Pink peppermint, cv. Spotlight dark red and cv. V10 Amy red were grown in E2 to facilitate crossing with *Euphorbia* species and also in E1 for all other crosses.

Poinsettias and related species (Figures 6.1 - 6.5) were grown according to standard commercial practices detailed in Experiment 3.3. Species were generally amenable to this protocol, but were manually watered as required rather than drip irrigated via computer control.

Table 6.1. List of *Euphorbia* species used for interspecific hybridisation including subgenus, chromosome number, source of germplasm and pedigree (continued over page)

Euphorbia species	Subgenus	Basic chromosome numbers		Initial propagule(s)	Source of germplasm and pedigree
		n	2n		
E. colorata†	*Poinsettia*	-	-	tubers	V. Steinmann, Santa Ana Botanic Garden, California U.S.A.
E. cornastra	*Poinsettia*	14[1]	28[1]	seeds	A. Le Duc, Louisiana State University, U.S.A.
E. cyathophora.1	*Poinsettia*	14[1], 28[1]	28[1], 56[1] 56[4]	seeds	R. Kobayashi, Paul Ecke Poinsettias Inc., California, U.S.A.
E. cyathophora.2	*Poinsettia*	14[1], 28[1]	28[1], 56[1]	seeds	P. Forster, Queensland Herbarium, Australia
E. davidii	*Poinsettia*	28[1]	56[1]	seeds	A. Storrie, Department of Agriculture, Tamworth, Australia
E. fulgens	*Agaloma*	-	28[3]	plant	The plant Place, Gosford, Australia
E. hormorrhiza†	*Poinsettia*	14[1]	28[1]	tubers	V. Steinmann, Santa Ana Botanic Garden, California, U.S.A.
E. leucocephala	*Agaloma*	-	28[4]	cuttings	A. Bernuetz
E. leucocephala cv. Pink finale	*Agaloma*	-	-	cuttings	Fosters Nursery, NSW Australia
E. marginata	*Agaloma*	28[2]	56[3]	seeds	A. Bernuetz
E. pulcherrima 97/24.1	*Poinsettia*	-	28[4]	seed	A. Bernuetz – seedling: cv. Freedom white x cv. V10 Amy red
E. pulcherrima 97/54.1	*Poinsettia*	-	-	seed	A. Bernuetz – seedling: cv. V10 Amy red x self
E. pulcherrima 97/85.2	*Poinsettia*	-	-	seed	A. Bernuetz – seedling: V10RxL x cv. Lemon drop
E. pulcherrima 97/96.1	*Poinsettia*	-	-	seed	A. Bernuetz – seedling: cv. FR x cv. V10 Amy red
E. pulcherrima 97/143	*Poinsettia*	-	-	seed	A. Bernuetz – seedling: cv. FR x cv. V10 Amy red
E. pulcherrima 97/144	*Poinsettia*	-	-	seed	A. Bernuetz – seedling: cv. PP x cv. V10 Amy red
E. pulcherrima 97/172.2	*Poinsettia*	-	-	seed	A. Bernuetz – seedling: cv. Freedom red x self
E. pulcherrima 97/176.2	*Poinsettia*	-	28[4]	seed	C. Underhill, Grafton, NSW – self seed from wild poinsettia
E. pulcherrima 97/176.3	*Poinsettia*	-	-	seed	C. Underhill, Grafton, NSW – self seed from wild poinsettia
E. pulcherrima 97/176.8	*Poinsettia*	-	-	seed	C. Underhill, Grafton, NSW – self seed from wild poinsettia
E. pulcherrima cv. Angelika	*Poinsettia*	-	28[4]	cuttings	A.J. Newport and Son Pty. Ltd., NSW, Australia. Mutation of a seedling[5]

† Species require quarantine in Australia, grown in environment E1. (-) = unknown or not counted. [1] Mayfield 1997, [2] Urbatsch *et al.* 1975, [3] Hans 1973, [4] Bernuetz.

Table 6.1 (continued). List of *Euphorbia* species used for interspecific hybridisation including subgenus, chromosome number, supplier and pedigree

Euphorbia species	Subgenus	Basic chromosome numbers		Initial	Supplier and pedigree
		n	2n	propagule(s)	
E. pulcherrima cv. Freedom red	*Poinsettia*	-	28[4]	cuttings	A.J. Newport and Son Pty. Ltd., NSW, Australia. Induced sport of a seedling[5]
E. pulcherrima cv. Freedom white	*Poinsettia*	-	-	cuttings	A.J. Newport and Son Pty. Ltd., NSW, Australia. Induced mutation of cv. Freedom red[5]
E. pulcherrima cv. Hot pink	*Poinsettia*	-	-	cuttings	A.J. Newport and Son Pty. Ltd., NSW, Australia. Mutant of Annette Hegg Dark red[6]
E. pulcherrima cv. Lemon drop	*Poinsettia*	-	-	cuttings	A.J. Newport and Son Pty. Ltd., NSW, Australia. Seedling[6]
E. pulcherrima cv. Pink peppermint	*Poinsettia*	-	-	cuttings	A.J. Newport and Son Pty. Ltd., NSW, Australia. Seedling[5]
E. pulcherrima cv. Spotlight dark red	*Poinsettia*	-	-	cuttings	F. & I. Baguley, Clayton South, Victoria, Australia. cv. Red Sails x cv. Angelika[5]
E. pulcherrima cv. Supjibi	*Poinsettia*	28[4]	-	cuttings	A.J. Newport and Son Pty. Ltd., NSW, Australia. Likely tetraploid of cv. Angelika[7]
E. pulcherrima cv. V10 Amy red	*Poinsettia*	-	28[4]	cuttings	A.J. Newport and Son Pty. Ltd., NSW, Australia. Seedling[5]
E. pulcherrima S2.3	*Poinsettia*	-	-	cuttings	N. Derera, ASAS, NSW Australia
E. pulcherrima S4	*Poinsettia*	-	-	cuttings	N. Derera, ASAS, NSW Australia
E. pulcherrima DxPP	*Poinsettia*	-	-	seed	A. Bernuetz – seedling: cv. Annette Hegg Diva x cv. Pink peppermint
E. pulcherrima V10RxL	*Poinsettia*	-	-	seed	A. Bernuetz – seedling: cv. V10 Amy red x cv. Annette Hegg Lady
Euphorbia radians.1†	*Poinsettia*	-	-	tubers	V. Steinmann, Santa Ana Botanic Garden, California U.S.A.
Euphorbia radians.2†	*Poinsettia*	-	-	plants/tubers	Rare plant research, Oregon, U.S.A.

† Species require quarantine in Australia, grown in environment E1. (-) = unknown or not counted. [1] Mayfield 1997, [2] Urbatsch *et al.* 1975, [3] Hans 1973, [4] Bernuetz, [5] United States Patent and Trademark Office, [6] Ecke *et al.* (1990), [7] Starman and Abbitt (1997).

Pollinations

Pollinations were performed depending upon flowering times and rate of production of anthers and pistils. Fresh pollen was collected prior to midday from most plants. Pollen was applied liberally to all areas of the ½ open receptive stigmatic surface (refer to Experiment 4.1). The number of pollinations conducted for each combination was recorded. Pollinations were performed as shown in Tables 6.2 and 6.3. A total of 1093 pollinations were performed between poinsettias (♀) and *Euphorbia* species (♂) during this study. Therefore, 3279 ovules were expected to be pollinated..

Table 6.2. Number of ovules pollinated for *E. pulcherrima* x *Euphorbia* sp. crosses

♂ ♀ (*E. pulcherrima*)	*E. colorata*	*E. cornastra*	*E. cyathophora.1*	*E. cyathophora.2*	*E. davidii*	*E. fulgens*	*E. hormorhiza*	*E. leucocephala*	cv. Pink finale *E. leucocephala*	*E. marginata*	*E. radians.1*	*E. radians.2*
97/24.1	6	33	30	12	-	63	30	15	15	48	111	12
97/54.1	-	30	-	-	-	-	-	-	-	-	-	-
97/85.2	-	9	-	-	-	-	-	-	-	-	-	-
97/96.1	-	3	-	-	-	-	-	-	-	-	-	-
97/143	-	15	-	-	-	-	-	-	-	-	-	-
97/144	-	198	-	-	-	-	-	-	-	-	-	-
97/172.2	-	18	-	-	-	-	-	-	-	-	-	-
97/176.2	-	30	15	18	12	9	15	-	-	9	36	-
97/176.3	15	30	-	-	12	-	-	-	-	-	-	-
cv. Angelika	-	42	-	-	-	-	-	-	-	-	-	-
DxPP	-	-	-	-	-	30	-	-	-	-	-	-
cv. Freedom red	39	102	15	15	27	39	24	-	123	15	36	-
cv. Freedom white	-	39	-	-	-	6	-	9	75	-	-	-
cv. Hot pink	-	21	-	-	-	-	-	12	3	-	36	-
cv. Pink peppermint	15	75	-	-	-	-	24	-	-	-	-	-
S2.3	-	84	-	-	-	30	-	-	-	-	-	-
S4	-	-	-	-	-	15	-	-	-	-	-	-
cv. Spotlight dark red	-	21	-	-	-	-	-	-	-	-	36	-
cv. Supjibi	-	33	-	-	-	54	-	-	66	-	-	-
cv. V10 Amy red	33	357	36	60	33	123	90	60	27	75	183	45
V10RxL	-	-	-	-	-	45	-	21	81	-	-	-
TOTAL	108	1140	96	105	84	414	183	117	390	147	438	57

(-) = not performed.

A total of 731 pollinations were performed (Table 6.3) between *Euphorbia* sp. (♀) and *E. pulcherrima* accessions (♂). Therefore, 2193 ovules were potentially pollinated.

Table 6.3. Number of ovules potentially pollinated for *Euphorbia* sp. x *E. pulcherrima* crosses

(*E. pulcherrima*) ♂ ♀	97/24.1	97/176.2 and 3	cv. Freedom red	cv. Freedom white	cv. Lemon drop	cv. Supjibi	cv. V10 Amy red	Total
E. colorata	9	-	9	-	-	-	12	30
E. cornastra	27	66	132	-	-	30	102	351
*E. cyathophora.*1	-	12	297	-	-	-	-	309
*E. cyathophora.*2	12	45	18	-	-	-	21	96
E. davidii	-	-	36	-	-	-	9	45
E. fulgens	30	-	12	-	-	93	60	195
E. hormorrhiza.	42	18	159	-	-		108	327
E. leucocephala cv. Pink finale	-	-	144	75	108	66	60	453
E. leucocephala	-	33	156	-	-	-	30	219
E. marginata	-	24	51	-	-	-	33	108
*E. radians.*1	6	18	18	-	-	-	18	60
*E. radians.*2†	-	-	-	-	-	-	-	-

(-) = not performed.

Emasculation of anthers was not conducted on genotypes that, during preliminary studies, did not self pollinate to produce seeds in insect-free greenhouses. Among those genotypes that readily selfed, emasculation prior to manual pollination was considered as a high risk as it could lead to ovary damage due to the requirement of removal of the cyathial wall. Therefore, among these species, heavy pollination was conducted prior to anthesis, followed by removal of anthers and stamens for 2 - 5 days after pollination.

To verify male and female fertility during the study, control pollinations (known successful self and cross combinations) were performed and seed production was noted.

Post pollination

Following pollinations, ovaries were observed approximately every 2 - 3 days. Developing ovaries, ovary abortion and ovary and seed collection date were recorded. Where seeds were produced, the number was recorded.

Seed sowing

Seeds from fifteen interspecific crosses were sown soon after collection onto moistened filter paper in petri dishes or *in vitro* (the latter similar to Experiment 4.1(b)). Seed number and type (solid or empty) and the results of sowing (germination, non-germination, death post-germination) were recorded. Among the remainder of crosses, embryo rescue was performed to enable the further development of immature embryos under *in vitro* conditions.

Embryo rescue *in vitro*

Cyathia with swollen ovaries were deemed to possess ovules containing fertilised egg cells and were collected prior to abortion. This stage was reached when an abscission layer formed on the cyathium pedicel. Ovaries were disinfested for 10 minutes in 4% NaOCL with one drop of Tween 20, then rinsed three times in autoclaved distilled water and allowed to dry in a laminar flow cabinet. Upon dissection, all ovules were removed, longitudinally measured and bisected and plated cut-side upwards onto tissue culture media containing MS basal salts (Murashige and Skoog 1962), 1g/l activated charcoal, 1g/l casein hydrolysate, 4g/l sucrose and 7g/l agar. Culture jars were placed inside the growth environment described in Experiment 3.2. Developing embryos were subcultured onto the abovementioned regeneration medium or a proliferation media containing MS basal salts, 0.3 mg/l benzyl adenine, 1g/l casein hydrolysate, 4g/l sucrose and 7g/l agar. Subsequent subculturing was performed at approximately 3 - 4 week intervals onto fresh media of either composition depending upon growth. Parents such as *E. colorata*, *E. cornastra*, *E. cyathophora*.1 *and E. cyathophora*.2, *E. davidii*, *E. fulgens*, *E. radians*.1 *and E. radians*.2, E. *hormorrhiza* and *E. pulcherrima* line 97/24.1 and cultivars Freedom red, Freedom white, V10 Amy red and Lemon drop were also established *in vitro* using shoot tip explants as detailed in Experiment 3.2.

Plantlets developed *in vitro*, were deflasked in the greenhouse by either planting the regenerated plantlets emerged directly from embryos, or by cutting and dipping developed shoots in 2000 mg/l IBA for 5 seconds, prior to placement in expanded Jiffy® propagation plugs. A constant water fog was initially applied and later gradually reduced to facilitate acclimatisation once plantlets had developed roots. The number of ovules exhibiting organogenesis and regeneration of plants/shoots was recorded. Furthermore, the efficiency of plant production was determined by dividing the number of ovules or seeds that produced plants by the number of ovules pollinated (three ovules per pollination).

Figure 6.1. *Euphorbia* species used for interspecific hybridisation - *E. radians*.1 (top) and *E. radians*.2 (below)

Figure 6.2. *Euphorbia* species used for interspecific hybridisation - *E. hormorrhiza* (also showing environment E2 - top) and *E. cyathophora* (below)

Figure 6.3. *Euphorbia* species used for interspecific hybridisation - *E. cornastra* (top) and *E. davidii* (below)

Figure 6.4. ***Euphorbia*** **species used for interspecific hybridisation -** ***E. colorata*** **(top) and** ***E. fulgens*** **(below)**

Figure 6.5 *Euphorbia* species used for interspecific hybridisation - *E. leucocephala* (top) and *E. marginata* (below)

Plant growth

Plants developed either via sowing seeds or *in vitro* culture (embryo rescue) were planted into 150 mm pots containing NFP potting mix with supplementary Osmocote®Plus 3 - 4 month slow release fertiliser applied to the potting mix surface at the recommended rate. Pots were placed at approximately 300 mm interpot distance as measured from the centre of the pots. Water was applied manually and plants were grown under a long photoperiod environment (light intensity >2 μmol m^{-2} s^{-1} for 4 h commencing at 10 pm) at approximately 25°C in a greenhouse. Putative hybrids derived from crosses of quarantined plants were grown in the quarantine environment detailed previously (E2).

Putative hybrids from the *E. pulcherrima x E. cornastra* cross (25 hybrids) and two parental controls (cv. V10 Amy red and *E. cornastra*) were removed from *in vitro* culture. These plants were acclimatised to the greenhouse environment as described previously and then grown under long photoperiod conditions (light intensity >2 μmol m^{-2} s^{-1} for 4 h commencing at 10 pm).

Characterisation of putative F_1 progeny

Putative F_1 progeny were characterised for various morphological characteristics such as leaf, stem and branch size and shape, hairiness, pubescence and colour as compared to their parents. Plants exhibiting characteristics similar to those of the maternal parent were noted and recorded as likely self pollinations. Plants exhibiting at least some characteristics of both parents were classified as putative hybrids.

Height and node number were assessed for 25 putative hybrids of the *E. pulcherrima* x *E. cornastra* cross and two parental controls (cv. V10 Amy red and *E. cornastra*) after 151 days under long photoperiod conditions. Height (mm) was measured from the first elongating node to the tip of each plant. Nodes were counted from the first elongating node to the uppermost node subtending a leaf greater than 30 mm long.

Nineteen putative hybrids from the *E. pulcherrima x E. cornastra* cross (one 97/144 x *E. cornastra* hybrid, one 97/54.1 x *E. cornastra* hybrid and 17 cv. V10 Amy red x *E. cornastra* hybrids) and two parental controls (cv. V10 Amy red and *E. cornastra*) were placed under short photoperiod (10 h) conditions in environment E1 after approximately 3 months from deflasking. Plants were placed at an interpot distance of 300 mm as measured from the centre

of the pots and drip irrigated. Approximately 7 weeks afterwards, bract development in relation to controls was noted (Figure 6.8) for these 19 hybrids.

Infection of *E. pulcherrima* x *E. cornastra* putative hybrids with PoiBI

Vegetative cuttings were harvested from all stockplants of *E. pulcherrima x E. cornastra* hybrids (putative) and propagated according to standard practices for poinsettias. Cuttings of cv. V10 Amy red containing PoiBI without PnMV were also propagated to enable graft transferral of PoiBI to be conducted. After acclimatisation, each cutting of cv. V10 Amy red was planted in a 150 mm pot adjacent to a cutting of a putative hybrid. NFP potting mix was used, slow release fertiliser was applied at the recommended rate and plants were manually watered and maintained at 25°C +/- 2 °C under long photoperiod conditions (light intensity >2 μmol m^{-2} s^{-1} for 4 h commencing at 10 pm) for several weeks. When the height of both plants was approximately 100 mm, plants were approach grafted (refer to Experiment 3.1 for methodology). Upon development of sufficient growth post-grafting, cuttings were removed from grafted putative hybrids and propagated according to standard protocols. Cuttings with roots were planted into 150 mm pots and grown in a commercial greenhouse according to standard poinsettia production methodology (from vegetative growth to flowering). Observations were made during the production period for suitability to commercial production and morphological changes such as increased branch number.

Results

Part 1. *E. pulcherrima* x *Euphorbia* sp. pollinations

A total of 1093 pollinations resulted in 3279 ovules being pollinated. Unequal pollination numbers resulted among crosses due to different rates of cyathia and anther production. Pollinations between poinsettias and the 12 species yielded at least one swollen ovary, from each cross combination, indicating possible fertilisation. Swollen ovaries numbered 243 and contained 689 ovules (Table 6.4) and 40 solid seeds (Table 6.5). All ovules and seeds were *in* vitro cultured (Table 6.5). Only ovules greater than 3 mm in length became organogenic (regenerated structures such as callus, embryos or shoots) when *in vitro* cultured. Ovules greater than 3 mm in length numbered 370 of which, 119 became organogenic. Ovules 3 mm or less were identical in size and appearance to unpollinated ovule controls and did not exhibit organogenesis *in vitro* (when harvested just prior to cyathia abortion). The number of ovules in swollen ovaries and the number of ovules greater than 3 mm in length are detailed in Table 6.5.

97/24.1 x E. radians.1 and cv. V10 Amy red x E. radians.1

From a total of 146 crosses conducted among poinsettias and *E. radians.1*, 42 swollen ovaries containing 126 ovules resulted (Table 6.4). From these ovaries, 36 ovules greater than 3 mm in length were obtained. Of these ovules, 4 exhibited organogenesis (Tables 6.5 and 6.6).

E. pulcherrima x E. cornastra

Among 380 crosses conducted between poinsettias and *E. cornastra*, 110 swollen ovaries were observed (Table 6.4). From a total of 177 ovules greater than 3 mm in length cultured *in vitro*, 76 ovules showed organogenesis (Table 6.5). The efficiency of production based on number of plants generated from number of ovules pollinated ranged from 0 – 27.3% (Table 6.6). Higher levels of efficiency were broadly related to fertility of female poinsettia parents. For example, 97/24.1 and 97/54.1 produced 27.3% and 16.7% plants/ovule pollinated respectively. These two were the most fertile female parents in Experiment 4.4. These were also two of the shortest poinsettias developed (Experiment 4.4).

***E. pulcherrima* lines 97/176.2 and 97/176.3 x *Euphorbia* sp.**

Crosses between wild poinsettias (97/176.2 and 97/176.3) and *Euphorbia* species often yielded seeds (Tables 6.4 and 6.5). Upon germination and growth, examination of the morphology of these progeny indicated they were likely to have been products of self-pollinations and not interspecific hybrids (Table 6.6). Therefore, attempts to prevent self pollination of these lines via liberal application of pollen and removal of anthers daily for 2 - 5 days after pollination, were not successful.

Ovules exhibiting organogenesis regenerated several possible structures. i. – undifferentiated callus, ii. – pre-embryogenic callus, iii. – somatic embryos derived from the embryo or iv. – the embryo directly germinated (Figures 6.6 and 6.7). In one instance somatic embryos developed from callus. The results of plant regeneration and morphological characterisation (evidence of hybridity) for ovules exhibiting organogenesis are presented in Table 6.6.

Table 6.4. ***E. pulcherrima*** **x** ***Euphorbia*** **sp. pollination numbers and pollinations exhibiting ovary swelling (in parentheses)**

♂ (*Euphorbia* sp.) / ♀ (*E. pulcherrima*)	*E. colorata*	*E. cornastra*	*E. cyathophora*.1	*E. cyathophora*.2	*E. davidii*	*E. fulgens*	*E. hormorrhiza*	*E. leucocephala*	*E. leucocephala* cv. Pink finale	*E. marginata*	*E. radians*.1	*E. radians*.2	Self pollinated seed production§
97/24.1	2 (1)	11(10)	10(0)	4(2)	-	21(10)	10(2)	5(0)	5(3)	16(0)	37(19)	4(1)	nil
97/54.1	-	10(10)	-	-	-	-	-	-	-	-	-	-	nil
97/85.2	-	3(2)	-	-	-	-	-	-	-	-	-	-	nil
97/96.1	-	1(1)	-	-	-	-	-	-	-	-	-	-	nil
97/143	-	5(0)	-	-	-	-	-	-	-	-	-	-	nil
97/144	-	66(6)	-	-	-	-	-	-	-	-	-	-	nil
97/172.2	-	6(0)	-	-	-	-	-	-	-	-	-	-	nil
97/176.2	-	10(2)	5(1)	6(0)	4(2)	3(2)	5(2)	-	-	3(3)	12(12)	-	yes
97/176.3	5(0)	10(6)	-	-	4(1)	-	-	-	-	-	-	-	yes
cv. Angelika	-	14(1)	-	-	-	-	-	-	-	-	-	-	nil
DxPP	-	-	-	-	-	10(3)	-	-	-	-	-	-	nil
cv. Freedom red	13(3)	34(6)	5(0)	5(0)	9(0)	13(0)	8(0)	-	41(0)	5(0)	12(2)	-	nil
cv. Freedom white	-	13(0)	-	-	-	2(0)	-	3(0)	25(0)	-	-	-	nil
cv. Hot pink	-	7(0)	-	-	-	-	-	4(0)	1(0)	-	12(0)	-	nil
cv. Pink peppermint	5(0)	25(4)	-	-	-	-	8(0)	-	-	-	-	-	nil
S2.3	-	28(5)	-	-	-	10(0)	-	-	-	-	-	-	nil
S4	-	-	-	-	-	5(0)	-	-	-	-	-	-	nil
cv. Spotlight dark red	-	7(0)	-	-	-	-	-	-	-	-	12(0)	-	nil
cv. Supjibi	-	11(0)	-	-	-	18(0)	-	-	22(0)	-	-	-	nil
cv. V10 Amy red	11(10)	119(57)	12(4)	20(1)	11(1)	41(21)	30(5)	20(1)	9(0)	25(3)	61(9)	15(2)	nil
V10RxL	-	-	-	-	-	15(5)	-	7(0)	27(2)	-	-	-	nil
TOTAL	36(14)	380(110)	32(5)	35(3)	28(4)	138(41)	61(9)	39(1)	130(5)	49(6)	146(42)	19(3)	1093(243)
% swollen ovaries	38.8	28.9	15.6	8.6	14.3	29.7	14.7	2.6	3.8	12.2	28.8	15.8	

(-) = not performed. § Natural self-seed production in insect free environment.

Table 6.5. Ovules from swollen ovaries and ovules greater than 3 mm in length displaying *in vitro* organogenesis for *E. pulcherrima* x *Euphorbia* sp. pollinations

♂ (*Euphorbia* sp.) / ♀ (*E. pulcherrima*)	*E. colorata*		*E. cornastra*		*E. cyathophora*.1		*E. cyathophora*.2		*E. davidii*		*E. fulgens*		*E. hormorrhiza*		*E. leucocephala*		*E. leucocephala* cv. Pink finale		*E. marginata*		*E. radians*.1		*E. radians*.2	
	Ov.	Org.	Ov.	Org.	Ov.	Org.	Ov.	Org.	Ov.	Org.	Ov.	Org.	Ov.	Org.	Ov.	Org.	Ov.	Org.	Ov.	Org.	Ov.	Org.	Ov.	Org.
97/24.1	1/3	0	23/30	13	-	-	1/6	1	-	-	14/30	3	0/6	0	-	-	0/9	-	-	-	17/57	3	3/3	0
97/54.1	-	-	16/30	6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
97/85.2	-	-	5/6	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
97/96.1	-	-	1/3	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
97/144	-	-	9/18	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
97/176.2§	-	-	5/6	1	1s/3	0	-	-	2s/6	0	4s/6	2	4s/6	0	-	-	-	-	*1+4s/9	1	25s/36	22	-	-
97/176.3§	-	-	7/18	3	-	-	-	-	1/3	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
cv. Angelika	-	-	1/3	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DxPP	-	-	-	-	-	-	-	-	-	-	¥5/9	0	-	-	-	-	-	-	-	-	-	-	-	-
cv. Freedom red	3/9	0	2/18	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3/6	0	-	-
cv. Pink peppermint	-	-	3/12	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S2.3	-	-	0/15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
cv. V10 Amy red	16/30	5	105/171	47	6/12	0	3/3	0	0/3	-	37/63	3	6/15	0	1/3	1	-	-	4/9	0	16/27	1	6/6	0
V10RxL	-	-	-	-	-	-	-	-	-	-	¥6/15	0	-	-	-	-	3/6	0	-	-	-	-	-	-
TOTAL		5		76		0		1		1		8		0		1		0		1		26		0

Ov. = Number of ovules produced greater than 3 mm in length / total ovules from swollen ovaries. Org. = Number of ovules greater than 3 mm in length exhibiting organogenesis. n.b. ovules <3 mm never regenerated. s = seed(s) produced. § Genotype self pollinates in insect free environment. ¥ ovules not *in vitro* cultured. * One ovule produced greater than 3 mm in length and four seeds, single organogenic event from a seed.

Table 6.6. Types of *in vitro* regeneration from *E. pulcherrima* x *Euphorbia* sp. ovules and seeds, number of plants produced and plant production efficiency

♀	♂	Method of regeneration: Callus	Somatic embryos from zygote	Direct germination	Plants regenerated	Morphological classification of hybridity‡	Plant production efficiency (%)§
cv. V10 Amy red	*E. colorata*	5†	-	-	n.a.w.	-	-
97/144	*E. cornastra*	-	3	-	2 (1 died)	hybrids	1.0 (2/198)
97/176.2	*E. cornastra*	-	1	-	died	-	-
97/176.3	*E. cornastra*	-	3	-	3	hybrids	10.0 (3/30)
97/24.1	*E. cornastra*	-	10	-	9 (1 died)	hybrids	27.3 (9/33)
97/54.1	*E. cornastra*	-	5	1	5 (1 died)	hybrids	16.7 (5/30)
cv. Angelika	*E. cornastra*	-	1	-	1	hybrid	2.4 (1/42)
cv. Freedom red	*E. cornastra*	-	1	-	1	hybrid	1.0 (1/102)
cv. Pink peppermint	*E. cornastra*	-	1	-	died	-	-
cv. V10 Amy red	*E. cornastra*	-	47	-	44 (3 died)	hybrids	12.3 (44/357)
97/176.3	*E. davidii*	-	-	1	1	non-hybrid	-
97/176.2	*E. fulgens*	-	-	2 seed	2	non-hybrids	-
97/176.3	*E. fulgens*	-	-	1	1	non-hybrid	-
97/24.1	*E. fulgens*	1†	2¥	-	n.a.w.	-	-
cv. V10 Amy red	*E. fulgens*	2†	1	-	n.a.w.	-	-
cv. V10 Amy red	*E. leucocephala*	-	-	1	died *in vitro*	-	-
97/176.2	*E. marginata*	-	-	1 seed	1	non-hybrid	-
97/176.2	*E. radians*.1	-	2	20	18 (4 died)	non-hybrids	-
97/24.1	*E. radians*.1	-	1	2	2 (1 died)	hybrids	1.8 (2/111)
cv. V10 Amy red	*E. radians*.1	1†	-	-	1	hybrid	0.5 (1/183)

‡ Morphology based identification of hybrids *in vitro* and in a greenhouse, 'hybrids' possess morphological features of both parents. 'Non-hybrids' are most likely self pollinations and always appear similar to the female parent. § Efficiency based on number of plants regenerated per number of ovules pollinated. † Pre-embryogenic callus.. ¥ Somatic embryos developing from callus. n.a.w. = not at time of writing.

Characterisation of putative F_1 progeny from *E. pulcherrima* x *Euphorbia* sp. pollinations

97/24.1 x E. radians.1 and cv. V10 Amy red x E. radians.1

All plants generated from these crosses displayed characteristics (*in vitro*) of the male parent, namely, thin hairy stems and hairy leaves. When shoots were cut and subcultured, they subsequently showed signs of reduced growth with necrosis at the cut surface. None survived to be deflasked into the greenhouse.

Figure 6.6. Pre-embryogenic callus (top) and direct embryo growth of a putative hybrid from *E. pulcherrima* x *Euphorbia* sp. ovules

Figure 6.7. Somatic embryo proliferation (top) and germination of somatic embryos (bottom) from *E. pulcherrima* x *Euphorbia* sp. ovules

E. pulcherrima* x *E. cornastra

All generated F_1 plants displayed characteristics of both poinsettia and *E. cornastra* parents. In visual comparison to the female poinsettia parents, leaf size was smaller, stem diameter was reduced and internode length was shorter (Figure 6.7). Of the 25 lines characterised for vegetative growth (21 were hybrids between V10 Amy red and *E. cornastra*) 23 exhibited reduced height (less than *E. cornastra*) and 15 exhibited increased node number, when compared to cv. V10 Amy red (Table 6.7). The remaining four lines that were not hybrids with cv. V10 Amy red were from crosses with lines 97/144, 97/54.1 and 97/176.3 and were similar in appearance to the other plants.

Table 6.7. Height and node data for putative hybrids between cv. V10 Amy red x *E. cornastra*

	Means and their standard errors		
	E. cornastra (n=3)	cv. V10 Amy red (n=5)	Putative hybrids (n = 25, range of heights)
Height (mm)	522 ± 51	537 ± 28	199 – 620
Node number	26.0 ± 2.9	33.0 ± 0.71	22 – 47
H/N (mm)	20.3 ± 2.0	16.3 ± 0.63	7.41 – 17.3

Characterisation of 19 putative hybrids (one 97/144 x *E. cornastra* hybrid, one 97/54.1 x *E. cornastra* hybrid and 17 cv. V10 Amy red x *E. cornastra* hybrids) grown under a 10 h short photoperiod environment showed that 17 exhibited earlier bract development compared to cv. V10 Amy red. The male parent *E. cornastra* was the earliest to flower, but exhibited rapid bract loss after reaching anthesis. This species also produced numerous seeds, apparently due to self pollination, indicating short photoperiod may be required for seed production. Seed production during long photoperiod conditions was not observed for this species, although it did flower under such conditions.

All putative hybrids exhibited pink bract colour, with varying degrees of colour intensity (Figure 6.8). Of the 19 putative hybrid lines observed, 9 exhibited male and female sterility (lack of reproductive structures) and the remaining 10 possessed stamens only.

Considering *E. cornastra* flowered under long photoperiod conditions, hybrid plants were observed under artificial long photoperiod for 18 months to determine if they would flower. Some lines appeared to commence long photoperiod flowering as indicated by partial colour development of leaves/bracts, but complete development was never observed.

When approach grafted to cv. V10 Amy red containing PoiBI, putative hybrids developed swollen buds followed by branches within 2 - 3 weeks of grafting. Cuttings harvested from these free-branching plants appeared to possess increased branching and improved ornamental appearance when grown under commercial conditions (Figures 6.9 and 6.10) indicating transmission of PoiBI. Profuse branching occurred from all nodes regardless of the number of nodes remaining on the primary stem after an apical decapitation (from 6 - 12 nodes). Cuttings produced roots rapidly, plants grew vigorously and within 14 - 16 weeks, the crop was ready for sale. This preliminary trial indicated some lines had immediate commercial potential.

Figure 6.8. *E. pulcherrima* x *E. cornastra* putative hybrids (four plants in centre) with male parent *E. cornastra* - left and female parent V10 Amy red - right, displaying reproductive phenotype after seven weeks under a short photoperiod environment

Figure 6.9. Vegetative stockplants of a *E. pulcherrima* x *E. cornastra* putative hybrid infected with PoiBI (right) and non-PoiBI infected (left)

Figure 6.10. *E. pulcherrima* x *E. cornastra* putative hybrids infected with PoiBI at completion of commercial production

Part 2. *Euphorbia* species x *E. pulcherrima* pollinations

A total of 731 pollinations were performed between *Euphorbia* sp. (♀) and *E. pulcherrima* accessions (♂, Table 6.8). Therefore, 2193 ovules were pollinated. Six cross combinations produced 85 swollen ovaries and these contained 121 ovules/seeds/empty seeds (Table 6.9). The contents of six combinations exhibited organogenesis (with 73 organogenic events observed) however, plants derived appeared to be from self-pollinations (Table 6.9).

Table 6.8. *Euphorbia* sp. x *E. pulcherrima* pollination numbers and swollen ovaries

♂ / ♀	97/24.1	97/176.2 and 3	cv. Freedom red	cv. Freedom white	cv. Lemon drop	cv. Supjibi	cv. V10 Amy red	Total	Self pollinated seed production§
E. colorata	3(0)	-	3(0)	-	-	-	4(0)	10(0)	yes
E. cornastra	7(0)	22(0)	44(0)	-	-	10(0)	34(0)	117(0)	yes in short photoperiod rare in long photoperiod
*E. cyathophora.*1	-	4(4)	99(50)	-	-	-	-	103(54)	yes, frequent
*E. cyathophora.*2	4(2)	15(16)	6(0)	-	-	-	7(0)	32(18)	yes, frequent
E. davidii	-	-	12(5)	-	-	-	3(0)	15(5)	yes, frequent
E. fulgens	10(0)	-	4(0)	-	-	31(0)	20(0)	65(0)	yes, rare
E. hormorrhiza.	14(0)	6(0)	53(1)	-	-		36(4)	109(5)	yes
E. leucocephala *cv. Pink finale*	-	-	48(0)	25(0)	36(1)	22(0)	20(0)	151(1)	yes, rare
E. leucocephala	-	11(0)	52(2)	-	-	-	10(0)	73(2)	yes, rare
E. marginata	-	8(0)	17(0)	-	-	-	11(0)	36(0)	yes, rare
*E. radians.*1	2(0)	6(0)	6(0)	-	-	-	6(0)	20(0)	no
*E. radians.*2†	-	-	-	-	-	-	-	0(0)	no

§ Untouched self-seed production in insect free environment. † female sterile.

Table 6.9. Types of *in vitro* and *in vivo* regeneration from *Euphorbia* sp. x *E. pulcherrima* ovules and seeds, number of plants regenerated and evidence of F_1 hybridity

♀	♂	Seeds		Empty seeds		Swollen ovules		Plants regenerated	Morphological classification of hybridity§
		No.	Org.	No.	Org.	No.	Org.		
E. cyathophora.1	97/176.3	4†	1	1†	0	-	-	1	non-hybrid
E. cyathophora.1	cv. Freedom red	61†	61	16†	2	-	-	61	non-hybrids
E. cyathophora.2	97/176.2	6‡	2	-	-	-	-	2	non-hybrids
E. cyathophora.2	97/24.1	5†	0	1†	0	2‡	1	1	non-hybrid
E. davidii	cv. Freedom red	13†	5	-	-	-	-	5	non-hybrids
E. hormorrhiza	cv. Freedom red	-	-	1‡	-	-	-	0	-
E. hormorrhiza	cv. V10 Amy red	2‡	2callus	2‡	0	-	-	2 shoots ex. callus	non-hybrids
E. leucocephala	cv. Freedom red	-	-	4‡	0	2‡	0	0	-
E. leucocephala cv. Pink finale	cv. Lemon drop	1†	1	-	-	-	-	1 (died)	-

† sown *in vivo*, ‡ sown *in vitro*. § Morphology based identification of interspecific hybrids *in vitro* and in greenhouse, 'hybrids' possess morphological features of both parents. 'Non-hybrids' are most likely self pollinations and always appear similar to the female parent.

Discussion

The primary aim of this experiment was to produce dwarf poinsettia-like plants through interspecific hybridisation. This aim was met with the development of hybrid progeny from the poinsettia x *E. cornastra* cross. Numerous putative dwarf F_1 hybrids were produced, and these constitute a new dwarf, short photoperiod flowering crop that grows in a similar fashion to poinsettias. Future molecular studies (e.g. AFLP, RFLP) may be desired to verify the hybridity of these new plants. However, the morphological evidence of hybridity (traits from both parents) is very substantial.

It was hypothesised that long photoperiod flowering plants could be produced from the *E. pulcherrima* x *E. cornastra* cross due to the male parent flowering in both long and short photoperiod. Commencement of long photoperiod flowering was observed in some F_1 lines, but bract development was never complete. Only partial bract colouration occurred. Further breeding studies should aim to produce long photoperiod flowering plants which could enable further expansion of the market and production of the crop throughout the year without the need for an artificial short photoperiod. Production for the Christmas market in Australia requires artificial short day manipulation via covering plants with a light impenetrable black cloth. Production without black cloth covering would be beneficial because better control of

night and early morning temperature could be obtained, thus resulting in reduced crop height, which is a serious problem under Australian conditions during this season.

Preliminary testing (Table 6.7) showed many lines were shorter than both *E. cornastra* and cv. V10 Amy red, which is one of the shortest commercial cultivars available. Further studies could be performed to identify the exact reduction in height, but this was not undertaken due to time constraints and the obvious morphological differences, which remained constant over a 24 month period.

Considering PoiBI reduces height in cv. Freedom red during commercial production, and in *E. cornastra* stockplants grafted to cv. V10 Amy red, the introduction of this phytoplasma into the putative F_1 hybrids was expected to do likewise. After grafting all of the hybrids to cv. V10 Amy red, highly branched plants were obtained. A preliminary commercial trial utilising cuttings from these highly branched plants showed several hybrids could be immediately commercialised. All hybrids could be grown within the current framework for poinsettias.

One potential limiting factor to the commercial exploitation of these hybrids is the short post-production period (approx. 2 weeks) that has been observed in some lines. This trait may be obtained from the *E. cornastra* parent, which displayed complete bract loss after reaching anthesis under short photoperiod. Future breeding work should centre on increasing post-production performance, either through backcrossing to poinsettias with superior post-production characteristics (e.g. Lilo or Freedom series) or via mutation or other means. The presence of PoiBI in poinsettias improved post-production performance, and when introduced into these putative hybrids improved this parameter slightly as shown in preliminary studies.

Further studies are required to elucidate the male and female fertility of the hybrids for future breeding. All lines were female sterile (lacking female reproductive structures) however, some possessed anthers. Therefore, the hybrids generated in this study can only be utilised as male parents. Future studies should concentrate on determining the pollen viability of these lines, through crossing and via aniline blue staining. Some preliminary studies (not presented) indicated pollen germination was low. A larger population of hybrids needs to be developed and tested to increase the chances of producing plants that are both male and female fertile. One method of potentially increasing fertility (if lack of fertility is due to chromosome pairing

problems) is to produce tetraploids of the putative hybrids, possibly via treatment with colchicine (Experiment 7.1).

The putative hybrids were distinct because they possessed pink bracts (with white undersides). Pink bracts were produced from both white (97/144, 97/54.1) and red bracted poinsettia parents crossed with the white bracted *E. cornastra*. This result is unusual given the genetics of flower colour inheritance for poinsettias. Investigations by Stewart (1960) revealed a single completely dominant gene, designated *WH* responsible for red bract colour in poinsettias. The homozygous recessive *wh/wh* conferred white bract colour. Modifying factors appeared to affect the phenotypes, as there were a variety of reds and whites produced. Petiole colour was a pleiotropic effect. Later, Stewart and Arisumi (1966) demonstrated that a second independent locus, termed *pk* was responsible for genetic pink bract colour in poinsettias when in the homozygous recessive form, and having the genotype *WH/--* at the *WH* locus. The genotype *pkpk* reduced the amount of anthocyanin pigment formed when *WH/--* (red) was established.

Observations of *E. cornastra* during two years of growth showed that anthocyanin was sometimes produced in the normally white bracts. Therefore, perhaps a gene(s) for anthocyanin production are suppressed or have reduced effect in this species. When intermated with white poinsettias, the gene(s) is expressed due to the new nuclear and cytoplasmic environment.

The use of ovary swelling as an indicator of successful zygote formation to identify potential successful crosses can be recommended for future studies in the species investigated. Although probably not 100% accurate, this method reduces the number of ovules that need to be cultured and provided the most rapid and easiest method to use given the lack of knowledge of growth and flowering conditions for the species. Future studies may utilise this data to determine the number of crosses that need to be performed to produce a given number of plants, or to develop time series embryo rescues for improved efficiency. However, culturing embryos at or close to abortion may allow the maximum level of development of the embryo, thus increasing efficiency of regeneration. Additionally, collection of ovules after a few days from pollination may pose the problem of somatic embryo development from non-zygotic tissues, a method which was utilised in Experiment 3.2.

Ovary swelling data indicated all 12 species could potentially form interspecific hybrids using poinsettias as female parents. The frequency of ovary swelling was however, low for some crosses. The production of swollen ovaries indicates that crosses displaying this characteristic may have produced embryos. It is likely that because ovaries were not removed soon after pollination, putative embryos did not survive or were not able to regenerate at the ovary collection time (close to abortion). Poinsettias with high intraspecific seed production developed the greatest numbers of putative hybrids (i.e. V10 Amy red, 97/24.1 and 97/54.1). Further studies should concentrate on such plants (that do not self pollinate in an insect free environment) for increased efficiency of production.

Attempts to prevent self pollination of lines that naturally self pollinate (97/176.2 and 97/176.3) via liberal application of pollen were not successful. This is a limiting factor when using these lines as female parents and therefore, other methods of emasculation such as chemical application need to be given consideration.

Success with interspecific hybridisation may be related to pollen morphology. Pollen morphology is considered useful in determining evolutionary relationships and for establishing taxonomical groupings (El-Ghazaly and Chaudhary 1993). Such information aids in selection of species for interspecific hybridisation. Three species in *Poinsettia*, namely *E. pulcherrima*, *E. radians* and *E. heterophylla*, and two related species, *E. fulgens* and *E. ariensis* exhibit pollen homogeneity, characterised by coarsely reticulate sexine (Nair 1961, El-Ghazaly and Chaudhary 1993). In this experiment, crosses Between *E. pulcherrima* and *E. radians* and *E.* pulcherrima and E. *fulgens* resulted in organogenic development. Therefore, future experiments could rely on pollen type as an indicator for selecting suitable species with which to hybridise.

During the course of this experiment 1207 self and cross pollinations (data not presented) were also performed between species (not including *E. pulcherrima*). These rarely produced seeds. Only one combination *E. hormorrhiza* x *E. cyathophora.1* produced plants. These were morphologically intermediate between both parents.

Development of interspecific hybrids could lead to the introgression of desirable novel genes into the poinsettia genepool. In addition, utilisation of *Euphorbia* species could produce poinsettias that are not influenced by factors such as mutational load or inbreeding depression, which are both currently prevalent in commercial intraspecific hybrids.

Chapter 7. Enabling technologies for further genetic improvement

General introduction

Breeding for dwarf poinsettias could be assisted by the manipulation of chromosome ploidy. Three techniques are demonstrated in this chapter for altering the ploidy of poinsettias. First, doubling the chromosome number of diploids could be expected to produce tetraploids with unique characteristics such as thicker stems, larger bracts, bracts that are elevated thus resisting drooping and with shorter internodes (Sander pers. comm. 1999, Trees pers. comm. 1999). Additionally, detrimental effects of mutational load/IBD on fertility could be reduced via the buffering effect obtained by duplicated heterozygous loci (if chromosome pairing between homologues is random) (Husband and Schemske 1997). Random chromosome pairing could be expected in recently synthesised tetraploids that have not evolved methods of preferential bivalent pairing (Husband and Schemske 1997) thereby creating recombinants. Doubling the chromosome number has successfully restored fertility in many plant species developed as a result of wide hybridisation (e.g. Triticale). Therefore, the development of an effective chromosome doubling technique could enable development of fertile *E. pulcherrima* x *E. cornastra* hybrids for further genetic improvement. At present, the use of this germplasm is limited due to the lack of female reproductive structures, and low pollen viability. In addition, production of tetraploids from current commercial cultivars could directly produce new cultivars.

The second section of this chapter involves the production of triploid plants. Triploids could be expected to express sterility due to a lack of bivalent pairing. Sterility might result in reduced pollen shedding and thus reduce the incidence of *Botrytis* infection and/or unwanted pollen on bracts. Sterility would also limit use of the material by other breeders in intraspecific hybridisation breeding programs. Triploids may have immediate commercial application if they can be developed from current commercial cultivars. Two methods are explored for the induction of triploids in this chapter. These are i. - crossing tetraploid and diploid plants and ii. - development of shoots from triploid endosperm tissue adjoining the normal diploid embryo.

Experiment 7.1. Colchicine chromosome doubling for the production of tetraploid poinsettias

Introduction

Developing a reliable, routine method for chromosome doubling is essential for future breeding work in poinsettias. Tetraploids could be developed for use as i. - parents in crosses with diploids to produce triploids, ii. - as a means of restoring fertility in recently synthesised interspecific hybrids, iii. - to improve seed production efficiency by the buffering effect of mutational load and/or IBD and iv. - as a method of producing new cultivars.

Seeds can be produced through crosses among tetraploid parents (Stewart and Pryor 1961, Sander pers. comm.1999). Stewart (1951) produced tetraploids via the application of a 0.1% lanolin emulsion of colchicine to the newly emerging buds of the diploid cv. Mrs. Paul Ecke. Plants were selected from several thousand shoots for distorted growth, thicker, shorter and broader leaves, darker bracts and larger flowers, larger pollen and larger seeds. A few of the older flowers (on the tetraploids) were selfed and then produced 13 seed, of which 9 germinated.

The experiment presented here studied colchicine concentration and treatment time in relation to plant survival and production of tetraploid plants via application to vegetative cuttings with roots.

Materials and methods

Production of cuttings for colchicine treatment

Stockplants of cv. Freedom red and cv. V10 Amy red infected with PoiBI (but not PnMV) were maintained under a long photoperiod environment in a greenhouse according to conventional practices (Experiment 3.1). Cuttings for colchicine treatment were harvested from stockplants and the lower leaves were removed at the stem with a knife. Approximately two mature leaves were left on each cutting. Basal ends of cuttings were dipped in liquid IBA (2000 mg/l) solution for five seconds prior to being placed into the propagation medium. This medium consisted of sphagnum peat and coarse grade perlite (1:1) in standard seedling trays. Approximately 30 cuttings were planted in each tray. Trays were placed in a constant fog of water generated by a Unifogger^{TM}. After 3 - 4 weeks, cuttings produced sufficient roots and

for the next 5 - 7 days, they were gradually acclimatised to the greenhouse environment by gradual reduction in humidity. A general liquid fertiliser was applied as required.

Colchicine preparation

Colchicine solutions were prepared by dissolving the required amount of colchicine powder in dimethylsulfoxide (DMSO). The amount of DMSO used was 1% of the final solution. The final solution consisted of colchicine, DMSO and de-ionised water. Three concentrations, 0.3%, 0.15%, 0.1% and a control were made. Two drops of Tween 20 were added for every 200 ml of solution. Two hundred millilitres of colchicine solution was sufficient for treating 60 cuttings with roots.

Colchicine treatment of cuttings with roots

Colchicine treatment concentrations and treatment times were adapted from Ahmed (pers. comm. 1998). Cuttings were gently removed from the propagation substrate and washed in water to remove all potting mix. Care was taken to avoid root breakage. Basal leaves were removed at the stem with a knife to facilitate colchicine treatment. Cuttings were sorted into two grades (type 1 = large, type 2 = small) according to size and root number, and were randomly allocated among treatments (Table 7.1.1). Cuttings were labelled and bundled together with wire. Approximately 10 - 20 mm of root length was excised from all root tips prior to placing the bundled cuttings into the appropriate colchicine solutions for 5 or 7 hours in a fume hood in a laboratory. Post treatment, roots were washed in running tap water for 30 - 40 minutes by placing all treated cuttings (including controls) in a bucket under a tap. Cuttings were planted into NFP media immediately after treatment and thoroughly watered in. Plants were treated as conventionally grown from this point onwards (long photoperiod maintained, manual watering, standard fertilising, 25°C ± 2°C temperature, etc). Stem apices were decapitated to facilitate axillary branching.

Post-treatment measurements of colchicine treated plants

i. - Nine days after colchicine treatment, the number of leaves (>10 mm) remaining on each plant were scored to obtain an indication of the toxicity of treatments.

ii. – Thirty weeks after treatment, the numbers of dead and alive plants (1 = alive, 2 = dead) were recorded to obtain an indication of the toxicity of treatments.

iii. – The number of plants that possessed enlarged morphological features (petioles, leaves and stems) was also noted at thirty weeks.

Table 7.1.1. Colchicine treatment of cultivars Freedom red and V10 Amy red

	Vegetative cuttings							
Cultivar	Freedom red				V10 Amy red			
Time	5 hr treatment		7 hr treatment		5 hr treatment		7 hr treatment	
Cutting Type	Type 1	Type 2	Type 1	Type 2	Type 1	Type 2	Type 1	Type 2
Colchicine concentration								
0 (water)	8	5	8	5	10	5	10	5
0.1%	8	7	8	7	10	5	10	5
0.15%	8	7	8	7	10	5	10	5
0.3%	8	7	8	7	10	5	10	5
Total	32	26	32	26	40	20	40	20

Type 1 = large cutting with roots, Type 2 = small cutting with roots.

Chromosome counts of putative tetraploids and controls

Shoot tip chromosome counts (as detailed in Appendix 3) were made on apical meristems derived from morphologically distinct branches (i.e. larger leaves, distorted growth and larger petioles). However, this proved to be difficult and did not provide clear chromosome counts. Root meristem counts were performed thereafter. This technique involved harvesting cuttings from branches of relevant plants and propagating them according to standard practices. After 2 - 4 weeks, roots emerging from the propagation substrate were cut and immediately placed into glass vials containing 3 parts absolute alcohol to 1 part glacial acetic acid (Appendix 3). Meristem squashes were performed, chromosomes were counted and further propagation was practiced from those plants with 56 chromosome cells (twice the number recorded for parental control plants, $2n = 28$).

Flowering of putative tetraploids and controls

Putative tetraploid plants and controls were grown according to conventional practices under a short photoperiod of 10 h duration in a commercial nursery. Plants were allowed to flower, and were observed weekly for differences between controls and putative tetraploids. When hermaphrodite cyathia were produced, self and cross pollinations were performed in an attempt to produce seeds. Temperature was maintained between 18°C (night) and 29°C (day).

Data analyses

Data analyses were performed with the MINITAB® software package (MINITAB® for windows release 12.22, Minitab Inc. 1998) utilising the general linear model command (GLM) and Tukey pairwise comparisons.

Results

Post-colchicine treatment measurements of plants

Nine days post-treatment

All treatment factors significantly influenced leaf number remaining after nine days (Table 7.1.3). V10 Amy red had higher mean leaf loss than Freedom red for all treatment factors and levels. For both cultivars, leaf loss increased as colchicine concentration increased and as cutting grade decreased (big to small). Increased treatment time resulted in significantly greater leaf loss for Freedom red, but did not influence V10 Amy red.

Table 7.1.2. Means and standard errors for leaves remaining after nine days post-treatment as influenced by cultivar, colchicine concentration, treatment time and cutting grade

	Freedom red			V10 Amy red		
Treatment /cv	n	Mean	SE	n	Mean	SE
Colchicine concentration						
0	26	4.00a	0.34	30	3.23a	0.32
0.1%	30	3.77a	0.29	30	1.80 b	0.20
0.15%	30	3.40a	0.29	30	1.63 b	0.21
0.3%	30	1.73 b	0.30	30	0.700 c	0.15
Treatment time						
5 hrs	58	3.76a	0.25	60	1.80a	0.20
7 hrs	58	2.64 b	0.22	60	1.80a	0.20
Cutting grade						
Type 1	64	3.92a	0.22	80	2.11a	0.18
Type 2	52	2.31 b	0.22	40	1.30 b	0.19

‡Means with common letter are not significantly different.

Table 7.1.3. ANOVA for effect of colchicine concentration, treatment time, cultivar and cutting grade on leaf number remaining at nine days post treatment

Source	df	MS
Colchicine concentration	3	56.81***
Treatment time	1	15.20**
Cultivar	1	136.0***
Cutting grade	1	77.72***
Error	229	1.780

*, **, ***: $p < 0.05, 0.01, 0.001$ respectively.

30 weeks post-treatment

At 30 weeks, all treatment factors significantly influenced the number of plants alive (Table 7.1.5). An identical trend was found for both cultivars; the number of dead plants increased as concentration increased, treatment time increased and cutting size decreased (Table 7.1.5).

Table 7.1.4. Number of plants alive and percent of plants dead, 30 days post-treatment as influenced by cultivar, colchicine concentration, treatment time and cutting grade

Cultivar	Freedom red			V10 Amy red		
Treatment	No. of plants treated	No. of plants remaining at 30 weeks	Dead plants (%)	No. of plants treated	No. of plants remaining at 30 weeks	Dead plants (%)
Colchicine concentration						
0	26	21	19	30	25	17
0.1%	30	26	13	30	18	40
0.15%	30	24	20	30	14	53
0.3%	30	11	30	30	2	93
Treatment time						
5 hrs	58	46	21	60	31	48
7 hrs	58	36	38	60	21	65
Cutting grade						
Type 1	64	49	23	80	45	44
Type 2	52	33	36	40	14	65
Total	116	82	29	120	59	49

Table 7.1.5. ANOVA for number of plants alive 30 weeks after treatment date, as influenced by colchicine concentration, treatment time, cultivar, and cutting grade

Source	df	MS
Colchicine concentration	3	4.240***
Treatment time	1	0.720*
Cultivar	1	3.391***
Cutting grade	1	1.440**
Residual	229	0.170

Morphological features at 30 weeks post-treatment

Table 7.1.2. Number of plants possessing enlarged morphological features and respective treatments at 30 weeks post-treatment

Cultivar	Cutting type	Colchicine concentration	Treatment time	No. of plants with potential tetraploid sectors
Freedom red	1	0.1%	7	1
Freedom red	1	0.15%	7	2
Freedom red	1	0.3%	5	3
Freedom red	1	0.3%	7	1
V10 Amy red	1	0.15%	7	2

The petiole diameter of potential tetraploid Freedom red leaves was approximately 4.0 mm. Control plants had petiole diameters of approximately 2.5 mm.

Chromosome counts of putative tetraploids and controls

Root tip chromosome counts are presented in Table 7.1.7. Several cells from Freedom red and V10 Amy red roots were recorded with 2n = 56 chromosomes (Figure 7.1.2). Controls recorded 2n = 28 (Figure 7.1.1). Some plants exhibited mixoploidy, with some doubled sectors recording 56 and others recording 28 chromosome cells. Some appeared aneuploid, but this may have been due to the difficulty in obtaining cells with good, countable chromosome spreads. Due to the difficulty with obtaining well spread cells and the observed mixoploidy, a limited number of root tip chromosome counts were performed.

Table 7.1.3. Root tip chromosome counts from roots developed on vegetative cuttings of putative tetraploid shoots derived via colchicine treatment

Cultivar and shoot accession	Root tip chromosome counts and number of cells counted	Number of roots counted
Freedom red (pot. 4x.4 sh3)	~28(x4), 28(x6)	2
Freedom red (pot. 4x.3 sh3.1)	56(x3)	2
Freedom red (pot. 4x.8 sh2)	28(x3)	4
Freedom red (pot. 4x.4 sh2.1)	28(x3)	2
V10 Amy red (pot. 4x.1 sh2.2)	28(x3)	3
V10 Amy red (pot. 4x.4 sh1)	28(x3)	3
	56(x2), ~56(2)	2
	~56, 41, ~53, ~25, ~24, ~51	3

~ = approximately.

Flowering of putative tetraploids and controls

Putative tetraploids and diploid controls reached anthesis after approximately 8 weeks under a short photoperiod environment. Putative Freedom red tetraploids possessed larger bracts with a ruffled appearance, and larger cyathia (Figure 7.1.3). Putative V10 Amy red plants possessed larger cyathia and broader bracts (Figure 7.1.4) that often had raised portions either side of the midrib. These bract 'protrusions' have been noted previously in progeny derived from V10 Amy red crosses.

Approximately 50 pollinations were performed (both self and cross for Freedom red and V10 Amy red putative tetraploids) but none produced seed. High temperatures (approximately 30°C) during this period may have influenced seed production. Alternatively, plants could have been female sterile. Pollen was produced from all putative tetraploids. The viability of this pollen requires testing for future studies.

Figure 7.1.1. Mitotic metaphase cell of cv. Freedom red displaying 2n = 28 chromosomes

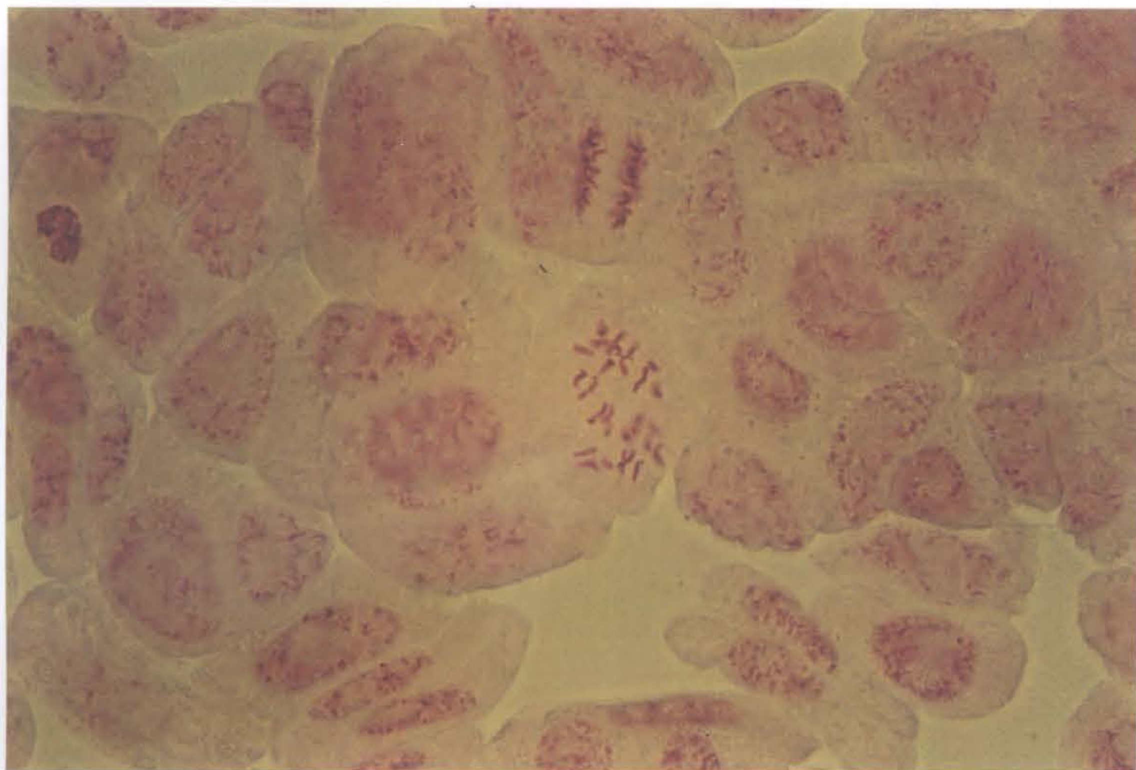


Figure 7.1.2. Mitotic metaphase cell of cv. Freedom red displaying 2n = 56 chromosomes

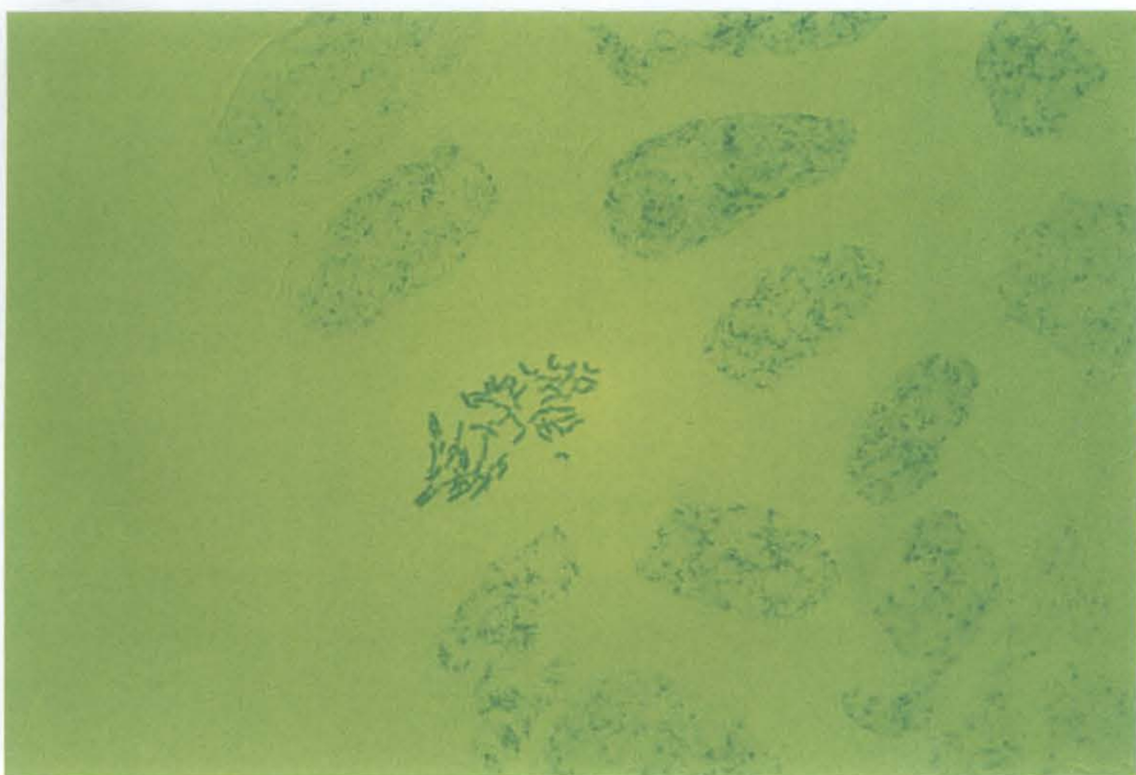


Figure 7.1.3. Morphology of a putative tetraploid Freedom red plant (left) compared to the diploid cultivar (right)

Figure 7.1.4. Morphology of a putative tetraploid V10 Amy red plant

Discussion

The production of tetraploid poinsettias (sectors) was achieved (as demonstrated by root chromosome counts) however, due to the difficulty in counting chromosomes and chimeric (mixoploid) nature of the plants produced, further studies are required to verify beyond doubt if chromosome doubling has occurred. Morphological characterisation indicated seven plants of Freedom red and two of V10 Amy red could be tetraploid due to increased petiole diameter. Leaves also appeared larger on these plants. Attempts at measuring stomata size were unsuccessful and require further development. At flowering, putative tetraploids possessed notably larger bracts and cyathia compared to diploid controls. These characteristics are considered desirable in poinsettia cultivars and could lead to the development of new cultivars. Future work should aim to develop stable tetraploid lines for vegetative propagation and identify their merit under commercial production conditions.

Further colchicine treatments should be based on the results of this study. These results indicate treating large cuttings for 5 - 7 hours with 0.3% colchicine is an appropriate combination for the production of tetraploid sectors/plants. This combination resulted in 25% of Freedom red plants producing morphologically (putative) tetraploid sectors. The combination of 0.15% colchicine and 7 h treatment of large V10 Amy red cuttings resulted in 13% of plants with morphological characteristics associated with tetraploids. Plants with a similarity to V10 Amy red may require lower concentrations due to the apparent reduced tolerance of this plant type to colchicine.

Experiment 7.2. Characterisation of plants produced via tetraploid (2n = 56) x diploid (2n = 28) crosses

Introduction

Triploid plants have been produced via intraspecific hybridisation at a very low frequency (0.1%) by Bempong and Sink (1968b) and (0.2%) by Milbocker and Sink (1969a). Both authors were successful in producing seed from the diploid x tetraploid cross. Reciprocal pollinations did not succeed. Most triploid embryos aborted during seed development, as evidenced by the high ratio of embryos with triploid chromosome counts found in abscised ovules. Milbocker and Sink (1969b) proposed that the determining factor in setting of seed in the diploid x tetraploid cross was a lack of development of the endosperm.

It was therefore postulated that embryo rescue techniques utilised for interspecific hybridisation could improve the efficiency of triploid production if the low level of seed production was due to abnormal endosperm development and not due to lack of embryo production. Several pollinations were performed between tetraploid (female) and diploid (male) poinsettias. This experiment details the results of embryo rescue and characterisation of two plants derived from these crosses.

Materials and methods

Plant materials and *in vitro* culture of ovules

Plants used for crossing were grown as per standard practices (Experiment 4.1) in Environment E1. Crosses (15) were conducted between cv. Supjibi and line 97/176.3. Crosses (5) were conducted between cv. Supjibi and cv. Capri red. Swollen ovaries from four cv. Supjibi x 97/176 and three swollen ovaries from cv. Supjibi x cv. Capri red crosses were collected just prior to abortion. These were subjected to *in vitro* culture procedures applied in Chapter 6. Upon regeneration of embryos and multiplication, small shoots were deflasked and acclimatised to the greenhouse environment as described previously in Experiment 3.1. Plants were then grown according to standard practices described in Experiment 3.1.

Cytology of progeny from tetraploid x diploid crosses

Mitotic chromosome counts were performed on root meristems of cuttings as described in Appendix 3.

Vegetative characterisation of putative triploid plants

Putative triploid plants and their parents were observed for leaf size, stem diameter, height, and other features that were distinguishing.

Reproductive characterisation of putative triploids

One plant of each putative triploid was placed under artificial short photoperiod conditions as described in Experiment 4.1 (Environment E1). Plants were observed for date of anthesis, and production of male and female reproductive structures. Self pollinations (manually performed) and reciprocal crosses between the putative triploid plants and cv. V10 Amy red were undertaken. The number of swollen ovaries/seeds produced was recorded.

Results

Two embryos germinated and were grown in the greenhouse, their pedigrees were cv. Supjibi x 97/176.3 and cv. Supjibi x cv. Capri red.

Cytology of putative triploid progeny

Obtaining cells with well spread non-overlapping chromosomes at pre-metaphase was difficult, as explained previously (Experiment 7.1). However, three cells of Supjibi x 97/176.3 were counted and these clearly displayed 42 chromosomes (Figure 7.2.1). This plant was therefore a triploid. Three cells of Supjibi x Capri red clearly had 35 chromosomes (Figure 7.2.2). This plant is aneuploid. Photographs could not be taken to clearly show each separated chromosome in a cell due to chromosome overlapping and differences in focus across the cell.

Vegetative and reproductive characterisation of plants with 42 and 35 chromosomes

Supjibi x 97/176.3

Morphologically, this line was similar in appearance to the male parent, but it had larger leaves and thicker stems. Leaves were upright and bract size was larger than the male parent, but similar to Supjibi. Cyathia were of comparable size to Supjibi. Hermaphrodite cyathia were produced, and both pistils and pollen appeared normal. Self pollinations and reciprocal pollinations with V10 Amy red did not yield seed or swollen ovaries. Thirty self pollinations were performed, and ten each of the reciprocal with V10 Amy red.

Figure 7.2.1. Mitotic root cell of Supjibi (2n = 56) x 97/176.3 (2n = 28) displaying 42 chromosomes at metaphase

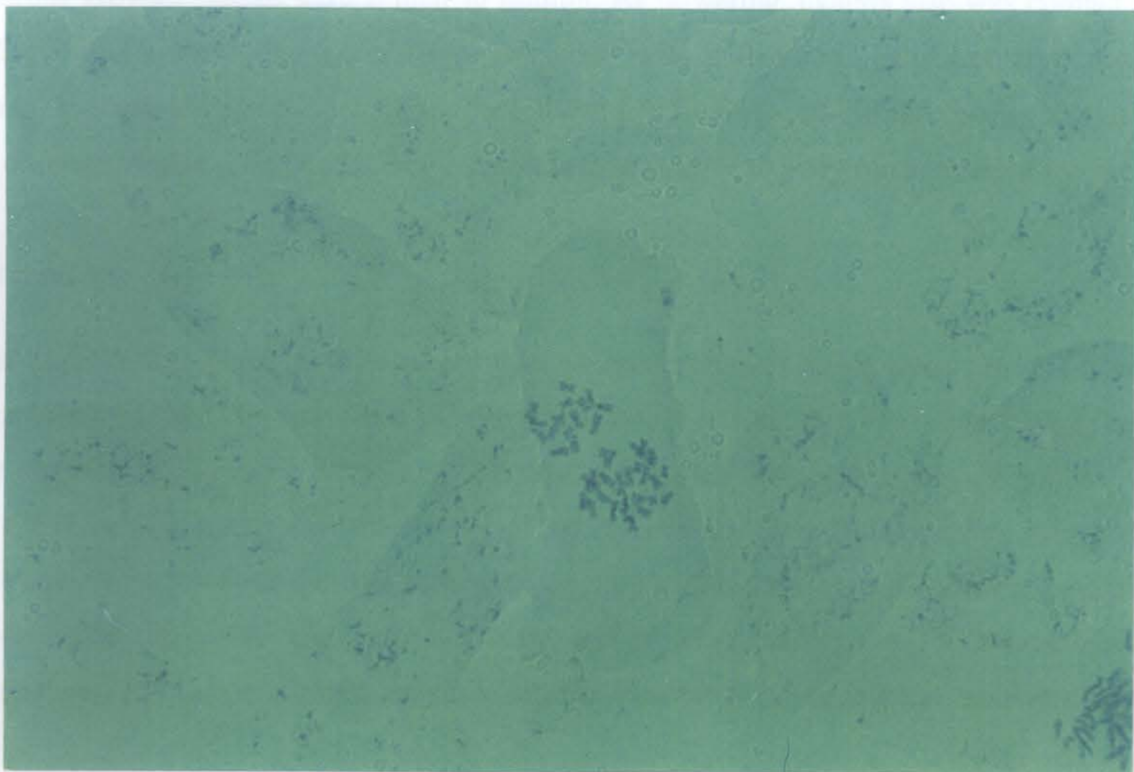
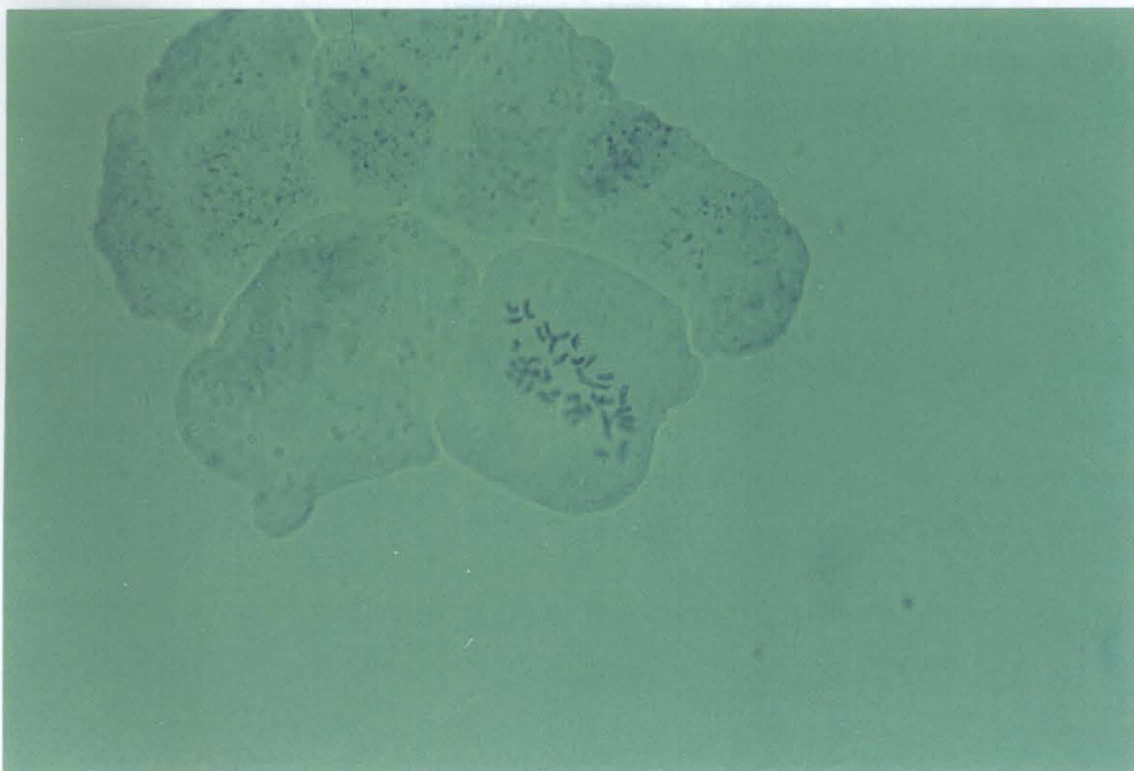


Figure 7.2.2. Mitotic root cell of Supjibi (2n = 56) x Capri red (2n = 28) displaying 35 chromosomes at metaphase



Supjibi x Capri red

This line appeared similar to the female parent. It possessed thick stems, upright leaves and vigorous growth. Leaves were similar to Supjibi. At flowering, cyathia size was comparable to Supjibi. Hermaphrodite cyathia were produced, with pollen and pistils similar to normal plants. Manual self and reciprocal pollinations with V10 Amy red did not result in any seed set or ovary swelling. Self and reciprocal pollinations with V10 Amy red numbered ten each.

Discussion

This experiment reports the first record of development of a triploid plant (2n = 42) via a tetraploid x diploid pollination and embryo rescue/ovule culture. Previous reports have shown that plants have only been derived via the reciprocal cross. Only a few (15 pollinations with 97/176.3 and 5 with Capri red) were required to achieve one plant from each. Further studies are required to elucidate the true efficiency of production. The 2n = 42 chromosome plant produced was vigorous and exhibited many of the characteristics of its female parent, indicating production of triploids may be useful as a means of producing new cultivars.

The production of a 2n = 35 chromosome plant from the Supjibi x Capri red cross could also be expected, because when plants of different ploidy are intermated, abnormalities of chromosome pairing can occur. If mispairing occurs some chromosomes can be lost during cell division, resulting in aneuploids (Simmonds 1981). This plant was similar to its female parent and exhibited vigorous growth, unlike plants produced by Bempong 1967. Chromosomal stability may be a factor to consider in the production of triploid plants.

Experiment 7.3. Characterisation of plants derived from an embryo and endosperm of the same seed

Introduction

Triploid plants are often produced as a result of crossing tetraploids with diploids. Another method of triploid plant production is to culture endosperm tissue from diploid seeds. Endosperm tissue is triploid because it results from fusion of a haploid male gamete with a diploid female gamete. The production of triploid plants from endosperm tissue has been previously reported in the Euphorbiaceae (Sehgal *et al.* 1994, Sehgal and Abbas 1996) but not for poinsettia. During Experiment 4.2 (where seeds were sown *in vitro*) a seed that regenerated a shoot from its endosperm was noted. This same seed also carried an embryo that germinated. Shoots from both tissues were subsequently separated and multiplied *in vitro*. The plants derived were grown in the greenhouse under the same environment and morphologically characterised. In addition, cytological observations were performed.

Materials and methods

Plant materials

The accession 97/91 with pedigree [(Annette Hegg Diva x Pink peppermint) x Freedom red] developed a shoot each from both endosperm and the embryo of the same seed, which were separately multiplied on MPM (Experiment 3.2) *in vitro* according to standard practices detailed in Chapter 3. Regenerants were deflasked and gradually acclimatised to the greenhouse environment as described previously (Chapter 3).

Plant growth and further multiplication

Acclimatised *in vitro* derived shoots with roots were planted into NFP potting mix in 150 mm pots and grown under a long photoperiod (>2 μmol m^{-2} s^{-1} incandescent light for 4 h from 10 pm) at 25 ± 2°C in a standard 'microclimate' room. Between 6 and 10 stockplants were developed of each plant type (embryo or endosperm derived). The recommended rate of Osmocote®Plus 3 - 4 month slow release fertiliser was applied to the potting mix surface and plants were drip irrigated.

Five uniform 60 mm long cuttings were derived from the established stockplants for each plant type and propagated according to standard practices. After 3 weeks, the cuttings with roots were acclimatised to the greenhouse environment and planted as described previously

into 150 mm pots. Plants in pots were grown completely randomised with approximately 300 mm interpot distance as measured from the centre of the pots in the 'microclimate' environment described previously.

Vegetative characterisation of embryo and endosperm derived plants

After 36 days from planting cuttings with roots, height (mm) and node number were measured and plants were morphologically characterised (leaf size, stem diameter, internode length, etc). ANOVA was performed with the MINITAB® software package (MINITAB® for windows release 12.22, Minitab Inc. 1998).

Reproductive characterisation of embryo and endosperm derived plants

Two plants of each plant type were placed into artificial short photoperiod conditions as described in Experiment 4.1. Plants were observed for date of anthesis and production of male and female reproductive structures. Pollinations were performed between plants producing reproductive structures and two other poinsettias, 97/176 and cv. V10 Amy red. Seed was collected and sown according to standard practices and the resulting plants were observed.

Cytology of embryo and endosperm derived plants

Mitotic chromosome counts were performed on shoot and root meristems as described in Appendix 3.

Results

Plants within each plant type (embryo or endosperm derived) were visually identical and were therefore considered as clones of the initial single shoots developed from either the embryo or endosperm. It could be assumed that they were not derived from more than one regenerative *in vitro* event.

Vegetative characterisation

The phenotype of embryo and endosperm derived plants is shown in Figure 7.3.1. Embryo derived plants were similar to most normal seedlings derived via intraspecific pollination. Endosperm derived plants possessed 'zigzag' stems, smaller and rounded leaves, swollen nodes, less root growth and a slower rate of vegetative growth. Significant differences were found between the two plant types for node number and height (Table 7.3.1) with endosperm derived plants being significantly shorter with fewer nodes.

Table 7.3.1. ANOVA for effect of plant type (endosperm or embryo derived) on plant height and node number with means and standard errors

		Height (mm)		Node number	
	n	Mean	SE	Mean	SE
Endosperm derived	6	243	14	16.2	0.40
Embryo derived	6	376	7.4	19.7	0.21
Source	df	Mean square			
Line	1	53067***		36.75***	
Error	10	729		0.617	

*, **, ***: $p < 0.05, 0.01, 0.001$ respectively.

Reproductive characterisation

Embryo derived plants reached anthesis after 8 weeks under the short photoperiod environment and appeared similar to most normal seedlings derived via intraspecific hybridisation. Hermaphrodite cyathia were produced and 7 seeds were produced from 5 pollinations with 97/176.2. All self pollinations (5) aborted within 28 days and resulted in no ovary swelling or seed set. Endosperm derived plants developed bracts and cyathia after approximately 9 weeks, but cyathia were small, male and female sterile (Figure 7.3.3). Figure 7.3.2 displays the two plant types after growth under the short photoperiod environment.

Cytology

Obtaining cells with well spaced non-overlapping chromosomes was difficult, as explained previously (Experiments 7.1 and 7.2). Counts of chromosomes from cells of embryo derived plants were approximately $2n = 28$, but none could be counted with absolute certainty. Counts of endosperm derived plants were either $2n = 36$ or $2n = 39$, indicating aneuploidy. (Figure 7.3.4)

Figure 7.3.1. Vegetative phenotype of endosperm (left) and embryo (right) derived plants

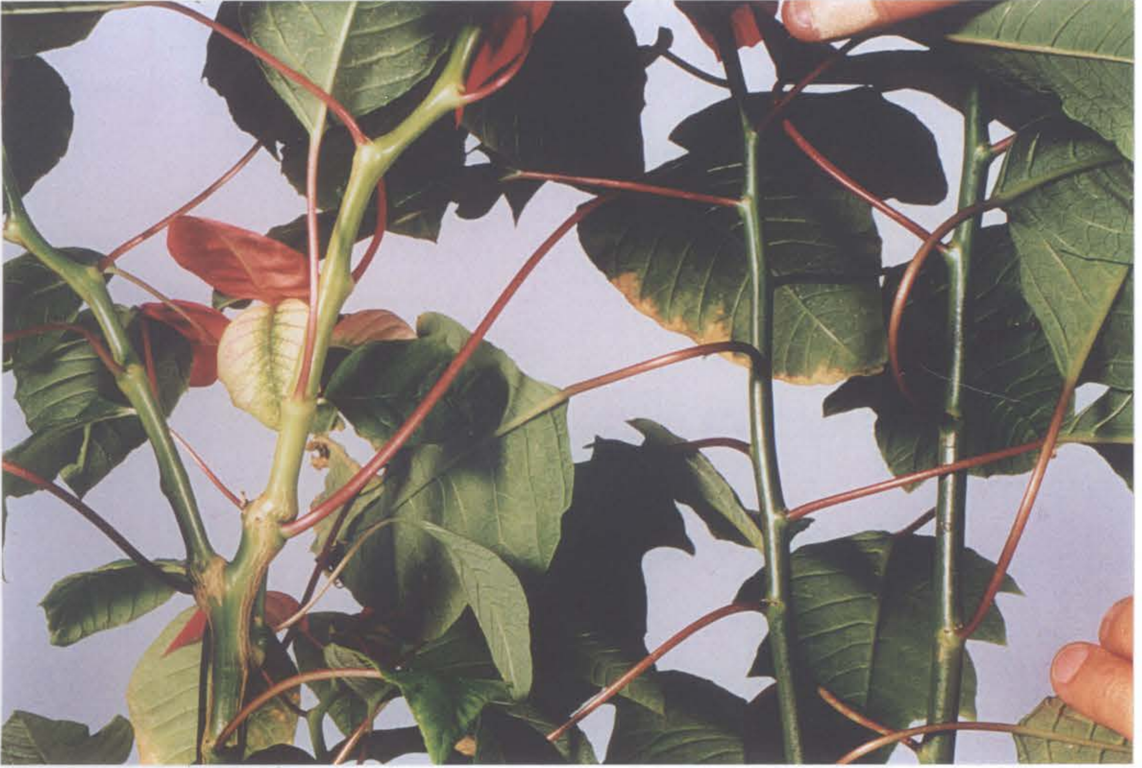
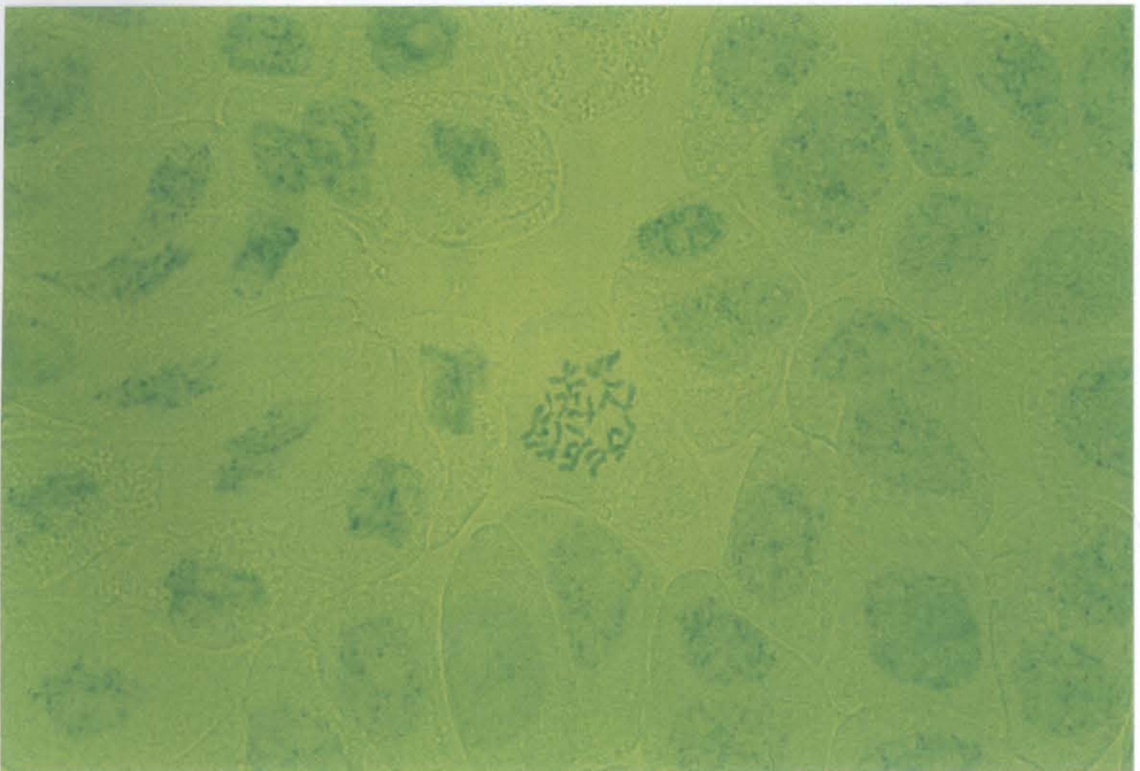


Figure 7.3.2. Reproductive phenotype of endosperm (left) and embryo (right) derived plants

Figure 7.3.3. Reproductive development of endosperm (left) and embryo (right) derived plants

Figure 7.3.4. Mitotic root cell of endosperm derived plant displaying 2n = 39 chromosomes at metaphase



Discussion

The production of plants from endosperm is a novel method for production of triploid plants in poinsettias. However, the plant regenerated from endosperm in this study possessed less than the expected 2n = 42 chromosome complement. Chromosome numbers were most frequently recorded as 2n = 36 or 39, indicating aneuploidy. This technique may therefore enable the development of plants with unusual chromosome numbers. This is the first report of endosperm derived plants of poinsettias.

Seeds were readily produced from embryo derived plants. It is probable that these were of the chromosome complement 2n = 28, as indicated but not verified with certainty by cytology. Endosperm derived plants displayed a lack of reproductive structures, which could be expected due to the altered chromosome number. Endosperm plants were slower growing, and shorter than embryo derived plants, and are therefore not especially useful for commercial production.

Chapter 8. General discussion

This project was undertaken with the aim of producing dwarf, ornamentally desirable poinsettias. Several experiments were conducted to investigate various methodologies that could be used to develop genetically dwarf cultivars. Two methods, namely intraspecific and interspecific hybridisation were considered as optimal for developing dwarf germplasm for the project. However, prior to any crossing program implementation, the influence of external factors such as viruses (PnMV, PnCV) and a phytoplasma (PoiBI), which were thought to infect the majority of poinsettia cultivars, needed to be evaluated.

During the study of PnMV, PoiBI and PnCV in poinsettias, it was found that PnMV infected all commercial cultivars present in Australia. In addition, PoiBI was found to infect all commercial cultivars tested and this is the first report of the presence of this phytoplasma in Australia. PnCV was identified in all commercial cultivars tested and this result is the first report of this virus in Australia. Therefore, it was shown that all three infective agents are present in current commercial cultivars grown in Australia. Considering these three infective agents could have implications on breeding, height and morphology of poinsettias, various techniques were employed to gain an understanding of the mode(s) of transmission of each infective agent and to develop material of a number of cultivars free of infective agents.

The transmission of PnMV and PoiBI had been examined by previous researchers (both graft transmissible, but not seed transmissible) therefore, the mode of transmission was investigated briefly to verify and confirm published results under Australian conditions. However, PnCV transmission had to be ascertained. PnCV was found to be both graft and seed transmissible. The implication of these results is that it would be difficult to control the spread of PnCV, as any seed derived poinsettias could be infected with this agent. Although PnCV produces no observable symptoms, a minor impact on poinsettia morphology cannot be ruled out and should be further investigated with increased replication in further experiments. Further studies are required to develop PnCV-free poinsettias and compare these with PnCV infected plants of the same genotype. A source of PnCV infected germplasm without PnMV and PoiBI is available at present, and plants free from all three infective agents (PnCV, PnMV and PoiBI) are already available (seed derived). These materials were developed during this study. Therefore, PnCV infected and non-infected plants of the same genotype could now be developed via grafting, to gain a better understanding of the effects of PnCV on poinsettia morphology.

It is considered desirable to produce poinsettias that are virus-free (Bradel *et al.* 2000) therefore, further studies should be conducted to produce poinsettias free of PnCV and PnMV. A difficulty that is encountered when attempting to produce virus-free poinsettias via the use of pathogen removal techniques (such as meristem culture and heat treatment) is that PoiBI is also often easily removed as well. PoiBI is a beneficial organism as it increases branching, and thereby the number of harvestable cuttings on stockplants of commercially produced plants. However, a source of poinsettia carrying PoiBI but not PnMV was developed during the study and this is one step towards producing virus-free but PoiBI infected poinsettias.

Further studies were conducted to investigate the effects of PoiBI and PnMV, on height and morphology because their singular and combined effects are a subject of controversy. The effects of PnMV on plant height and morphology were limited. The most common morphological alteration was the presence of an angular mosaic on leaves produced at temperatures below 20°C. A synergistic effect was observed when this pathogen, in conjunction with PoiBI, infected cv. Freedom red plants. Plants with the PnMV and PoiBI infection had significantly greater stem diameter than all other infection types (Experiment 3.4). The presence of PnMV alone in cv. V10 Amy red resulted in an approximate 10% reduction in bract size compared to non-infected plants. Therefore, considering the effects of this virus could be both advantageous (increased stem diameter) and deleterious (angular mosaic, reduced bract size) removal of PnMV from current commercial cultivars requires further consideration based upon its influence on individual genotypes.

The main effect of PoiBI was to significantly reduce plant height in cv. Freedom red in three consecutive commercial production experiments conducted throughout the year. However, PoiBI infection did not significantly reduce height in one experiment performed with cv. V10 Amy red. In addition, all four selected short lines grown with and without PoiBI in Experiment 4.4 were not significantly different for height at the completion of this experiment. It could be suggested that a genotype x phytoplasma interaction existed because PoiBI had a lower effect on reducing plant height in genetically short cultivars (the four elite lines were selected for short height and cv. V10 Amy red is also considered short). Cultivar Freedom red is considered taller than cv. V10 Amy red, and had a significant response to infection with PoiBI. Other symptoms, such as increased branching and rounded leaves and bracts resulted from PoiBI infection in all lines/cultivars. In addition, PoiBI significantly improved post-production performance in cv. Freedom red, and appeared to do the same in

cv. V10 Amy red. Improvement of post-production performance is desirable to increase shelf-life.

Considering PoiBI consistently induced branching, frequently decreased plant height, and improved post-production performance, alteration of endogenous phytohormone levels was thought to be a likely cause of these changes. Results from the study in Experiment 3.5 demonstrated that this was in fact the case. PoiBI infection resulted in reduced IAA concentration and increased concentration of several CKs at the first sampling. The mechanism by which PoiBI infection resulted in these alterations in hormone levels is unknown, but could be due in part to utilisation of IAA by PoiBI, production of CKs by PoiBI, interaction of PoiBI on enzymes involved in the production of CKs and IAA, or more directly upon genes coding for the production of these compounds. Further studies are required in this area and could include a time series experiment for hormone testing, which could identify precisely the time of maximum CK and minimal IAA presence in infected plants. Data from such studies could be useful in establishing a CK spray regime, where non-infected poinsettias could be promoted to develop higher numbers of branches as in a PoiBI infected plant by a single spray application. Alternatively, IAA inhibitors could be utilised alone or in conjunction with CKs. Plants could also be transformed with a gene coding for CK or an IAA inhibitor. Branched, non-PoiBI infected plants produced via such a method would negate the need for PoiBI infection. Currently, PoiBI infection is a mandatory requirement to obtain good branching resulting in a high number of cuttings harvested from stockplants.

PoiBI may also be unevenly distributed within each plant and could result in variability within a commercial cultivar population. It is also heat labile at temperatures above 37°C. Therefore, reduced morphological symptoms could result in plants grown at high temperatures. However, no reduction in branching or morphology was observed in the cv. Freedom red study performed during summer with a temperature range from 16 - 46°C.

Seed production and fertility could also be affected by PoiBI. A preliminary study in Experiment 4.1 showed that a difference of approximately 10% was found for seed production efficiency between two lines infected (lower seed production) and non-infected (higher seed production) with PoiBI. Further work needs to be conducted in this area to determine conclusively if PoiBI influences fertility across a number of cultivars/lines and cross combinations.

The phytoplasma PoiBI, at present, can only be maintained *in vivo* in poinsettia plants or the wild species *E. cornastra*. If methods to isolate and culture PoiBI could be established then injection of this agent into poinsettia seedlings could reduce the cost of cultivar production. In addition, sap of PoiBI infected poinsettia plants could be injected into non-infected plants to transfer PoiBI. Further studies are required in this area to develop faster, more efficient methods of PoiBI inoculation, which reduce the labour and material cost of grafting to transmit PoiBI.

It would also be desirable to investigate if phytoplasmas other than PoiBI could infect poinsettia and related species, beginning with those most closely related and present in Australia. Such studies could result in novel poinsettias being produced, perhaps due to different morphological changes induced and elucidate the relationships between closely related phytoplasmas on hosts. Further studies on the infection of other *Euphorbia* species with PoiBI are also necessary, as PoiBI infection may result in desirable morphological alteration.

Intraspecific hybridisation was investigated to determine if dwarf poinsettias could be produced through intermating the available cultivar genepool. The primary goal was to produce poinsettias within a set height criteria, without growth regulator application during all seasonal periods of the year in Australia. Initially, the breeding system and fertility of several cultivars and lines was investigated to efficiently plan the breeding program. Results from these studies showed many cultivars were influenced by mutational load and in part, by IBD.

In the population of cultivars and lines studied, major genes for short height appeared to be present in cultivars such as Freedom red and V10 Amy red. When segregation analysis was performed on progeny from Freedom red and cv. V10 Amy red derived from self pollination, the possibility of several genes of minor effect determining genetic plant height in these two cultivars was noted. Height could also be affected by IBD, and/or mutational load, due to reduced vigour. IBD can result in reduced seed production and acts similar to an incompatibility mechanism.

A method for testing female fertility was established using wild-type tester pollen and could find an application in commercial breeding projects for testing new germplasm for seed production capability. Such data is crucial to the development of genetic recombinants via intraspecific hybridisation. The wild-type plant is ideal for such investigations because it has

no (human-induced) mutational load and has a reduced chance of producing progeny homozygous for deleterious genes (from IBD) due to a distant relatedness with commercial cultivars.

In the study of intraspecific hybridisation, two plants were selected from 216 based upon ornamentally excellent phenotype and on height and node criteria. In addition, numerous plants were selected based solely upon good or excellent ornamental appeal. All of these plants were then infected with phytoplasma and grown as a commercial crop. This showed that many of the lines selected based on ornamental appeal were shorter than cv. Freedom red and cv. V10 Amy red when grown during summer, but not when initially selected as single plants. This result should be noted prior to establishing any selection procedures in future experiments. That is, it may be beneficial to infect seedlings with PoiBI and harvest cuttings from these plants for use in the first single plant selection process. The increased replication could result in improved robustness of subsequent selection. The process of PoiBI infection would be labour intensive if performed via grafting, but would allow selection of plants based upon their final appearance. The period from selection to stock build-up would also be shortened. Further development of increasing efficiency in this area is required.

Interspecific hybridisation was performed as a third option for inducing dwarfness in poinsettias. There are many opportunities within the closely related species for increasing the *E. pulcherrima* gene pool. Novel poinsettia-like plants were produced from the cross between *E. pulcherrima* and *E. cornastra*. These hybrids grew and flowered very quickly, which could reduce the production period and increase efficiency/income for growers. Further development and selection among these plants is required to improve post production performance. Assessment of the hybrids developed, and construction of further hybrids could enable the selection of female and male fertile progeny, which is important for further breeding. Crosses could be performed with poinsettias to improve post-production performance and to develop colours other than pink and increase bract size, if desired. In addition, it may be desirable to re-assess the original *E. cornastra* and *E. pulcherrima* parents, and repeat crosses of more desirable combinations (e.g. *E. cornastra* plants that have larger bracts, or flower more profusely, etc).

Crosses with *E. cornastra* could be expected to produce progeny that flower under a long photoperiod, because *E. cornastra* flowers under both long and short photoperiods. Partial long photoperiod flowering was observed for some hybrids, but full bract colour development

did not occur. If poinsettia-like plants could be established that flower under long photoperiod, market expansion could result through the virtue of year-round production.

Germplasm collections are necessary to enhance and widen the genepool for interspecific hybridisation. Some species deserve breeding on their own merit (intraspecific) and this is suggested as a means to develop new poinsettia-like plants. Further species should be collected and utilised to produce novel interspecific hybrids with poinsettias. Different plant forms and flowering times could be developed. One important limitation to the use of some *Euphorbia* species in Australia is their classification by the Australian Quarantine and Inspection Service. Many *Euphorbia* species are considered as weeds, as such, it is desirable to focus on those species that are allowed entry into Australia, such as *E. cornastra*.

In addition to the three primary studies conducted, triploid production was investigated. Most of the traits of the female 4x parent were maintained in the progeny. Therefore, further crossing between desirable 4x and 2x poinsettias is suggested to develop new cultivars with benefits of genes from both ploidy types (cultivars of different ploidy).

Tetraploids were produced via colchicine application, and these could be used for the development of triploids, or for direct tetraploid seed production when intermated. Considering crosses between Freedom red and V0 Amy red in the diploid condition resulted in some very good progeny, crosses between these two cultivars at the tetraploid level could be investigated to obtain an understanding of seed production efficiency and the extent of desirability of such progeny. In addition, tetraploid poinsettias may be useful for crossing with tetraploid species (such as *E. hormorrhiza*, triploid progeny were produced when crossing between this species and *E. cyathophora* with 2n = 28 chromosomes). Producing tetraploids of newly synthesised hybrids could restore fertility.

Future directions

In future breeding studies it is suggested that pedigree data be used or where this data is not available, AFLP, RFLP or other molecular techniques are used to determine relatedness. Such information will aid in the structuring of breeding programs to improve seed production and assist in harnessing heterotic effects.

Efforts should be made to develop inbred lines fixed for certain desirable characters, which will assist in directed crossing for set criteria. Intermating inbred lines could also result in

heterosis. Development and selection of inbred lines will also remove deleterious genes from the genome, which could result in improved seed set from crosses.

To breed dwarf, vigorous, ornamentally desirable poinsettias the following protocol could be followed. i. - intermate short desirable plants with good vigour and distant relatedness, ii. - grow as single plants and infect each with PoiBI, iii. - apically decapitate to induce branching and re-instate juvenility to reduced the effects of variability in germination period and PoiBI infection, iv. - take several cuttings from each plant and trial as per commercial practices with cultivar controls infected with PoiBI and v. - select, based upon mean ornamental appeal score, height and node number compared to controls, the best plants.

Another avenue that could be exploited is the development of seed lines from wild type crosses or highly fertile lines. If doubled haploids could be produced and combining analysis performed, an F_1 hybrid seed produced poinsettia cultivar could be produced. The establishment of such a system would negate the need for growers to have stock plants for cutting production, thereby increasing growing space and reducing establishment and maintenance costs. Special propagating facilities (misting, bottom heat, etc) would not be required. Because the seed is an F_1 hybrid, growers could not successfully utilise seeds developed from the F_1s due to non-uniformity resulting from segregation. Seeds are also easier to handle (no sap). In addition, the seed producer would know exactly how many seeds have been sold, and therefore the royalty that is required to be received from growers. If growers decided to perform vegetative propagation of the F_1 they would require a large number of stockplants because the seeds would not be infected with phytoplasma, thus making vegetative propagation unattractive. Another benefit of a seed cultivar is that seeds could be planted directly into their final pots (similar to direct propagation of poinsettia cuttings which can be performed).

Another potential method to speed the development of new cultivars would be to utilise embryo rescue. Poinsettias require from 4 - 5 months to produce ripe seeds from a cross. If this could be reduced to 1 - 2 months a significant improvement in speed of production would result. This technique may be of greater cost than conventional production, but may be useful for speeding crucial crosses or crosses that fail to fully ripen.

Selections made in Chapter 5 require post-production testing, followed by trials of selected lines (red and white) in various regions and times of the year prior to being accepted as

deserving of plant protection (PBR or plant patenting) and distribution within Australia and overseas. Following the determination of the best red plants, mutation breeding could be undertaken to produce a colour series (pink, white, marble, jingle bells) from the initial red plant. If the initial red plant is heterozygous for red bract colour, this could make the process easier as only a single strand mutation would be theoretically required, compared to a double strand mutation of the same locus coding for anthocyanin production.

The diversity of morphology in selected lines could be increased through intermating newer cultivars such as cv. Winter Rose, cv. Carousel, cv. Monet, etc., and thereby expand the market. In addition, continual trialing of any new cultivars released should be undertaken to determine if these are desirable for use in reducing plant height or heat tolerance in Australia.

Considering somatic embryos were produced from the *E. pulcherrima* x *E. cornastra* hybrids, it may be easier to develop a somatic embryo propagation system for this crop compared to normal poinsettias. Such a system is highly space efficient and has many benefits over conventional propagation systems.

Development of a rapid method of PoiBI infection of seedlings, possibly via sap injection or PoiBI culture injection is required to speed cultivar development. In addition, a source of PoiBI that is both PnCV and PnMV-free is desirable and should be developed.

The usefulness of tetraploids in breeding new cultivars should also be assessed, as these could have the desirable attribute of thicker stems, which could result in minimal branch breakage.

It could be concluded that avenues for the development of dwarf poinsettias are numerous. Therefore, there are many options for future breeding of dwarf poinsettias, and for developing poinsettias suited to growth under high temperature environments. In addition, development of new and different poinsettias and poinsettia-like plants could expand the market and result in increased grower and seller profitability with concurrent consumer satisfaction.

Chapter 9. References

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Appendix 1. NFP media composition

Components	
coir fibre	22.5%
sphagnum peat	7.5%
composted pine bark fines	55%
active Mineral	15%

Base Fertiliser Added	**Kg/m^3**
ferrous sulphate	0.3
potassium nitrate	0.05
micromax	0.75
lime	1.7
dolomite	2.9
fine superphosphate	1.5
nutricote orange*	2.0
wetting agent	0.7
air filled porosity	15%
water holding capacity	62%

*Nutricote No. 7 orange for flowering plants. Controlled release fertiliser. Manufactured by: Chisso Asahi Fertiliser Co. Ltd. Tokyo, Japan. Distributor in Australia: Arthur Yates and Co. Limited. 244 - 254 Horsley Road, Milperra NSW 2214.

Analysis of Nutricote No. 7 orange Controlled release fertiliser for flowering plants

13.0% nitrogen (N)

    7.5% nitrate nitrogen

    5.5% ammonium nitrogen

5.7% phosphorus (P)

    4.4% citrate soluble

    1.3% water soluble

9.1% potassium (K)

6.0% calcium (Ca)

    As phosphates

5.7% inert coating ingredients

Soil temperature and nutrient release time corresponding to the release of 80% nitrogen

Soil temperature	10°C	15°C	20°C	25°C	30°C	35°C
Release time	145 days	110 days	90 days	70 days	55 days	45 days

Suggested application rates:

Top dressing

150 mm pots 4 g (medium) 8 g (high)

Incorporated into mix

Per cubic metre 2kg (medium) 4kg (high)

Particle Size Analysis

Sieve (mm)	% of total
4.75	9.5
2.36	26.6
Coarse	**36.1**
1.18	21.8
0.60	19.0
Medium	**40.8**
0.30	12.9
0.15	6.5
0.075	2.8
pan	0.9
Fine	**23.1**

Appendix 2. Osmocote®Plus fertiliser specifications

Scotts Osmocote®Plus Controlled Release Fertiliser, 3 - 4 month 15 + 4.8 + 10.8 + 1.2 Mg + all essential trace elements (B, Cu, Fe, Mn, Zn)

15.0% nitrogen (N)

    7.7% nitrate nitrogen

    7.3% ammonical nitrogen

4.8% phosphorus (P)

    soluble in neutral ammonium citrate and water

    3.6% P water soluble

10.8% potassium (K)

    Water soluble (Chloride free)

3.0% sulphur (S)

    present as sulphates

1.2% magnesium (Mg)

0.02% boron (B)

    present as boric acid

0.05% copper (Cu)

    present as copper sulphate

0.4% iron (Fe)

    0.20% present as iron sulphate

    0.20% present as iron EDTA

0.6% manganese (Mn)

    present as manganese sulphate

0.02% molybdenum (Mo)

    present as sodium molybdate

0.015% zinc (Zn)

    present as zinc sulphate

5.0% organic residue coating

    (vegetable oils)

Osmocote®Plus is effective in all commonly used substrates and the release of nutrients is not influenced by pH, salt concentration or water quality.

Soil temperature and longevity

Average soil temperature	Longevity
16°C	4 - 5 months
21°C	3 - 4 months
31°C	1.5 - 2 months

Suggested application rates

Crop	Light feeding crops	Heavy feeding crops
Flowering crop	1.0 - 2.5kg/m3	2.5 - 4.0 kg/m^3*
Pot plants	1.5 - 2.0 kg/m3	2.0 - 4.0 kg/m^3
Container/nursery stock	1.5 - 3.5 kg/m3	3.5 - 5.0 kg/m^3

* Rate used in experiments, i.e. approximately 10 g / 150 mm diameter pot

Appendix 3. Cytology methodology

Root tips. Cuttings were struck according to conventional propagation practices. Roots were not allowed to desiccate. Healthy, white, rapidly growing 5 mm long root tips were selected.

Shoot tips were taken from uppermost shoots of vigorously growing plants. Immature leaves were removed.

Cyathia were collected from vigorously growing flowering plants. Cyathial walls were removed.

Plant material (root tips, shoot tips and cyathia) was collected between 7:00 - 8:00am in summer months, prepared as described above and then placed into test tubes filled with 4 ml of ice cold 3:1 ethanol : glacial acetic acid. Test tubes were immersed in ice water in a beaker and maintained at 4°C after collection. After 5 hours, (or up to 24hrs) all materials were transferred to 1N HCL and treated as follows.

1) Preheat 1N HCL for 1 min.
2) Add plant material and maintain @ 60°C for 16 - 17 mins.
3) Remove to Feulgen stain.
4) Stain for several minutes to approximately 1hr @ 21°C, until tips turn purple.
5) When tips/cyathia are purple, perform chromosome counts.
6) For root and shoot tips remove very small amounts of tissue, by use of a pointer to stroke out meristematic cells while avoiding epidermal layers. Maceration may not be necessary. If HCL treatment has been successful, tips should be very soft and easily break up, use only the smallest portion and remove the root cap. For cyathia, remove anthers below the least mature anther showing yellow pollen until the desired stage is found.
7) Dry off excess stain, add a drop of 45% acetic acid and place on a cover slip.
8) Tap cover slip gently with the end of a pencil (if necessary) to break apart cells underneath.
9) Firmly press slide between cardboard or folded filter paper (5 - 10sec).
10) Heat over a flame for 1 - 2 sec if required.

Storage of root tips was not successful, either freezing or otherwise.
Blurry, lighter staining chromosomes result from shoot tips compared to root tips.

Appendix 4. Details of species in *Euphorbia* subgenus *Poinsettia*

Description, location, cytology, height and alliance of species in *Euphorbia* subgenus *Poinsettia* (Mayfield 1997)

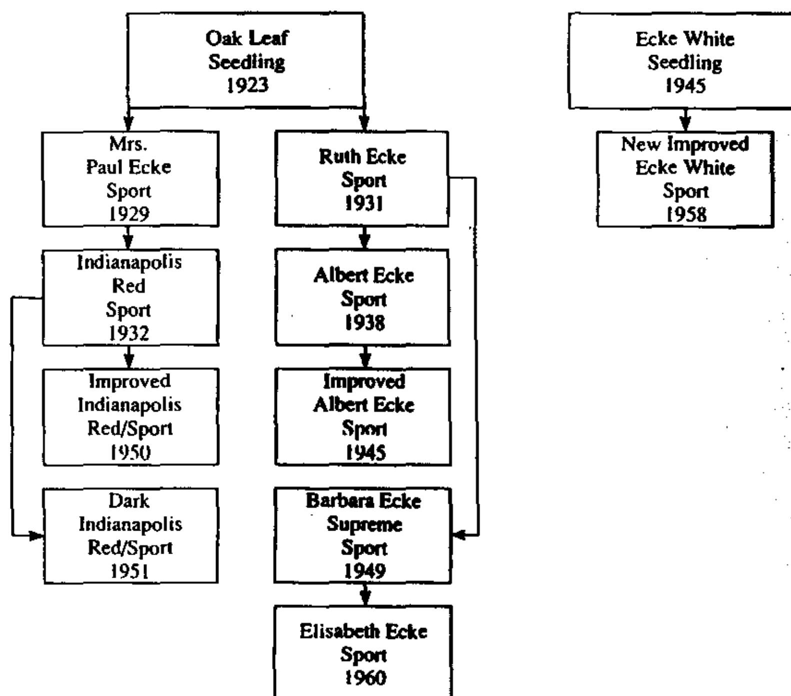
Species	Habit (Annual/Perennial/ Tuberous)	Location	n	2n	Height and notes	Alliance D = Dentata P = Poinsettia
*Euphorbia abscondita**	A	Baja California			400 mm	P
Euphorbia colorata	PT	Mexico			200 – 400 mm. Bright scarlet bracts	P
Euphorbia cornastra	P	Mexico	14	28	2 m. White bracts	P
Euphorbia cuphosperma	A	Mexico, Arizona	28	56 (?)	200 – 800 mm	D
Euphorbia cyathophora	A	Eastern U.S.A., Mexico, central America, Venezuela, Ecuador	14, 28	28, 56	250 – 1800 mm	P
Euphorbia davidii	A	U.S.A., Mexico, Argentina	28	56	250 – 700 mm	D
Euphorbia dentata	A	U.S.A., Mexico	14	14, 28	150 -700 mm	D
Euphorbia elliptica	A	Chile, Peru, Lima, Bolivia			150 – 650 mm	P
Euphorbia heterophylla	A	American tropics, subtropics. Introduced widely in old world tropics	14	28, 56	500 - 1300 mm	P
Euphorbia hormorrhiza	PT	Mexico	14	28	300 – 750 mm	P
Euphorbia kurtzii	A	Paraguay, Argentina, Bolivia			300 - 500 mm	P
Euphorbia pentadactyla	A	Bolivia, Argentina	28	56	250 – 750 mm	D
Euphorbia pinetorum	P	Florida			250 – 500 mm	P
Euphorbia pulcherrima	P	Guatemala, Mexico	14, 15, 21, 28	21, 26, 28, 30, 42, 56	10 m	P

Description, location, cytology, height and alliance of species in *Euphorbia* subgenus *Poinsettia* (Mayfield 1997)

Species	Habit (Annual/Perennial/Tuberous)	Location	n	2n	Height and notes	Alliance D = Dentata P = Poinsettia
Euphorbia pumicicola	A	Baja California, Mexico			80 – 750 mm	P
Euphorbia radians	PT	Mexico			100 – 350 mm	P
Euphorbia radians var. *radians*	PT	Mexico				P
Euphorbia radians var. *stormieae*	PT	Mexico				P
Euphorbia restiacea	PT	Mexico	14	28	500 - 1300 mm. White bracts	P
*Euphorbia richardsonii**	P	Mexico	14	28	200 – 400 mm	P
*Euphorbia schiedeana**	A	Guatemala, Mexico	14	28	300 – 1200 mm	D
Euphorbia strigosa	PT	Mexico			150 – 900 mm. Similar to E. colorata.	P
*Euphorbia subilsae**	A	Argentina	28	56	100 – 450 mm	D
*Euphorbia tiarata**	PT	Mexico			400 – 700 mm	P
*Euphorbia tubadenia**	A	Mexico			200 – 500 mm	D
Euphorbia zonosperma	A	Brazil			to 1500 mm	P

* Indicates the species has not been published outside of Mayfield (1997).

Appendix 5a. Pedigrees of prominent commercial cultivars from 1923 to 1960 (Ecke *et al.* 1990)



Appendix 5b. Pedigrees of prominent commercial cultivars from 1963 to 1990 (Ecke *et al.* 1990)