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Developing resistance to whitefly in poinsettia (Euphorbia pulcherrima) using Agrobacterium-mediated transformation

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DEVELOPING RESISTANCE TO WHITEFLY IN POINSETTIA (*Euphorbia pulcherrima*) USING *Agrobacterium*-MEDIATED TRANSFORMATION

By

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DEVELOPING RESISTANCE TO WHITEFLY IN POINSETTIA

(*Euphorbia pulcherrima*) USING *Agrobacterium*-MEDIATED

TRANSFORMATION

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The broad objective of this research was to develop transgenic poinsettia that express tryptophan decarboxylase (TDC) capable of protecting poinsettia against whitefly. An effective and efficient *in vitro* micro propagation and proliferation technique of poinsettia 'Prestige Red' was successfully developed in this study and this protocol can be used for potential development of transgenic poinsettia. Poinsettia 'Prestige Red' was successfully infected by *Agrobacterium rhizogenes* producing hairy roots at the site of infection. Investigations of more effective PGR concentrations are necessary in order to develop transgenic poinsettia through hairy roots. Stem disks of poinsettia 'Eckespoint Pollys Pink' developed into somatic embryos when they were transformed by *A. tumefaciens* harboring TDC. *A. tumefaciens*-mediated transformation of poinsettia through somatic embryogenesis is cultivar dependent. Additional research into more effective PGR combinations, antibiotic concentrations and antinecrosis

chemicals is required in order to develop transgenic poinsettia harboring TDC through somatic embryogenesis using *A. tumefaciens*.

DEDICATION

To my parents

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CHAPTER 1

INTRODUCTION

Euphorbia pulcherrima Willd. ex Koltz, poinsettia, is one of the most important potted flowering plants in the United States. Sales of poinsettia were rated number one among all flowering potted plants in the United States during the past decade (Pickens et al. 2005). It is the most popular plant during the winter holiday season and is often referred to as winter rose and Christmas star. The correlation of poinsettia bract colors and leaves, red and green, make this a popular plant for use during the holiday season.

The whitefly, *Bemisia tabaci*, belonging to family Aleyrodidae, is a serious primary pest of several cropping systems worldwide and represents a major threat to horticulture (Head et al. 2004). A new strain of *B. tabaci* named 'B' biotype is now scattered worldwide and the occurrence of 'B' biotype has strengthened the importance of its economic impact (Lin and Ren 2005). The 'B' bio-type, which was later named as a new species (i.e. *Bemisia argentifolii*), is more resistant, more cold tolerant, develops faster and is five times more prolific than the 'A' biotype (i.e. *Bemisia tabaci*) (Heather 2000). *Bemisia argentifolii* is characterized by a broad host range and especially by an association with poinsettia, causing distinct damage through direct sap-feeding, honeydew-associated problems, and phototoxic disorders (Lin and Ren 2005). Significant economic losses have occurred due to geminiviruses transmitted by whitefly (Lin and

Ren 2005). Therefore, development of resistant poinsettia varieties against whitefly attack would be important for integrated pest management.

Though there are several whitefly control measures, including chemical control, biological control and cultural control, each method has drawbacks. The eradication of *B. tabaci* populations is difficult to achieve as chemical insecticides often vary in effectiveness and also use of pesticides is not effective especially because of the development of resistance among biotypes of whitefly (Head et al. 2004). Whiteflies proceed to be significant pests of poinsettia in the Northeastern United States, even following the development of more effective pesticides in the mid 1990s (Van Driesche et al. 2002). The major problem with biological control methods of whitefly is the high cost. Further, release of insect parasitoids such as *Encarsia formosa* may lead to the decrease in pest populations but the use of these agents alone is improbable to attain total pest mortality and would be prohibitively expensive (Head et al. 2004). Cultural control alone is not effective enough to avoid economic loss of the crop especially with commercial poinsettia production. Development of whitefly resistance in poinsettia through genetic transformation would be highly beneficial.

Insect choice of a host plant for feeding and reproduction starts with the sensing of structural and metabolic constituents of the plant (Thomas et al. 1995). Previous research has demonstrated that plant alkaloids act upon insect recognition, feeding, and oviposition interfering with the insect's life cycle and causing reduction in insect population (Thomas et al. 1995; Smith et al. 2006). For example, the effect of tryptophan decarboxylase (TDC) and tryptamine on whitefly reproduction was investigated by Thomas et al. (1995) who found that transgenic tobacco expressing TDC had a 97%

reduction in whitefly reproduction. TDC, a cytosolic enzyme, catalyzes the terpenoid indole alkaloid (TIA) biosynthetic pathway by decarboxylation of L-tryptophan to produce tryptamine which is an important protoalkaloid (Di Fiore et al. 2002). Current levels of tryptamine in poinsettia are estimated at 5 µg per g leaf fresh weight: if concentrations were increased as much as 10 fold, poinsettia may be protected from whitefly (Smith et al. 2006). The objectives of this study were:

- to develop an effective and efficient *in vitro* proliferation technique for poinsettia.
- to develop a successful *Agrobacterium*-mediated transformation technique for poinsettia and to transform poinsettia with the TDC gene construct using *Agrobacterium*.

LITERATURE CITED

Di Fiore, S., Q. Li, M.J. Leech, F. Schuster, N. Emans, R. Fischer and S. Schillberg. 2002. Targeting tryptophan decarboxylase to selected subcellular compartments of tobacco plants affects enzyme stability and *in vivo* function and leads to a lesion-mimic phenotype. Plant Physiol. 129(3):1160-1169.

Head, J., A.J. Lawrence and K.F.A. Walters. 2004. Efficiency of the entomopathogenic nematode, *Steinernema feltiae*, against *Bemisia tabaci* in relation to plant species. J. Appl. Entomol. 128:543-547.

Heather J.M. 2000. Featured Creatures: silverleaf whitefly. 6 May 2009. http://entomology.ifas.ufl.edu/creatures/veg/leaf/silverleaf_whitefly.htm

Lin, L. and S. Ren. 2005. Development and reproduction of 'B' biotype *Bemisia tabaci* (Gennadius) (Homoptera: Aleyrididae) on four ornamentals. Insect Sci. 12:137-142.

Pickens, K.A., Z.M. Cheng and R.N. Trigiano. 2005. Axillary bud proliferation of *Euphorbia pulcherrima* Winter Rose™. In vitro Cell Dev. Biol.-Plant 41:770-774.

Smith, F., T.S. Chou, R. Eisenreich, J. Sanford, A. Blowers and J. Van Eck. 2006. Production of transgenic poinsettia. United States Patent 7119262. 23 December 2007. http://www.patentstorm.us/ patents/7119262.html

Thomas, J.C., D.G. Adams, C.L. Nessler, J.K. Brown and H.J. Bohnert. 1995. Tryptophan decarboxylase, tryptamine, and reproduction of the whitefly. Plant Physiol. 109:717-720.

Van Driesche, R.G., S. Lyon, K. Jacques, T. Smith and P. Lopes. 2002. Comparative cost of chemical and biological whitefly control in poinsettia: is there a gap? Fla. Entomol. 85:488-493.

CHAPTER II

LITERATURE REVIEW

Poinsettia and its significance in the horticulture industry

Botany of poinsettia

Euphorbia pulcherrima Willd. ex Klotzsch, poinsettia is a member of the Euphorbiaceae (spurge family) family and the *Euphorbia* genus (USDA n.d.). The *Euphorbia* consists of about 1,600 to 2,000 species including *E. fulgens*, scarlet plume; *E. marginata*, snow-on-the-mountain; *E. splendens,* crown-of-thorns; and several other herbaceous perennials and annuals (Ecke et al. 2004).

In the native habitat, poinsettia is a tropical deciduous shrub which grows up to 3m tall (Wilkerson et al. 1999). Flowers of poinsettia are monoclinous monoecious complimented by showy bracts for attracting pollinators. The floral morphology promotes cross pollination. During flowering, the top upper leaves, closest to apical flower cluster called the cyathium are modified into showy colorful bracts in shades of red, white, pink and maroon or combinations of these colors (Ecke et al. 2004; Kessler n.d.). In the industry, poinsettia is produced using rooted cuttings.

Poinsettia market

Since the introduction of poinsettia over 170 years ago, it has become the primary potted flowering plant produced and sold in the US with the leading producer being Paul Ecke Poinsettias, Encinitas, California since 1909 (Smith et al. 2006). With the introduction of long-lasting and earlier blooming cultivars, the poinsettia market has expanded from small scale flower shops, garden centers and nurseries to mass market outlets such as supermarkets and chain stores (Ecke et al. 2004). Poinsettia is popular primarily during the winter holiday season and dominates the ornamental horticulture market with sales starting in mid-November: the highest demand is for red cultivars then white, pink and others respectively (Ling et al. 1997). According to the national Floriculture Crops Report, about 47.5 million potted poinsettia plants were produced in the 15 states surveyed for 2007, with a wholesale value of about $181 million (USDA, NASS 2008). Global production of poinsettia has out grown hundreds of millions and is still spreading out, witness to its economic and market impact on the floral industry (Clarke et al. 2008).

Poinsettia breeding

During the mid-1950s, poinsettia breeding programs were started in several institutions including the USDA Research Center at Beltsville, Maryland and Paul Ecke Poinsettias, Encinitas, California (Ecke et al. 2004). ‘Barbara Ecke Supreme’ was the first tetraploid introduced to the trade in 1949 having sturdier stems and bracts (Ecke et al. 2004). The first long-lasting cultivar with stiff stems and good foliage was ‘Paul Mikkelsen’ and upon its introduction a new era of poinsettia began with the primary

market of poinsettia changing from cut flowers to potted plants. After introduction of 'C-1 Red' having an attractive long-lasting showy red bract in 1968, the C-1 color series ('C-1 Pink', 'C-1 Marble', 'C-1 White', 'C-1 Hot Pink' and 'C-1 Jingle Bells') was released (Ecke et al. 2004). In 1988, innovation of Eckespoint 'Lilo' with resistance to epinasty started a new trend of dark green foliage that continues to be popular in the 21st century. In 1991 the introduction of an early blooming cultivar, Eckespoint 'Freedom Red', advanced the poinsettia market to early November (Ecke et al. 2004).

Though traditional breeding is still important in the development of new cultivars, mutation breeding has been employed to develop cultivars with various bract colors (Starman et al. 1999). In recent years, breeders have introduced many new cultivars with novel or improved morphological traits such as new bract colors, larger bracts, stiff stems, free branching, earliness to flower and long lasting qualities. Other traits that breeders are selecting for include both growers' traits: greenhouse performance and crop timing and market traits: consumer appeal, consumer recognition and long shelf life (Ecke et al. 2004).

Before breeding of the interspecific hybrid 'Dulce Rosa' which can be grown for fall or black-clothed for flowering during spring or summer, all cultivars were developed from *Euphorbia pulcherrima*, an extinct species in nature (Huang and Chu 2008). Another interspecific hybrid is Prestige^{TM} Red (Eckespoint® Prestige™) which was bred by the Paul Ecke ranch in Encinitas, California. Prestige^{TM} Red is a high performance cultivar with showy red or maroon bracts and dark green leaves in a strong upright stem: It is exceptionally well branched, with extremely sturdy stems that resist stem breakage during shipping and handling (Ecke et al. 2004; Ecke.com 2008).

Whitefly and its impact on crop plants

Whiteflies are insects belonging to the family Aleyrodidae (Jones 2003). They are highly polyphagous, attacking many field crops that are economically important throughout the tropics and sub-tropics. They have recently become a significant pest of protected horticulture where intensive management practices are carried out inside greenhouses in temperate regions (Denholm et al. 1998). About 1300 whitefly species in over 120 genera have been described, but only whiteflies in the *Bemisia* (*Bemisia tabaci*) and *Trialeurodes* (*T. vaporariorum*, *T. abutilonea* and *T. ricini*) genera are virus vectors (Jones 2003).

The Whitefly, *Bemisia tabaci* or sweet potato whitefly is a serious primary pest of several cropping systems worldwide (Head et al. 2004). A new strain of *B. tabaci* termed 'B' biotype (*Bemisia argentifolii* or silverleaf whitefly) which was described in 1994 as a new species has an intrinsically high tolerance to a broad range of insecticide groups and the occurrence of 'B' biotype has reinforced its economic impact (Heather 2000; Lin and Ren 2005; Dennehy et al. 2007). The *B. tabaci* 'B' biotype is more resistant, more cold tolerant, develops faster and is five times more prolific than 'A' bio-type (i.e. *Bemisia tabaci*) and it is characterized by a wide host range including plants belonging to families: Fabaceae, Malvaceae, Solanaceae and Euphorbiaceae (Jones 2003).

Impact of whitefly in poinsettia and whitefly management in poinsettia

Whitefly (*Bemisia tabaci*) is the most serious insect pest of poinsettia resulting in particular economic concern (Ecke et al. 2004). *B. tabaci* 'B' biotype which is also called 'poinsettia' biotype is highly fertile and it causes pronounced damage to poinsettia

through direct sap-feeding, honeydew-associated problems and phytotoxic disorders (Lin and Ren 2005). Moreover, honeydew produced by whitefly promotes sooty mold and it reduces plant quality. Further, high whitefly infestation causes loss of aesthetic value of the plant and some physiological disorders: leaf chlorosis and bleaching of plant parts. In fact, significant economic loss has occurred due to whitefly-transmitted geminiviruses causing spread of viral diseases among plants (Lin and Ren 2005).

Though there are several whitefly control measures, including chemical control, biological control and cultural control, each method has its own drawback. The eradication of *B. tabaci* populations is difficult to achieve as chemical insecticides often vary in effectiveness depending on the geographical region (Head et al. 2004). Use of pesticides has become less effective due to development of resistance among biotypes of whitefly. Insecticide resistant strains appeared in 1986 in Florida and then in 1987 in Texas: since then resistance has spread rapidly throughout all 50 states (Wilkerson et al. 1999). Even after the development of more effective pesticides in the mid 1990s, whiteflies continue to be serious pests in poinsettia in the Northeastern United States (Van Driesche et al. 2002).

The major problem with biological control methods of whitefly is the high cost. When the profit margin is low, the cost of production should be minimized due to competition among many growers. Further, exposure of poinsettia to insect parasitoids such as *Encarsia formosa* may lead to the decrease of pest populations but the use of these biological control agents alone is unlikely to accomplish total pest mortality (Head et al. 2004). Cultural control methods such as sanitation and screening of greenhouses are not efficient enough to avoid economic loss of the crop: though cultural control methods

are helpful to lessen the impact of whitefly on the crop (Heather 2000). Therefore, an alternative management option for whitefly in poinsettia is necessary and development of genetically engineered (transgenic) poinsettia varieties which express polypeptides capable of protecting plants against whitefly attack would be beneficial.

In vitro proliferation of Euphorbiaceae

Members of the genus *Euphorbia* are succulent shrubs, secreting milky sap (latex) at the cut surfaces, distributed in subtropical regions in East Africa, South America, East Asia and India (Uchida et al. 2004). *Euphorbia* is rich in terpenoids and sterols used industrially as vitamins, anti-cancer drugs, insecticides and steroid compounds. Latex produced by members of Euphorbiaceae are rich in lower molecular weight hydrocarbon compounds and are similar to those now produced from petroleum (Tideman and Hawker 1982).

Several studies have been implemented to *in vitro* proliferate plants belonging to the family Euphorbiaceae (Tideman and Hawker 1982; Ripley and Preece 1986; Jakobek et al. 1986 and Uchida et al. 2004). The study of *in vitro* proliferation of *E. lathyris*, *E. peplus* and *E. tannensis* resulted in successful protocols for nodal, internodal and leaf explant propagation in the MS (Murishuge Skoog) basal medium supplemented with plant growth regulators (PGRs): cytokinine (BA- 6-benzylaminopurine) and auxin (NAA- naphthaleneacetic acid) at various concentrations. In these three species, addition of auxin (NAA) to the media suppressed shoot formation and increased callus production while the highest concentration of BA (10 μM/ without NAA) enhanced shoot formation (Tideman and Hawker 1982). In all three species root initiation was efficient with 0.5 μM

NAA in ½ MS basal medium: rooting was suppressed and callus was developed at high concentrations (5 μM) of NAA in ½ MS basal medium.

Jakobek et al. (1986) reported that *E. antisyphilitica* had optimum shoot proliferation in a medium supplemented with 0.13 μM NAA and 4.44 μM BA using shoot explants: rooting occurred with 0.49 μM IBA (indole-3-butyric acid) in a ½ MS basal medium. Ripley and Preece (1986) noted that *E. lathyris* is difficult to establish *in vitro* unless wounding is minimized and explants (shoot tips) are oriented vertically in a solid MS basal medium supplemented with BA: *E. lathyris* rooted in MS basal medium supplemented with 1 mg/L NAA. According to Uchida et al. (2004) *E. tirucalli* was successfully regenerated *in vitro* in LS (Linsmaier and Skoog) medium supplemented with thidiazuron (TDZ) and NAA using internodal explants. Adventitious buds from the internodal explants were efficiently induced on LS medium supplemented with 0.02 mg/L TDZ (Uchida et al. 2004). According to this study rooting was achieved in a LS medium containing 0.02 mg/L NAA followed by ½ LS medium without vitamins.

In summary, it was found that many members of Euphorbiaceae family have been successfully *in vitro* propagated using a combination of auxins and cytokinins. Successful *in vitro* culture for each species is varied by explant type and PGR combination.

In vitro proliferation of poinsettia

In vitro propagation is an important initial step of most genetic modification protocols in order to add valuable traits to the existing plants. Also, *in vitro* propagation of poinsettia could be an alternative strategy to fulfill the highest demand during the production season. Several studies have evaluated protocols for tissue culture of different

poinsettia cultivars through organogenesis or somatic embryogenesis (Nataraja et al. 1973; Nataraja 1974; De Langhe et al. 1974; Chu and Chao 2000; Roy and Jinnah 2001; Jasrai et al. 2003; Pickens et al. 2005 and Clarke et al. 2008). PGRs that are incorporated into the basal medium would vary depending on the poinsettia cultivar and the explant.

Organogenesis

An early study of poinsettia *in vitro* proliferation was conducted by Nataraja et al. 1973 and Nataraja 1974 using seed as explants. However, use of seed in poinsettia *in vitro* propagation is limiting since it is not a clonal propagation method. According to this study, seed grown in modified White's medium (BM/Basal Medium) with 2% sucrose and 10% coconut milk had robust seedling growth compared to BM alone. According to De Langhe et al. (1974), petiole explants from *E. pulcherrima* 'Paul Mikkelsen' produced more callus in ½ MS medium (with 3% sucrose) supplemented with NAA and 2ip (2-isopentenyl adenine): existing callus differentiated into buds with higher 2ip/NAA ratio. High NAA levels produced more grayish green calluses instead of red calluses. Internodal explants produced red calluses when the BM was supplemented with 6×10^{-5} M IAA (indole-3-acetic acid) and 5×10^{-6} M kinetin (Kn).

Roy and Jinnah (2001) obtained multiple shoot buds from stem explants (shoot tips and nodal explants) cultured on MS medium supplemented with 1.5 mg/L BA, 0.5 mg/L Kn and 15% coconut milk: repeated subculture on the same medium resulted in rapid shoot proliferation. Also, 100 mg/L casein hydrolysate increased shoot height and shoots were rooted on a ½ MS medium containing 1 mg/L IBA and 0.5 mg/L IAA. According to Pickens et al. 2005, leaf explants of poinsettia 'Winter Rose' developed into

plantlets by incorporating 8.8-13.3 μM BA and 17.1 μM IAA into the MS basal medium: presence of IAA produced adventitious shoots from existing red calluses.

In summary, auxin:cytokinin is critical in determining the type of the callus and organogenesis during the growth of an explant into a plantlet. High auxin:cytokinin ratio minimized red calluses and developed high amounts of grayish green calluses and some white calluses. Low auxin:cytokinin leads to the development of shoots whereas higher ratio of auxin:cytokinin is important for root initiation.

Somatic embryogenesis

According to Chu and Chao (2000), the most calluses induced from explants of poinsettia 'Nobel Star', 'Picacho', 'Red Splender' and 'Pepride' cultured on a full strength MS medium supplemented with 0.4 or 0.8 mg/L 2,4-D (2,4-dichlorophenoxyacetic acid) 0.2 or 0.4 mg/L BA. In this study it was further found that stems had the strongest response for callus formation among explants: leaf, petiole and stem. According to Jasrai et al. (2003) MS basal medium supplemented with 9.8 μM 2ip and 2.69 μM NAA stimulated somatic embryogenesis in red pigmented calluses. Also, maturation of somatic embryoids was achieved at reduced levels of NAA (0.54 μM) in the same medium. Clarke et al. (2008) induced callus from poinsettia 'Millenium' using internodal explants when MS basal medium was supplemented with 0.2 mg/L BA and 0.2 mg/L CPA (4-chlorophenoxy acetic acid): somatic embryos were induced with 0.3 mg/L NAA and 0.15 mg/L 2ip. The embryos matured when MS basal medium was supplemented with 0.05 mg/L BA and plantlets rooted in a ½ MS basal medium (2% sucrose) with 2 mg/L IAA.

In summary, all of the above studies primarily used several auxin and cytokinin combinations in poinsettia *in vitro* propagation through somatic embryogenesis. Different PGR treatments were used for individual poinsettia cultivars and explants.

Biotechnology in ornamental horticulture

Genetic engineering is a novel approach in breeding ornamental plants in order to enhance the commercial value of existing cultivars by enhancing specific traits such as flower color, photoperiod, post harvest longevity, fragrance, disease resistance, drought resistance, insect resistance, and reduction in leaf senescence (Chandler and Lu 2005). In contrast to traditional breeding and marker assisted selection programs, the direct integration of a small number of genes into the plant nuclear genome by genetic engineering seems to be a more effective and efficient tool to improve valuable traits (Toth et al. 2006). Genetic engineering has been successfully employed in the production of a number of ornamental crops such as blue roses, novel carnations, transgenic gladiolus and improvement of chrysanthemum (Clarke et al. 2008). To date, different transformation approaches developed transgenic ornamentals of over 30 genera (Clarke et al. 2008). Transformation methods including microprojectile bombardment (biolistic transformation), electroporesis and *Agrobacterium*-mediated transformation have been used for about 20 years in order to develop transgenic plants of different species.

Microprojectile bombardment

Intact plant tissue or protoplasts can be bombarded with microprojectile particles coated with foreign DNA using a gene gun: the particles could be Tungsten or Gold

beads and the current trend is the use of a burst of pressurized helium gas instead of the particles (Glick and John 1993). Some of the foreign DNA is washed off the particles and becomes incorporated into the host plant genome (Halford 2003). Bombarded plant cells are then developed into calluses and finally into transgenic plants harboring the foreign gene. Currently this new and promising technique is widely used in producing transgenic plants including monocotyledonous plants (such as cereals) which are difficult to transform using *Agrobacterium*-mediated transformation. Sanford et al. (1999) and Smith et al. (2006) used this technique to successfully develop transgenic poinsettia varieties having novel colors, fragrance and disease resistance. Some drawbacks of microprojectile/biolistic transformation are the tendency to generate transformants with a high transgene copy number, complex transgene loci and unpredictable silencing of the transgene (Clarke et al. 2008). Also, the original researchers who invented the gene gun technique benefit from the developed transformation protocol by collecting royalties.

Electroporesis

Plant protoplasts can be induced to accept naked foreign DNA directly by electroporesis also known as direct gene transfer or DNA-mediated gene transfer (Halford 2003). Electroporesis uses high voltage to pulse protoplasts with electricity causing pores in the cell membrane which facilitates foreign gene uptake directly into the protoplast and then integration of foreign genes into the plant nuclear genome. Protoplasts modified by foreign genes can then be developed into a callus and finally into a transgenic plant. Electroporesis can be applied not only to protoplasts but also to intact cells in pieces or in suspension. This technique has been successfully used in developing

transgenic plants including disease resistant poinsettia by Vik et al. (2001) and Clarke et al. (2006). Electroporesis as a method of producing transgenic plants has drawbacks: every plant species is not feasible for this method and the foreign gene may not express due to rearrangement of DNA.

Agrobacterium-mediated transformation

Currently *Agrobacterium*-mediated transformation is widely used in producing transgenic plants and the number of species susceptible to *Agrobacterium*-mediated transformation is increasing (Gelvin 2003). The basic principle behind *Agrobacterium*-mediated transformation is the use of the bacterium's natural mechanism of infection and modification of the plant nuclear genome, to introduce foreign genes into the host plant genome. When the bacterium successfully infects a host plant through a wound in the stem or roots, T-DNA (transferred DNA), non-chromosomal bacterial DNA, is transferred and integrated into the plant nuclear genome except the left and right border sequences of T-DNA which are necessary to transfer T-DNA into the plant genome (Ecke 2004). When the T-DNA of the bacterium is modified with a foreign gene, the foreign gene is integrated into the host plant's nuclear genome and the trait relevant to the foreign gene is expressed within the host (transgenic) plant. The bacterial T-DNA is modified by incorporating foreign genes into the T-DNA through binary vectors consisting of left and right borders of T-DNA but none of the genes present in "wild type" T-DNA (Halford 2003). The vectors can be native plasmids present in the bacterium or native (or modified) plasmids from other bacteria known to transport DNA into cells being transformed (Pickens 2004; CAMBIA 2008). The efficiency of T-DNA

transfer into a plant depends on the plant species/cultivar, type of tissue and the method of infection.

The genus *Agrobacterium* is a member of the family Rhizobiaceae. Primarily two *Agrobacterium* species are used in transformation: *A. rhizogenes* and *A. tumefaciens*. There are several *Agrobacterium*-mediated transformation studies of plants belonging to the family Euphorbiaceae including transformation of *Euphorbia lathyris* by *A. rhizogenes* (Cheetham et al. 1996), transformation of *Jetropha curcas* by *A. tumefaciens* (Li et al. 2008), transformation of *Manihot esculenta* by *A. tumefaciens* (Ihemere 2003) and also transformation of *Euphorbia pulcherrima* by *A. rhizogenes* (Vik et al. 2001) and *A. tumefaciens* (Clarke et al. 2008).

Agrobacterium rhizogenes

A. rhizogenes is a Gram-negative soil bacterium containing the Ri (root-inducing) plasmid and the bacterium causes hairy root disease in many plants. When the bacterium infects a susceptible plant, the T-DNA between left and right borders of the Ri plasmid is transferred and integrated into the nuclear genome of the plant (Ecke 2004). When Ri-TDNA is stably integrated into the plant genome, it encodes genes that direct the synthesis of auxin or increase the sensitivity of the transformed plant cells to auxin resulting in hairy roots at the site of infection (Hongwei et al. 2006). This property has been used to produce transgenic plants when a foreign gene is integrated into Ri-TDNA (Hu and Du 2006). Transgenic plants are developed from hairy roots harboring the foreign gene through the production of callus from hairy roots in a medium supplemented with various concentrations of PGRs and antibiotics (Weathers et al. 1994).

The first higher organisms transformed were tobacco plants created in 1973 using *A. rhizogenes* (Tepfer 1990). Currently most of the dicotyledonous plants and some monocotyledonous plants such as maize and rice and polycotyledonous plants are susceptible for *A. rhizogenes*-mediated transformation (Hongwei et al. 2006). The plants regenerated from hairy roots exhibit characteristic phenotypes such as wrinkled leaves, shorter internodes and altered flower morphology (Tepfer 1990), an extremely abundant and plagiotropic root system, reduced apical dominance, reduced fertility and an increased ability of leaf explants to differentiate roots in a medium free of PGRs (Hu and Du 2006). Hairy roots resulting from *A. rhizogenes*-mediated transformation are used primarily for development of transgenic plants and to produce secondary metabolites in commercial pharmaceuticals (Tepfer 1990).

Agrobacterium tumefaciens

A. tumefaciens is a Gram-negative common soil bacterium containing the Ti (tumor-inducing) plasmid and the bacterium causes crown gall disease inducing tumors near the junction of the root and the stem in many plants. Tumors are formed when the T-DNA, between left and right borders of the Ti plasmid, is transferred and integrated into the nuclear genome of the plant (Pickens 2004). Upon integration of the foreign gene into the plant genome, some of the genes of Ti-TDNA direct the production of PGRs causing proliferation of transformed plant cells and forming a tumor (CAMBIA 2008). Since only the border regions of Ti-TDNA are necessary to transfer foreign DNA into the plant genome, T-DNA can be "disarmed" by removing the tumor formation genes but not the border regions. This "disarmed" plasmid can then be used as a vector for transferring

foreign genes into plants (Pickens 2004). *A. tumefaciens* only infects dicotyledonous plants in the wild however monocotyledonous cereal crops have successfully been transformed with *A. tumefaciens* (Halford 2003).

Use of TDC gene in pest and disease resistance

High crop losses occur due to pest and disease attacks in most of the economically important crops including poinsettia even with control measures: chemical, biological and cultural control. Thus, producing a transgenic plant that expresses at least one macromolecule (e.g. protein) that confers resistant to pest and disease would be a best alternative strategy (Smith et al. 2006). Biosynthesis of the plant protoalkaloid, tryptamine from L-tryptophan (an amino acid) in *Catharanthus roseus* has drawn much attention of scientists who have been developing pest and disease resistant transgenic plants. Tryptophan decarboxylase (TDC) enzyme catalyzes the decarboxylation reaction of L-tryptophan into tryptamine and if a transgenic plant expresses TDC, tryptamine is accumulated in the plant leading to increased resistance in some species to pest and disease (Thomas et al. 1995).

Several studies demonstrated incorporation of TDC gene into agronomically and horticulturally important crops including tobacco, potato, canola, geranium, petunia, lisianthus and also poinsettia (Songstad et al. 1990; Thomas et al. 1995; Yao et al. 1995; Sanford et al.1999 and Smith et al. 2006). Songstad et al. (1990), noted that young fully expanded leaves of TDC transformed tobacco plants had 4 to 45 times greater TDC activity than did controls and the plants with highest TDC activity contained more than 1mg of tryptamine per g fresh weight, a 260 fold increase over control. Thomas et al.

(1995) extended the observation of Songstad et al. (1990) and demonstrated that tobacco plants transformed by *Agrobacterium tumefaciens* harboring TDC accumulated tryptamine, adversely affected the development and reproduction of *Bemisia tabaci*. According to Thomas et al. (1995) anti-whitefly effect(s) of tryptamine may exert during either larval and pupal development and/or adult selection of a leaf for feeding and oviposition. Thus, Thomas et al. (1995) found that transgenic tobacco expressing TDC had a 97% reduction in whitefly reproduction.

According to Yao et al. (1995) tryptamine accumulation of transgenic potato is tissue specific (i.e. leaves of transgenic potato produced high levels of tryptamine whereas tubers did not produce tryptamine) resulting in increased susceptibility of transformed potato tubers to *Phytophthora infestans*. Sanford et al. (1999) discovered that enhanced TDC expression can confer on plants resistance to a broad spectrum of bacterial, fungal and nematode phytopathogens in transgenic petunia, poinsettia, geranium and lisianthus. Transgenic petunia harboring TDC exhibited fungicidal and fungistatic activity, antibacterial effects and nematode inhibitory effects: transgenic poinsettia, geranium and lisianthus acquired resistance to *Botrytis cinerea* with higher tryptamine levels. Smith et al. (2006) demonstrated transgenic poinsettia, produced by microprojectile delivery method, which express macromolecules capable of protecting the plant against several phytopathogens and insects. Leaves of transformed poinsettia, with TDC cDNA fused to CaMV 35S promoter, accumulated 40-50 μg/g fresh weight of tryptamine which is toxic enough to repel whitefly compared to untransformed poinsettia having 5 μg/g fresh weight of tryptamine. Thus, Smith et al. (2006) found that increased

tryptamine levels in transgenic poinsettia lines exert deleterious effects on larval/pupal development in the whitefly's life cycle.

pBI121

The plasmid pBI121 is widely used as a binary vector in developing transgenic plants (Chen et al. 2003). pBI121, a derivative of binary vector Bin19, carries a kanamycin selectable marker gene that confers kanamycin resistance upon transformed plant cells (Songstad et al. 1990). According to Chen et al. (2003), pBI121 which is 14758 bp long is composed of T-DNA (6193 bp) including left and right boundary sequences. T-DNA which is the most important region of pBI121 is used to transfer foreign gene(s) into plants through direct transformation or *Agrobacterium*-mediated transformation. T-DNA is mainly composed of neomycin phosphotransferase gene (NPTII) conferring resistance to kanamycin, CaMV 35S promoter and β-Glucuronidase (GUS) gene in between left and right border sequences. Generally GUS is replaced by the foreign gene which is placed under the control of CaMV 35S. This transformation technique was successfully used in a wide range of transgenic plant protocols including transformation of TDC into tobacco by Songstad et al. (1990).

Transgenic poinsettia

As the natural variation in poinsettia has been explored extensively, use of genetic engineering is highly important in order to enhance the commercial value of poinsettia by controlling traits such as flower color, early flowering, day neutrality, free branching, dwarfness, fragrance, pest and disease resistance, and superior post harvest and shipping

qualities (Vik et al. 2001). There are few studies about genetic transformation of poinsettia: Sanford et al. (1999), Vik et al. (2001), Smith et al. (2006) and Clarke et al. (2006 and 2008).

According to Sanford et al. (1999) transgenic poinsettia 'Angelika', transformed through biolistic transformation, developed resistance to *Botrytis cinerea* with higher levels of tryptamine accumulated. Vik et al. (2001) noted that particle gun delivery on poinsettia 'Lilo Red' meristems yielded a high frequency of blue spots and *Agrobacterium*-mediated transformation resulted in low transformation rate. According to Vik et al. (2001), transformed poinsettia 'Lilo Red' by *in vivo* electroporesis resulted in high transformation rates while reducing lengthy tissue culture techniques. During the preliminary study of developing resistance to *Poinsettia mosaic virus*, Clarke et al. (2006) demonstrated transformation of poinsettia 'Millenium' apical meristems using electrophoresis: Out of 70 GFP (Green Fluorescent Protein) transformed plants, 22 were positive to GFP screening.

Smith et al. (2006) reported development of transgenic poinsettia 'Angelika', 'Freedom' and 'Jolly Red' through microprojectile mediated transformation: modified traits were inhibition of pest and diseases, growth habit modification, adding fragrance and modifying color. According to Smith et al. (2006) transgenic poinsettia encoded substances such as polypeptides, β-1, 3-glucanase, ribosome-inactivating protein, lytic peptide and plant defensin that confers resistance to disease caused by bacteria and fungi. Clarke et al. (2008) continued the preliminary study of developing resistance to *Poinsettia mosaic virus* using *Agrobacterium*-mediated transformation. As the first successful *Agrobacterium*-mediated transformation technique of poinsettia, this study

will progress the development of *Agrobacterium*-mediated transformation protocols for pest and disease resistant poinsettia varieties.

In summary, development of transgenic poinsettia against whitefly would be an effective and efficient strategy to control whitefly in poinsettia compared to other control measures. Since TDC has shown potential for pest and disease management in economically important crops including poinsettia (Songstad et al. 1990; Thomas et al. 1995; Yao et al. 1995; Sanford et al.1999 and Smith et al. 2006), anti-whitefly effects would be expected from TDC transformed poinsettia. Thus, next step should be development of a successful *in vitro* proliferation technique for poinsettia since it is a prerequisite for transformation of poinsettia with TDC gene.

LITERATURE CITED

CAMBIA. 2008. Agrobacterium-mediated transformation-Overview. 2 March 2009. http://www.patentlens.net/daisy/AgroTran/835.html

Chandler, S.F. and C. Lu. 2005. Biotechnology in ornamental horticulture. In Vitro Cell and Dev. Biol.-Plant 41:591-601.

Cheetham, R., E. Follansbee and P. Weathers. 1996. Transformation of *Euphorbia lathyris* by *Agrobacterium rhizogenes*. Acta Hort. 426:511-518.

Chen, P., W. Chen-Kuen, S. Shaw-Ching and T. Kin-Ying. 2003. Complete sequence of the binary vector pBI121 and its application in cloning T-DNA insertion from transgenic plants. Mol. Breeding 11:287-293.

Chu, C.Y. and Y.C. Chao. 2000. Somatic embryogenesis of poinsettia (*Euphorbia pulcherrima* Wild. Ex Klotzsch). Seed and Nursery 2(2):157-171.

Clarke, J.L., S.S. Klemsdal, E. Floistad, A.K. Hvoslef-Eide, S. Haugslien, R. Moe and D.R. Blystad. 2006. Genetic engineering of poinsettia with the aim of enhancing its resistance to poinsettia mosaic virus. Acta Hort. 722: 321-325.

Clarke, J.L., C. Spetz and S. Haugslien. 2008. *Agrobacterium tumefaciens*-mediated transformation of poinsettia, *Euphorbia pulcherrima*, with virus-derived hairpin RNA constructs confers resistance to *Poinsettia mosaic virus*. Plant Cell Rep. 27(6):1027-1038.

De Langhe, E., P. Debergh and R. Van Rijk. 1974. *In vitro* culture as a method for vegetative propagation of *Euphorbia pulcherrima*. Z. Pflanzenphysiol 71:271-274.

Denholm, I., M. Cahill, T.J. Dennehy and A.R. Horowitz. 1998. Challenges with managing insecticide resistance in agricultural pests, exemplified by the whitefly *Bemisia tabacai*. Philosophical Transactions: Biol. Sci. 353:1757-1767.

Dennehy T.J., B.A. DeGain, V.S. Harpold and R.J. Nichols. 2007. Biotype designations and insecticide susceptibility of southwestern *Bemisia tabaci*. Arizona Cotton Rep. (P-151):127-154.

Ecke.com. 2008. All poinsettias. 4 February 2009. http://www.ecke.com/Search/Variety_Results.asp

Ecke, P., J.E. Faust, J. Williams and A. Higgins. 2004. The Ecke Poinsettia Manual. Ball Publishing. Batavia, IL.

Geier T., A. Beck and W. Preil. 1992. High uniformity of plants regenerated from cytogenetically variable embryogenic suspension cultures of poinsettia (*Euphorbia pulcherrima* Willd. ex Koltz). Plant Cell Rep. 11:150-154.

Gelvin, S.B. 2003. *Agrobacterium*- mediated plant transformation: the biology behind the "gene-jockeying" tool. Microbiol. Mol. Biol. R.:16-37.

Glick, B.R. and E.T. John. 1993. Methods in Plant Molecular Biology and Biotechnology. CRC press, Inc. Boca Raton, FL.

Halford, N.G. 2003. Genetically Modified Crops. Imperial College Press. London, UK.

Head, J., A.J. Lawrence, and K.F.A. Walters. 2004. Efficiency of the entomopathogenic nematode, *Steinernema feltiae*, against *Bemisia tabaci* in relation to plant species. J. Appl. Entomo. 128:543-547.

Heather J.M. 2000. Featured Creatures: silverleaf whitefly. 15 May 2009. http://entomology.ifas.ufl.edu/creatures/veg/leaf/silverleaf_whitefly.htm

Hongwei, X., X. XZhou, J. Lu, J. Wang and X. Wang. 2006. Hairy roots induced by *Agrobacterium rhizogenes* and production of regenerative plants in hairy root cultures in maize. Sci. China Ser. C 49(4):305-310.

Huang, C.H. and C.Y. Chu. 2008. Methods to improve the fertility of poinsettia. Scientia Hort. 117:271- 274.

Hu, Z. and M. Du. 2006. Hairy roots and its application in plant genetic engineering. J. Integ. Plant Biol. 48(2):121-127.

Ihemere, U.E. 2003. Somatic embryogenesis and transformation of cassava for enhanced starch production. Ph.D. dissertation. The Ohio State University, Columbus, OH.

Jakobek, J.L., R.A. Backhaus and K. Herman. 1986. Micropropagation of candelilla, *Euphorbia antisyphilitica* Zucc. Plant Cell Tiss. Org. 7:145-148.

Jasrai, Y.T., K.N. Thaker and M.C. D'Souza. 2003. *In vitro* propagation of *Euphorbia pulcherrima* Willd. through somatic embryogenesis. Plant Tiss. Cul. 13(1):31-36.

Jones, D.R. 2003. Plant viruses transmitted by whiteflies. European J. Plant Path. 109:195-219.

Kessler, J.R. n.d. Poinsettia commercial greenhouse production. Auburn University. 15 September 2007. http://www.ag.auburn.edu/hort/landscape/poinset.htm

Li, M., H. Li and H. Jiang. 2008. Establishment of an *Agrobacterium*-meiated cotyledon disc transformation method for *Jatropha curcas*. Plant Cell Tiss. Org. 92:173-181.

Lin, L. and S. Ren. 2005. Development and reproduction of 'B' biotype *Bemisia tabaci* (Gennadius) (Homoptera: Aleyrididae) on four ornamentals. Insect Sci. 12:137-142.

Ling, J. T., S. Roger and N. Gawel. 1997. Identification of poinsettia cultivars using RAPD markers. HortScience 32(1):122-124.

Nataraja, K., M.S. Chennaveeraiah and P. Girigowda. 1973. *In vitro* production of shoot buds in *Euphorbia pulcherrima*. Curr. Sci. 42(16):577-578.

Nataraja, K. 1974. Morphogenesis in embryonal callus of *Euphorbia pulcherrima in vitro*. Curr. Sci. 44(4):136-137.

Pickens, K.A., Z.M. Cheng and R.N. Trigiano. 2005. Axillary bud proliferation of *Euphorbia pulcherrima* Winter Rose™. In vitro Cell Dev. Biol.-Plant 41:770-774.

Pickens, K.A. 2004. *In vitro* propagation, regeneration, attempted tetraploid induction, and *Agrobacterium*-mediated transformation of *Euphorbia pulcherrima* 'Winter Rose'(TM). Ph.D. dissertation, The University of Tennessee, United States - Tennessee. Retrieved May 21, 2008, from Dissertations & Theses: Full Text database. (Publication No. AAT 3156536).

Ripley, K.P. and J.E. Preece. 1986. Micropropagation of *Euphorbia lathyris* L. Plant Cell Tiss. Org. 5:213-218.

Roy, S.K. and M. Jinnah. 2001. *In vitro* micropropagation of poinsettia (*Euphorbia pulcherrima* Willd.). Plant Tiss. Cul. 11(2):133-140.

Sanford, J., A.D. Blowers, F. Smith and J. Van Eck. 1999. Transgenic plants using the TDC gene for crop improvement. World Intellectual property Organization. 20 December 2008. http://www.wipo.int/pctdb/en/wo.jsp?WO=1999%2F06581&IA=WO1999%2F06581&DISPLAY=DESC

Smith, F., T.S. Chou, R. Eisenreich, J. Sanford, A. Blowers and J. Van Eck. 2006. Production of transgenic poinsettia. United States Patent 7119262. 23 December 2007. http://www.patentstorm.us/ patents/7119262.html

Songstad, D.D., V. De Luca, N. Brisson, W.G.W. Kurz and C.L. Nessler. 1990. High levels of tryptamine accumulation in transgenic tobacco expressing tryptophan decarboxylase. Plant Physiol. 94:1410-1413.

Starman, T.W., D. Xiangrong and A. Shane. 1999. Nucleic acid scanning techniques distinguish closely related cultivars of poinsettia. HortScience 34(6):1119-1122.

Tepfer, D. 1990. Genetic transformation using *Agrobacterium rhizogenes*. Physiol. Plantarum 79:140-146.

Thomas, J.C., D.G. Adams, C.L. Nessler, J.K. Brown, and H.J. Bohnert. 1995. Tryptophan decarboxylase, tryptamine, and reproduction of the whitefly. Plant Physiol. 109: 717-720.

Tideman, J. and J.S. Hawker. 1982. *In vitro* propagation of latex-producing plants. Ann. Botany 49:273-279.

Toth, S., K. Csaba and S. Peter 2006. *Agrobacterium*-mediated genetic transformation of the desiccation tolerant resurrection plant *Rsmonda myconi* (L) Rchb. Plant Cell Rep. 25:442-449.

Uchida, H., O. Nakayachi, M. Otani, M. Kajikawa, Y. Kohzu, K. Yamato, H. Fukusawa, T. Shimada and K. Ohyama. 2004. Plant regeneration from internode explants of *Euphorbia tirucalli*. Plant Biotechnol. 21:397-399.

USDA. n.d. Plants Database. Plants Profile. 15 September 2007. http://plants.usda.gov/java/profile?symbol=EUPU9

USDA, NASS. 2008. Floriculture Crops: 2008 Summary.

Van Driesche, R.G., S. Lyon, K. Jacques, T. Smith, and P. Lopes. 2002. Comparative cost of chemical and biological whitefly control in poinsettia: Is there a gap? Fla. Entom. 85:488-493.

Vik, N.I., A.K. Hvoslef-Eide, H. Gjerde and K. Bakke. 2001. Stable transformation of poinsettia via electrophoresis. Acta Hort. 560:101-103.

Weathers, P.J., R.D. Cheetham, E. Follansbee, and K. teoh. 1994. Artemisinin production by transformed roots of *Artemisia annua*. Biotechnol. Lett. 16:1281-1286.

Wilkerson, D., L. Barnes, B. Drees and C. Hall. 1999. The Texas poinsettia producers guide.Texas Agricultural Extension Service, Texas A&M. 13 June 2008. http:// aggie-horticulture.tamu.edu/greenhouse/nursery/guides/poinsettia/

Yao, K., D. Vincenzo and B. Normand. 1995. Creation of a metabolic sink for tryptophan alters the phenylpropanoid pathway and the susceptibility of potato to *phytophthora infestans*. The Plant Cell 7:1787-1799.

CHAPTER III

POINSETTIA (*Euphorbia pulcherrima* 'PRESTIGE™ RED')

in vitro PROPAGATION

Abstract

Euphorbia pulcherrima, poinsettia, micropropagation and *in vitro* proliferation is extensively practiced by poinsettia breeders and researchers. Slow growth rate of plantlets, few micro shoots per explant, and slow root growth rate are restrictions of *in vitro* propagation of poinsettia. The objective of this experiment was to develop an effective and efficient *in vitro* proliferation technique for poinsettia 'Prestige™ Red'.

Explants (apical buds and axillary buds) obtained from greenhouse grown plants were placed on Murashige-Skoog (MS) basal medium supplemented with various concentrations (4 μM-12 μM) of 6-benzylaminopurine (BA) and 4 μM of indole-3-acetic acid (IAA) in order to optimize callus proliferation and organogenesis of poinsettia *in vitro*. Explants placed on media containing only BA and combinations of BA and IAA produced red callus at the base of plantlets after one month while explants in a medium without plant growth regulators (PGRs) produced no callus. Subculturing of red callus in a medium with BA (4 μM-12 μM) produced additional callus and micro buds. Regenerated micro buds produced the greatest number of micro shoots on a medium with BA alone. White callus did not give rise to micro buds or micro shoots. Four month old

shoots initiated roots on MS basal medium without any plant growth regulator (PGR); however addition of IAA into the medium increased rooting efficiency in terms of number of roots and quicker root initiation.

Incorporation of PGRs into poinsettia micropropagation media at different stages of *in vitro* plantlet development enhanced rapid callus formation and accelerated shoot and root growth. Optimization of PGR concentrations during poinsettia micropropagation helped resolve previous restrictions of *in vitro* poinsettia proliferation.

Introduction

Euphorbia pulcherrima Willd. ex Klotzsch, poinsettia, belongs to the family Euphorbiaceae and the genus *Euphorbia* consisting of close to 2,000 species: milky sap (latex) secreted at the cut surfaces is a common feature of the family Euphorbiaceae (Ecke et al. 2004). During flowering, upper leaves are modified into colorful bracts making poinsettia popular during the winter holiday season. As the number one flowering potted plant in the United States, it continues to increase in revenues annually (Clarke et al. 2008).

After initiation of poinsettia breeding programs in mid-1950s, most of the cultivars were developed from *Euphorbia pulcherrima*, an extinct species in nature (Huang and Chu 2008). The cultivar Prestige™ Red (Eckespoint® Prestige™) was developed in 2002 for increased greenhouse performance (Ecke et al. 2004). Characteristics of 'Prestige™ Red' include strong stems, upright growth habit, resistance to stem breakage and uniform shoot growth which facilitate floral display and make it a popular cultivar (Ecke.com 2008).

Few studies have reported *in vitro* organogenesis and axillary bud proliferation protocols of poinsettia (De Langhe et al. 1974; Roy and Jinnah 2001 and Pickens et al. 2005). De Langhe et al. (1974) first discovered the role of cytokinins in developing caulogenetic callus from internodal explants in poinsettia 'Paul Mikkelsen': higher cytokinin:auxin produced more micro buds from existing callus. According to Roy and Jinnah (2001) repeated subculture in a medium supplemented with 1.5 mg/L BA (6-benzylaminopurine), 0.5 mg/L Kn (kinetin) and 15% coconut milk resulted in rapid shoot proliferation from internodal explants: shoots were rooted on a ½ MS medium containing 1 mg/L IBA (indole-3-butyric acid) and 0.5 mg/L IAA (indole-3-acetic acid). Protocols for both axillary bud proliferation and shoot organogenesis were developed using terminal buds and leaf tissues of poinsettia 'Winter Rose' by Pickens et al. 2005: explants produced the greatest number of axillary buds on media containing between 2.2 and 8.8 µM BA.

The development of *in vitro* protocols for plant regeneration through either organogenesis or somatic embryogenesis is one of the main prerequisites for the potential applications of clonal propagation and genetic transformation (Vila et al. 2003). *In vitro* clonal propagation of poinsettia is important since conventional propagation of poinsettia by cuttings and seed has several limitations. Propagation through seed is difficult since seed lose viability upon storage and plants originating from seed have genetic variability which is not preferred in producing uniform plants for market demand (Jasrai et al. 2003). Propagation through cuttings is not feasible year-round because it is labor intensive and costly (Jasrai et al. 2003). Most transformation techniques demand *in vitro* proliferation of plants and especially *Agrobacterium*-mediated transformation requires *in vitro* plant

regeneration either through organogenesis or somatic embryogenesis. Therefore, the objective of this research was to develop an effective and efficient *in vitro* organogenesis technique for poinsettia 'Prestige Red' prior to *Agrobacterium*-mediated transformation.

Materials and Methods

Plant material and culture conditions

Apical buds and axillary buds (i.e. explants) with 1-2 cm of stem of *E. pulcherrima* 'Prestige™ Red' were excised from healthy plants grown in the greenhouse under long day photoperiods in order to maintain vegetative growth: long days were created during winter using night interruption lighting with incandescent bulbs from 10 PM to 2 AM. The buds were surface disinfected by placing them in a 1% Tween 20 solution for 5 min, followed by 70% ethyl alcohol for 1 min and then 20% bleach for 15 min (Pickens et al. 2005). Finally buds were dipped in sterile water thrice for 5 min. Basal medium (20 ml) containing Murashige and Skoog (MS) salts (4.4 g/L), myo-inositol (0.1 g/L), sucrose (30 g/L) and agar (7g/L) (pH 5.7-5.8) (Pickens et al. 2005) was poured into 100 ml baby food jars and autoclaved at a temperature of 121 ^{0}C at 15 psi pressure for 15 minutes. Various concentrations of PGRs were incorporated into MS basal medium depending on treatment type: BA was incorporated before autoclaving and filter sterilized IAA was incorporated into the liquid medium after autoclaving. Disinfected buds were placed on the autoclaved medium solidified with agar in aseptic conditions under a laminar hood and incubated in a growth chamber with 16 h photoperiod (125 μmol $m^{-2}s^{-1}$ illumination with fluorescent bulbs) at 25^0 C day and 22^0 C

night temperatures. In this experiment primarily two types of studies were carried out: callus study and rooting study.

Callus study

The combination of auxins and cytokinins was tested in this experiment since successful *in vitro* proliferation protocols have been reported for plants belonging to the family Euphorbiaceae including a few poinsettia cultivars using several types of auxins and cytokinins. The callus study was composed of two treatments: For the first treatment, BA was incorporated into the basal medium at concentrations of 4 μM, 6 μM, 8 μM, 10 μM and 12 μM (Table 3.1) and for the second treatment, the same concentrations of BA were incorporated in combination with 4 μM of IAA (Table 3.2). For both treatments, medium without PGR was used as a control. After one month, explants were evaluated for percentage of explants having red callus at the base and callus color. Further, the number of micro buds and micro shoots were examined (data not shown). Explants were subcultured monthly onto a new medium with the same ingredients.

Rooting study

Four month old *in vitro* grown poinsettias were transferred onto a rooting media of three treatments: full MS with 28.5 μM IAA (Roy 2001), ½ MS with 28.5 μM IAA and BA (4 μM) alone (Table 3.3). An experimental control with no PGRs was also included. The plants were evaluated for average number of days taken for root initiation and number of roots initiated per shoot. The experiment was terminated after five months.

Statistical analysis

Each experiment had five explants per treatment and each experiment was repeated once. All treatments were arranged in a Randomized Complete Block Design (RCBD) and data were analyzed by the General Linear Model (GLM) using SAS 9.1(Statistical Analysis Software). Treatment means were separated by Least Significant Difference (LSD) method at $\alpha = 0.05$.

Results and Discussion

Explant disinfection, plant material and growth conditions

Surface-disinfection of explants was difficult due to latex secreted at the cut surfaces of explants and the latex is a common problem with micro propagation of plants belonging to family Euphorbiaceae. Thus, at least 15-25% of the explants were infected per each micro propagation cycle even with cautious disinfection steps. Disinfection steps included use of proper disinfecting solutions at proper concentrations, through disinfection of tools by autoclaving and working under a disinfected laminar hood. Infections were greater (40%) without using 1% Tween 20 solution during the disinfection process of explants. Thus, Pickens et al. (2005) disinfection protocol was used in order to minimize infections encountered. Explants (apical buds and axillary buds) of poinsettia 'Prestige^{TM} Red' responded to the PGR combinations (i.e. IAA for auxin and BA for cytokinin) by developing into calluses prior to organogenesis. Monthly repeated subculture onto new medium was necessary in order to achieve organogenesis and finally a plantlet.

Callus study

Primarily two types of calluses were identified in poinsettia micropropagation: red callus (Fig. 3.1A) and white callus (Fig. 3.1B). With continuous subculturing in a medium with BA (4 μM-12 μM), red callus produced more micro buds and micro shoots (data not shown) and healthy plants whereas white callus remained unchanged. White callus is a result of recalcitrant cell clumps which are unproductive. Red callus is the most important in poinsettia proliferation: it proliferates fast and synthesizes into micro buds and micro shoots. The results observed were consistent with other experiments of poinsettia such as De Langhe et al. (1974) and Pickens et al. (2005). Apart from red and white calluses, grayish green callus was also observed with low BA levels (2-4 μM). This was consistent with the study of De Langhe et al. (1974), where dominant callus color was red with low IAA:kinetin (Kn) and the higher the ratio, the higher the grayish green callus produced.

Explants grown in a media supplemented with BA produced red callus at the base of the explant whereas explants without BA did not give rise to any callus (Table 3.1). There is a significant (α=0.05) difference between plants which are treated with BA and without BA. Therefore use of BA in poinsettia micropropagation is effective and increased red callus production. BA can be used to generate more red calluses, more number of micro buds (Fig. 3.1C) and micro shoots (Fig. 3.1D) in poinsettia micropropagation. The impact of cytokinins on poinsettia *in vitro* bud development was reported previously by De Langhe et al. (1974) who observed faster buds development with higher levels of 2iP (2-isopentenyl adenine).

Explants supplemented with both BA and IAA produced red callus at the base of the explant showing a significant (α=0.05) difference among treatments (Table 3.2). Continuous growth in a medium supplemented with BA (4 μM-12 μM) alone resulted in a greater number of micro buds and micro shoots compared to medium supplemented with both IAA (4 μM) and BA (4 μM-12 μM) (data not shown). Uchida et al. (2004) reported the impact of cytokinins on adventitious bud formation of *E. tirucalli*: adventitious buds were efficiently induced when the medium was supplemented thidiazuron (TDZ) compared to a medium with both TDZ and IAA. Incorporation of IAA and BA into the medium generated more red calluses but BA/IAA ratio is critical in generating red callus. The greater the BA:IAA, the greater the red callus produced in this study. Equimolar concentrations of cytokinins to auxins are generally used to sustain callus, whereas higher cytokinin to auxin ratios lead to stimulate shoot development (Pickens et al. 2005).

Rooting study

Four month old poinsettia transferred into the rooting medium rooted irrespective of IAA concentrations. Incorporation of BA had an inhibitory effect on rooting: plants treated with BA developed callus at the base of the plant instead of rooting. In our study, the best medium for poinsettia *in vitro* rooting was ½ MS with 28.5 μM IAA because it increased rooting efficiency (Table 3.3). Increased IAA concentrations had a negative impact on rooting leading to callus development at the base of the plant (data not shown).

Conclusions

BA (4 μM-12 μM) can be used to effectively generate more red calluses, micro buds and micro shoots from apical and axillary buds of poinsettia 'Prestige^{TM} Red'. Incorporation of IAA and BA into the medium generate more red callus but BA:IAA is critical in generating red callus: the greater the BA/IAA ratio, the greater the red callus production. Red callus is the most important for poinsettia proliferation compared to white callus. The best medium for *in vitro* rooting of poinsettia was ½ MS with 28.5 μM IAA for rooting efficiency. Findings from this research can be used as an effective and efficient *in vitro* micro propagation and proliferation technique of poinsettia 'Prestige^{TM} Red'. This protocol can be used in both poinsettia *in vitro* proliferation and as the first step in transgenic poinsettia production. Further research is necessary to determine how to minimize white callus production during *in vitro* poinsettia culture.

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Table 3.1 Red callus initiation from one month old poinsettia 'Prestige Red' explants (apical buds and axillary buds) supplemented with 6-benzylaminopurine (BA).

Treatments (BA) µM	% of explants with a red callus at the base
4	100 a[z]
6	100 a
8	100 a
10	100 a
12	100 a
0	0 b

[z]Treatments with the same letter are not significantly different. Means were separated using Least Significant Difference (LSD) at P_{α}=0.05.

Table 3.2 Red callus initiation from one month old poinsettia 'Prestige Red' explants (apical buds and axillary buds) supplemented with indole-3-acetic acid (IAA) + 6-benzylaminopurine (BA)

Treatments	% of explants with a red callus at the base
4 µM IAA and 4 µM BA	83.7 b[z]
4 µM IAA and 6 µM BA	83.7 b
4 µM IAA and 8 µM BA	100 c
4 µM IAA and 10 µM BA	100 c
4 µM IAA and 12 µM BA	100 c
0 µM IAA and 0 µM BA	0 a

[z]Treatments with the same letter are not significantly different. Means were separated using Least Significant Difference (LSD) at P_{α}=0.05.

Table 3.3 Root initiation from four month old poinsettia 'Prestige Red' micro shoots (4 months old) supplemented with plant growth regulators (PGRs)

Treatments	Average # of days taken for root initiation	# of roots initiated
No PGRs	35 a[z]	2 a[z]
4 µM BA	0 d	0 c
½ MS with 28.5 µM IAA	24 c	3 b
Full MS with 28.5 µM IAA	28 d	3 b

[z]Treatments with different letters are significantly different. Means were separated using Least Significant Difference (LSD) at P_{α}=0.05.

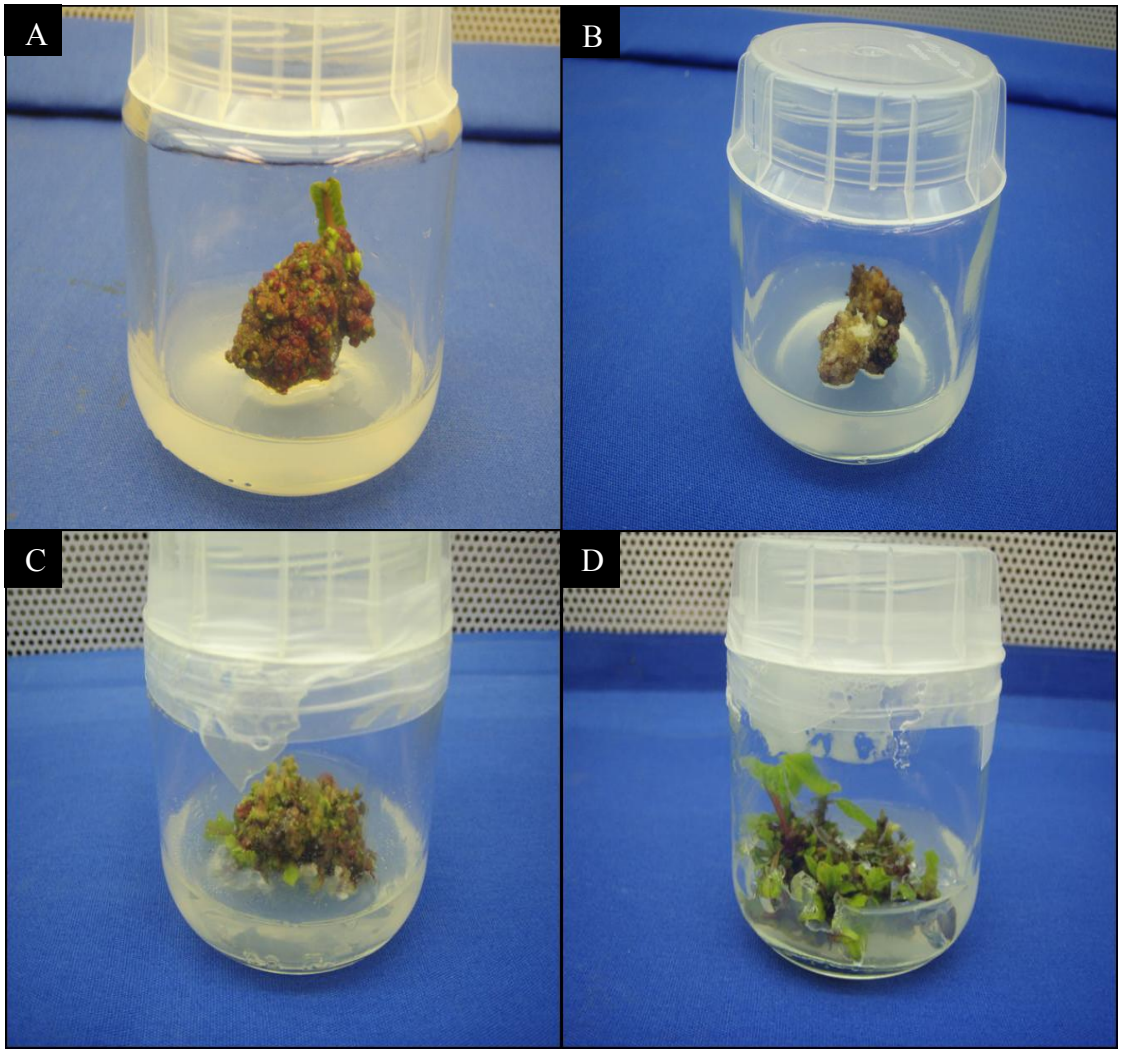

Fig. 3.1 Poinsettia ‘Prestige Red’ explants (apical buds and axillary buds) producing red and white calluses, micro buds and micro shoots in poinsettia micropropagation. A, Red callus (one month old); B, White callus (one month old); C, Micro buds (two months old); D, Micro shoots (three months old)

LITERATURE CITED

Clarke, J.L., S. Carl and H. Sissel. 2008. *Agrobacterium tumefaciens* – mediated transformation of poinsettia, *Euphorbia pulcherrima*, with virus-derived hairpin RNA construct confers resistance to *Poinsettia mosaic virus*. Plant Cell Rep. 27:1027-1038.

De Langhe, E., P. Debergh and R. Van Rijk. 1974. *In vitro* culture as a method for vegetative propagation of *Euphorbia pulcherrima*. Z. Pflanzenphysiol 71:271-274.

Ecke.com. 2008. All poinsettias. 4 February 2009. http://www.ecke.com/Search/Variety_Results.asp

Ecke, P., J.E. Faust, J. Williams and A. Higgins. 2004. The Ecke Poinsettia Manual. Ball Publishing. Batavia, IL.

Jasrai, Y.T., K.N. Thaker and M.C. D'Souza. 2003. *In vitro* propagation of *Euphorbia pulcherrima* Willd. through somatic embryogenesis. Plant Tiss. Cul. 13(1):31-36.

Pickens, K.A., Z.M. Cheng, and R.N. Trigiano. 2005. Axillary bud proliferation of *Euphorbia pulcherrima* Winter Rose™. In Vitro Cell Dev. Biol.-Plant 41:770-774.

Roy, S.K. and M. Jinnah. 2001. *In vitro* micropropagation of poinsettia (*Euphorbia pulcherrima* Willd.). Plant Tiss. Cul. 11(2):133-140.

Uchida, H., O. Nakayachi, M. Otani, M. Kajikawa, Y. Kohzu, K. Yamato, H. Fukusawa, T. Shimada and K. Ohyama. 2004. Plant regeneration from internode explants of *Euphorbia tirucalli*. Plant Biotechnol. 21:397-399.

Vila, S. G. Ana, R. Hebe and M. Luis. 2003. Somatic embryogenesis and plant regeneration from immature zygotic embryos of *Melia azedarach* (Meliaceae). In Vitro Cell Dev. Biol.-Plant 39:283-287.

CHAPTER IV

DEVELOPMENT OF TRANSGENIC POINSETTIA (*Euphorbia pulcherrima*) HARBORING TDC (TRYPTOPHAN DECARBOXYLASE) USING *Agrobacterium*-MEDIATED TRANSFORMATION

Abstract

Since natural variation of poinsettia has been explored extensively, development of transgenic poinsettia could improve existing poinsettia cultivars. The gene TDC (tryptophan decarboxylase), which converts tryptophan into tryptamine, has been used to develop pest and disease resistance cultivars of agronomically and horticulturally important crops. The objective of this research was to develop transgenic poinsettia using *Agrobacterium*-mediated transformation harboring TDC and to increase whitefly resistance in poinsettia.

Poinsettia 'Prestige Red', infected with *Agrobacterium rhizogenes* (ATCC 15834), produced hairy roots at the infection locus. Hairy roots failed to develop into plantlets when treated with various plant growth regulator (PGR) concentrations: 6-benzylaminopurine (BA: 1-1.8 mg/L), indole-3-acetic acid (IAA: 0.1-0.5 mg/L) or BA (1-1.8 mg/L) and IAA (0.4 mg/L). These results suggest that further study into PGR concentrations that are effective to develop callus from hairy roots is necessary in order to develop transgenic poinsettia through *A. rhizogenes.*

When poinsettia stem disks were grown *in vitro* with various concentrations of plant growth regulators (PGRs), 'Eckespoint Pollys Pink' produced the most pro-embryogenic masses (PEMs) compared to four other cultivars: 'Prestige Red', 'Novia Red', 'Elf Red' and 'Eckespoint Freedom Early Red'. Therefore, 'Eckespoint Pollys Pink' was used for somatic embryogenesis of both transformed (by *Agrobacterium tumefaciens* (LBA 4404)) and non-transformed stem disks. The PEMs of 'Eckespoint Pollys Pink' turned brown and started to senesce after five weeks of growth in somatic embryo induction (SEI) medium. Somatic embryos appeared in mostly necrotic PEMs after nine weeks in SEI medium. Excised somatic embryos were further grown in somatic embryo maturation (SEM) medium. Additional research into more effective PGR combinations, antibiotic concentrations and antinecrosis chemicals is required in order to develop transgenic poinsettia harboring TDC using *A. tumefaciens*.

Introduction

Though traditional plant breeding has played a vital role in plant selection for favorable traits, the modern technique of developing transgenic plants through genetic engineering has recently been successful. Genetic engineering is often quicker and can introduce characteristics not available in the native genera. Today genetic engineering serves as the primary means of genome modification in many plant genera. This popularity can be attributed to the reduction of production time for valuable traits compared to traditional breeding. Plant transformation has been practiced through several techniques including microprojectile bombardment (biolistic transformation), electroporesis and *Agrobacterium*-mediated transformation for twenty years.

Though genetic engineering has been used in more than thirty genera of ornamental crops, development of transgenic poinsettia has only started recently (Clarke et al. 2008). There are few studies of transgenic poinsettia using the different gene transformation techniques (Sanford et al. 1999; Vik et al. 2001; Smith et al. 2006 and Clarke et al. (2006 and 2008)). Sanford et al. (1999) developed transgenic poinsettia 'Angelika' resistant to *Botrytis cinerea* through biolistic transformation. Vik et al. (2001) explored three transformation techniques of poinsettia 'Lilo Red': *Agrobacterium*-mediated transformation, particle gun delivery and *in vivo* electroporesis. Vik et al. (2001) noted some drawbacks of the first two *in vitro* transformation techniques: particle gun delivery on the poinsettia meristems ended up with a high frequency of blue spots and *Agrobacterium*-mediated transformation resulted in low transformation rate. Smith et al. (2006) reported that transgenic poinsettia 'Angelika', 'Freedom' and 'Jolly Red', developed through microprojectile mediated transformation, showed a wide range of pest and disease resistance, modified growth habit and modified flower color and fragrance. Clarke et al. (2008) developed a successful *Agrobacterium*-mediated transformation technique for poinsettia 'Millenium' resistant to *Poinsettia mosaic virus*. *Agrobacterium*-mediated transformation protocols are highly species and cultivar dependent (Clarke et al. 2008). Presently there is no research regarding transformation of industry leading cultivars of poinsettia such as 'Prestige Red'.

The gene tryptophan decarboxylase (TDC) which converts L-tryptophan into tryptamine has become important with scientists who work on development of pest and disease resistance in transgenic plants. Several studies have evaluated development of pest and disease resistance in TDC transformed transgenic plants accumulating higher

concentrations of tryptamine (Songstad et al. 1990; Thomas et al. 1995; Yao et al. 1995; Sanford et al.1999 and Smith et al. 2006). According to Songstad et al. (1990), young fully expanded leaves of TDC transformed tobacco plants contained more than 1mg of tryptamine per g fresh weight, a 260 fold increase over controls. Thomas et al. (1995) found that the transgenic tobacco expressing TDC had a 97% reduction in whitefly reproduction. Sanford et al. (1999) observed transgenic petunia harboring TDC exhibits fungicidal and fungistatic activity, antibacterial effects and nematode inhibitory effects. Further, transgenic poinsettia, geranium and lisianthus with high tryptamine levels acquired resistance to *Botrytis cinerea*. Smith et al. (2006) reported that leaves of TDC transformed poinsettia, produced through microprojectile delivery method, accumulated 40-50 µg/g leaf fresh weight of tryptamine which is toxic enough for whitefly compared to untransformed poinsettia having 5 µg/g leaf fresh weight of tryptamine.

Currently, *Agrobacterium*-mediated transformation is widely used in producing transgenic plants and the number of species susceptible to the transformation is increasing (Gelvin 2003). Primarily two *Agrobacterium* species are used in *Agrobacterium*-mediated transformation: *A. rhizogenes* causing hairy root disease and *A. tumefaciens* causing crown gall disease in infected plants. *A. rhizogenes* is used mostly in transformation of dicotyledonous plants and some monocotyledonous plants such as cereals (Hongwei et al. 2006). When the bacterium infects a susceptible plant, the T-DNA (transferred DNA), between left and right borders of the Ri (root-inducing) plasmid of the bacterium, is transferred and integrated into the nuclear genome of the plant leading to hairy root formation at the site of infection (Gelvin 2003; Pickens 2004). In the process of developing transgenic plants from the hairy roots, the hairy roots are grown in

a medium supplemented with various concentrations of Plant Growth Regulators (PGRs) and antibiotics. *A. tumefaciens* is widely employed in development of both monocotyledonous and dicotyledonous transgenic plants (Gelvin 2003). The T-DNA, between left and right borders of the Ti (tumor-inducing) plasmid of the bacterium, is transferred and integrated into the nuclear genome of the plant upon infection of a susceptible plant, leading to tumor formation at/around the site of infection (CAMBIA 2008). Disarmed *A. tumefaciens* (i.e. Ti-TDNA is replaced by a foreign gene) is used in developing transgenic plants primarily through somatic embryogenesis or organogenesis (Pickens 2004). There are few *Agrobacterium*-mediated transformation studies of plants belonging to the family Euphorbiaceae including transformation of poinsettia by *A. rhizogenes* (Vik et al. 2001) and *A. tumefaciens* (Clarke et al. 2008). Currently, *Agrobacterium*-mediated transformation of poinsettia 'Millenium' by Clarke et al. (2008) is the only successful protocol reported for *Agrobacterium*-mediated poinsettia transformation.

Though there are few studies of transgenic poinsettia, no reported studies exist regarding development of TDC transformed poinsettia through *Agrobacterium*-mediated transformation .The objective of this research was to develop TDC transformed transgenic poinsettia expressing higher concentrations of tryptamine and enhancing whitefly resistance in poinsettia.

Materials and Methods

Transformation of poinsettia 'Prestige Red' with *A. rhizogenes*

In vitro plant material

Stock plants of poinsettia 'Prestige Red' were maintained in MS (Murashige and Skoog) basal medium containing MS salts (4.4 g/L), myo-inositol (0.1 g/L), sucrose (30 g/L) and agar (7 g/L) (pH 5.7-5.8) in baby food jars without any plant growth regulator (PGR) (Pickens et al. 2005). The MS basal medium was autoclaved at a temperature of 121 ^{0}C at 15 psi pressure for 15 minutes and cooled prior to transfer of *in vitro* plantlets to the disinfected solidified medium. The stock plants were grown in a growth chamber with 16 h photoperiods (125 µmol $m^{-2}s^{-1}$ illumination with fluorescent bulbs) at 25^0 C day and 22^0 C night temperatures.

A. rhizogenes culture

Agrobacterium rhizogenes ATCC 15834 was grown in YMB (yeast mannitol broth) medium containing K_2HPO_4 (0.5 g/L), $MgSO_4H_2O$ (2.0 g/L), NaCl (0.1 g/L), mannitol (10.0 g/L), yeast extract (0.4 g/L) and agar (15 g/L) (pH 7.0) at 25^{o}C (Weathers et al. 1994). Stock culture of *A. rhizogenes*, consisting of overnight broth culture and 15% (v/v) sterile glycerol, was stored at -80^{o}C in 1ml aliquots. *A. rhizogenes*, subcultured from the original stock culture in YMB liquid medium, was used for the infection: prior to infection the bacterial suspension was incubated on a shaker (150 rpm) at 25^{o}C for 48 hours.

Infection of poinsettia 'Prestige Red' with *A. rhizogenes*

Five month old *in vitro* grown poinsettia 'Prestige Red' was infected with *A. rhizogenes* under a laminar hood using three methods: injection of the bacterial suspension (25µl) into a stem node without prior wounding, injection of the bacterial suspension (25µl) into a wound on the stem node and direct application of a bacterial paste into a wound on the stem node. *In vitro* stem nodes were wounded with a sterilized scalpel blade and latex that secreted at the wound was swabbed with a disinfected cotton bud. Infected plantlets were maintained *in vitro* in the MS basal medium without PGRs in a growth chamber with 16 h photoperiods (125 µmol $m^{-2}s^{-1}$ illumination) at 25^0C day and 22^0 C night temperatures.

Hairy root culture

Hairy roots originated at the site of infection after a month and were excised from *in vitro* plantlets and grown *in vitro* in a medium containing Murashige and Skoog (MS) salts (4.4 g/L), sucrose (30 g/L), carbenicillin (250 mg/L) and agar (7 g/L) (pH 5.7-5.8) supplemented with various concentrations of PGRs. The PGRs, BA (6-benzylaminopurine) and IAA (indole-3-acetic acid) were used alone or in combination: BA alone (1 mg/L, 1.2 mg/L, 1.4 mg/L, 1.6 mg/L and1.8 mg/L), IAA alone (0.1 mg/L, 0.2 mg/L, 0.3 mg/L, 0.4 mg/L and 0.5 mg/L) and both BA (1 mg/L,1.2 mg/L, 1.4 mg/L, 1.6 mg/L and1.8 mg/L) and IAA (0.4 mg/L) together for a month. The MS basal medium was autoclaved at a temperature of 121 ^{0}C at 15 psi pressure for 15 minutes and poured into sterilized petri dishes after adding carbenicillin and PGRs into the medium at 45-50^0

C. The hairy roots were maintained *in vitro* in a growth chamber with continuous light (125 μmol $m^{-2}s^{-1}$ illumination with fluorescent bulbs) and dark at 25^0C for one month.

Transformation of poinsettia with *A. tumefaciens* harboring TDC

In this study, the basic protocol for *A. tumefaciens*-mediated transformation of poinsettia was based on the procedures of Clarke et al. (2008). In many species, it is important to combine auxin and cytokinin for initiation of embryogenic cultures (George et al. 2008). Thus, various types of auxins and cytokinins were used in different concentrations in this study depending on the growth stage such as callus induction (CI), somatic embryo induction (SEI) and somatic embryo maturation (SEM). Bi-weekly repeated subculture onto a new medium was necessary in order to gain excellent pro-embryogenic masses (PEMs) since PGRs deplete with time (Sumaryono et al. 2001).

Plant material and explant disinfection

In a preliminary study, five poinsettia cultivars: 'Prestige Red', 'Novia Red', 'Elf Red', 'Eckespoint Pollys Pink' and 'Eckespoint Freedom Early Red' were tested for response to PGR concentrations of CI medium and SEI medium. Upper most internodes, excised from healthy vigorously greenhouse grown poinsettia, were surface disinfected by placing them in a 1% Tween 20 solution for 5 min, followed by 70% ethyl alcohol for 1 min and then 20% bleach for 15 min (Pickens et al. 2005). Finally the nodes were dipped in sterile water thrice for 5 min. Sterilized internodes were cut into stem disks of 5-10 mm thickness and 10 stem disks of each cultivar were placed on CI medium under aseptic conditions under a laminar hood. These stem disks were further evaluated for

PEMs proliferation in SEI medium. Poinsettia 'Eckespoint Pollys Pink' was used as the primary cultivar in this study since it responded to PGR concentrations of SEI medium better than the other four cultivars. There were 3 replicates: in each replicate, 40 stem disks of 'Eckespoint Pollys Pink' were used. While half of the stem disks were treated with *Agrobacterium*, the remaining were not treated with *Agrobacterium* and used as controls.

Mobilizing TDC into *A. tumefaciens*

The plasmid pBI121 was used in this experiment in order to mobilize TDC into *Agrobacterium*. The plasmid pBI121, harboring TDC cDNA subcloned to the CaMV 35S promoter in place of the deleted β-glucuronidase gene, comprises a selectable marker gene that confers resistance to kanamycin upon transformed plant cells (Songstad et al. 1990). The resulting plasmid, CaMV 35S-TDC was introduced into *Agrobacterium* by electrophoresis (Cold Spring Harb. Protoc. 2006). Presence of TDC in *Agrobacterium* was confirmed by growing transformed *Agrobacterium* in a liquid medium supplemented with kanamycin (50 mg/L) and through gel electrophoresis.

A. tumefaciens culture

A. tumefaciens LBA 4404 was grown in LB (Luria Bertani) medium containing Bacto-tryptone (10 g/L), Bacto-yeast extract (5 g/L), NaCl (10 g/L) and Bacto-agar (15 g/L) (pH 7.0) (Gartland and Michael 1995). Stock culture of *A. tumefaciens* was prepared similar to *A. rhizogenes* in this study.

Infection

A. tumefaciens LBA 4404 harboring TDC was subcultured directly from stock solution maintained at -80^{o}C. The bacterium was grown in 25 ml liquid LB medium supplemented with 50 mg/L kanamycin on a shaker with a speed of 150 rpm at 28^{o}C for 18-20 hours until an OD_{600} (Optical Density) of 0.6-0.8 was reached. *A. tumefaciens* cells were collected by centrifugation at 2,700 rpm for 10 min, washed twice with MS basal medium supplemented with 2% sucrose (MS-2) and resuspended in 10 ml of MS-2 (Clarke et al. 2008). Disinfected stem disks of 5-10 mm thickness were inoculated with the *Agrobacterium* suspension for 5 min in a shaker at 100 rpm at 25^{o}C.

Co-cultivation

Inoculated stem disks were blotted with sterile filter paper and placed in co-cultivation medium containing MS salts (4.4 g/L), sucrose (30 g/L), agar (7 g/L) and PGRs (CPA (4-chlorophenoxy acetic acid) (0.2 mg/L) and BA (0.2 mg/L) in aseptic conditions under a laminar hood. Stem disks were co-cultivated with *Agrobacterium* on the co-cultivation medium at 24^{o}C in the dark for 72 hours.

Callus induction

After co-cultivation, stem disks were blotted on sterile filter paper and transferred onto CI medium containing MS salts (4.4 g/L), sucrose (30 g/L), agar (7 g/L) and PGRs (CPA (0.2 mg/L) and BA (0.2 mg/L)) in aseptic conditions under a laminar hood. Stem disks, inoculated with *Agrobacterium*, were grown in the CI medium supplemented with antibiotics (claforan (500 mg/L) and kanamycin (10 mg/L)) whereas stem disks which

were not inoculated with *Agrobacterium* were grown in the CI medium without the antibiotics at 25^{o}C with light (125 µmol $m^{-2}s^{-1}$) for 10 days.

Somatic embryo induction

Upon callus induction, stem disks were transferred into SEI medium containing MS salts (4.4 g/L), sucrose (30 g/L), agar (7 g/L) and PGRs (NAA-1-naphthaleneacetic acid (0.3 mg/L) and 2iP-2-isopentenyl adenine (0.15 mg/L)) in aseptic conditions under a laminar hood. Stem disks, inoculated with *Agrobacterium*, were grown in the SEI medium supplemented with antibiotics (claforan (400 mg/L) and kanamycin (25 mg/L)) whereas stem disks which were not inoculated with *Agrobacterium* were grown in the SEI medium without the antibiotics at 25^{o}C with light (125 µmol $m^{-2}s^{-1}$) for 3 months. The PGR concentrations in the study of Clarke et al. (2008) were modified at the latter part of this research because of unexpected negative results (i.e. necrosis in PEMs): concentration of NAA (i.e. 0.3 mg/L) was maintained constant while the 2iP concentrations were changed to 0.09 mg/L, 0.12 mg/L and 0.18 mg/L.

Somatic embryo maturation

After three months of growth in SEI medium, excised somatic embryos were transferred into somatic embryo maturation (SEM) medium containing MS salts (4.4 g/L), sucrose (30 g/L), agar (7 g/L), BA (0.05 mg/L), claforan (400 mg/L) and kanamycin (10 mg/L) in aseptic conditions under a laminar hood. Somatic embryos were grown *in vitro* at 25^{o}C with light (125 µmol $m^{-2}s^{-1}$) until they developed into micro shoots.

Results and Discussion

Transformation of poinsettia 'Prestige Red' with *A. rhizogenes*

Infection of poinsettia 'Prestige Red' with *A. rhizogenes*

Out of the three infection methods tested, injection of the bacterial suspension (25µl) into a wound on the stem of *in vitro* grown poinsettia was effective: hairy roots appeared at the site of infection after one month (Fig. 4.1). This is consistent with the study of transformation of *Euphorbia lathyris* using *A. rhizogenes* where wounded plants produced hairy roots more efficiently (Cheetham et al. 1996). Produced phenolic compounds of wounded plant cells have induced expression of *vir* genes of *A. rhizogenes* leading to hairy root initiation at the wounded site (Shah 2005). Thus, *A. rhizogenes* ATCC 15834 infects poinsettia 'Prestige Red' successfully by developing hairy roots at the site of infection due to increased auxin activity at the site upon infection (Hongwei et al. 2006). The two other infection methods evaluated were not effective: plants injected with bacterial solution without prior wounding and plants applied with a bacterial paste did not produce hairy roots: plants applied with a bacterial paste were compromised due to the over activity of the bacterium on the plant.

Hairy root culture

Excised hairy roots from the site of infection of *in vitro* grown plants did not respond to PGR concentrations and failed to develop into callus after one month. Development of transgenic poinsettia through hairy roots was not possible using this

technique. Use of *A. rhizogenes* was discontinued for the rest of the study. There are few studies regarding transformation of plants belonging to the family Euphorbiaceae using *A. rhizogenes*: hairy roots of *Euphorbia lathyris* successfully developed into transgenic plants in a medium with PGRs (Cheetham et al. 1996).

Transformation of poinsettia with *A. tumefaciens* harboring TDC

Explant disinfection

Pickens et al. (2005) disinfection protocol was used in this study in order to minimize contaminations. At the beginning of the micropropagation cycle, higher percentages (25%) of infections were observed even with cautious disinfection steps. This is primarily due to latex secreted at the cut surfaces of explants and frequent (once in two weeks) sub culturing. The latex is a common problem with micropropagation not only in poinsettia but also in other plants belonging to the family Euphorbiaceae.

Presence of TDC in *Agrobacterium*

Agrobacterium transformed with pBI121 harboring TDC grew in a liquid medium supplemented with kanamycin (50 mg/L) whereas non-transformed *Agrobacterium* (control) did not. This confirms acquired kanamycin resistance of transformed *Agrobacterium* through pBI121 harboring TDC. Presence of TDC in *Agrobacterium* was further confirmed by gel electrophoresis: the middle of 500 and 600 bp bands of 100 bp ladder (1st lane) was parallel to the DNA bands of other four lanes representing extracted TDC which is 554 bp in size from transformed *Agrobacterium* (Fig. 4.2).

Callus induction

There were no differences in callus induction among the cultivars when grown under equal concentrations (i.e. 0.2 mg/L) of auxin and cytokinin. Independent of the type of the cultivar, stem disks inoculated with *Agrobacterium* showed late callus induction apparently compared to non-inoculated stem disks (data not shown). This is because of anti-growth effects of antibiotics present in the medium with inoculated stem disks. This late growth of *Agrobacterium* inoculated stem disks was observed throughout the somatic embryogenesis study and it was obvious during SEI (Fig. 4.3). This is consistent with somatic embryogenesis of poinsettia 'Millenium' reported by Clarke et al. 2008.

Somatic embryo induction

In most species a low concentration of cytokinin tends to stimulate PEMs and higher concentration of cytokinin may have a negative effect: thus auxin:cytokinin of 2:1 was used during somatic embryo induction in this study (George et al. 2008). Poinsettia 'Eckespoint Pollys Pink' responded more to the PGR concentrations of the SEI medium by producing the most PEMs compared to the other four cultivars evaluated (Fig. 4.4): the protocol was continued with 'Eckespoint Pollys Pink' and the other cultivars were discontinued. Figure 4.5 shows the subsequent growth (i.e. increased diameter) of stem disks of 'Eckespoint Pollys Pink' with time. The PEMs were mostly green in color mixed with brown and some reddish cell clumps (Fig. 4.6A). PEMs of 'Eckespoint Pollys Pink' kept proliferating in SEI medium supplemented with NAA (0.3 mg/L) and 2iP (0.15 mg/L) until five weeks (Fig. 4.6A) and then PEMs started to turn brown and senesce (Fig.

4.6B). This brownish phenomenon is common for poinsettia somatic embryogenesis prior to the globular stage of somatic embryos becoming visible (Personal communication, Dr. Jihong L. Clarke, Norwegian Institute for Agricultural and Environmental Research, Norway). Somatic embryos (green in color) became visible in mostly necrotic PEMs after nine weeks on SEI medium (Fig. 4.6C). Clarkes' protocol on 'Eckespoint Pollys Pink' finally resulted in three somatic embryos (Fig. 4.6D) from one stem disk out of one hundred and twenty stem disks evaluated. In this study, the effectiveness of modified treatments during SEI should be further evaluated in order to select the best PGR combination for better induction of somatic embryos. Requirements for somatic embryogenesis may vary depending on the genotype and the cultivar since somatic embryogenesis has been described as being genetically determined (George et al. 2008). Thus, development of a particular somatic embryogenic protocol for a particular poinsettia cultivar is of paramount importance.

Conclusions

Poinsettia 'Prestige Red' can be successfully infected by *A. rhizogenes* producing hairy roots at the site of infection when prior wounded stem nodes were injected with a bacterial suspension. Out of three methods tested, injection of a bacterial suspension onto a prior wound on the stem helps successful infection of *A. rhizogenes* into poinsettia. Various concentrations of IAA and BA may not be effective enough to produce callus from hairy roots. Further studies into more effective PGR and antibiotic concentrations are necessary in order to develop transgenic plants through hairy roots resulting from *A. rhizogenes* infection of poinsettia.

Poinsettia ‘Eckespoint Pollys Pink’ can be employed for somatic embryo development in this study since it produced the most PEMs in response to PGR concentrations in SEI medium compared to the other four cultivars tested. *A. tumefaciens*-mediated transformation of poinsettia through somatic embryogenesis is cultivar dependent. Brownish phenomenon is common in poinsettia somatic embryogenesis in both *A. tumefaciens* transformed and non-transformed PEMs. However, *A. tumefaciens* transformed PEMs show delay in development compared to non-transformed PEMs. Although no TDC transformed transgenic poinsettia expressing higher concentrations of tryptamine were generated, PEMs and embryos were generated from *A. tumefaciens* infected poinsettia ‘Eckespoint Pollys Pink’ stem disks. Additional research into more effective PGR combinations, antibiotic concentrations and antinecrosis chemicals is required in order to develop transgenic poinsettia harboring TDC through *A. tumefaciens*.

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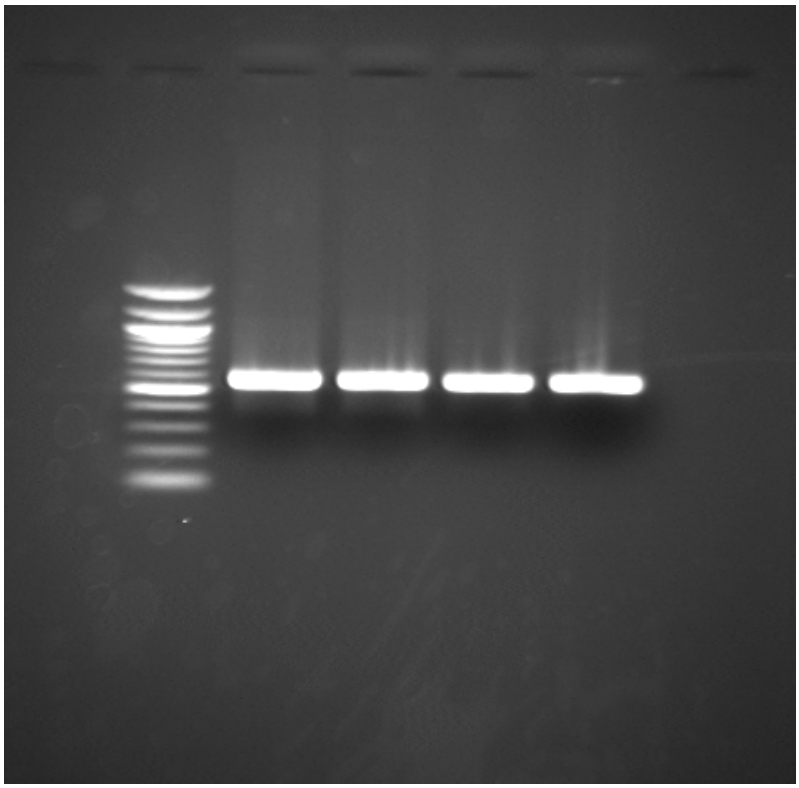
rhizogenes ATCC 15834 was provided from American Type Culture Collection, Manassas, VA.

Fig. 4.1 Hairy root initiation (after one month) at the site of *A. rhizogenes* infection in poinsettia 'Prestige Red'



1 2 3 4 5

Fig. 4.2 Gel picture confirming presence of TDC in transformed *Agrobacterium tumefaciens*

Lane 1 – 100 bp ladder
Lane 2, 3, 4 and 5 – plasmid DNA with TDC isolated from transformed *Agrobacterium tumefaciens*

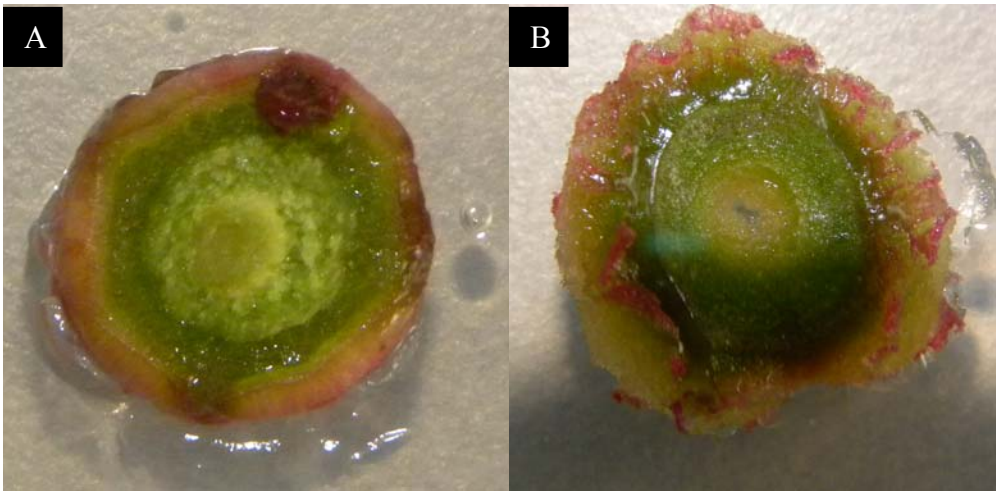


Fig. 4.3 Responsiveness of five weeks old poinsettia 'Eckespoint Pollys Pink' stem disks to plant growth regulators (PGRs) and antibiotics in somatic embryo induction (SEI) medium. A, stem disk inoculated with *Agrobacterium tumefaciens*; B, stem disk not inoculated with *Agrobacterium tumefaciens*

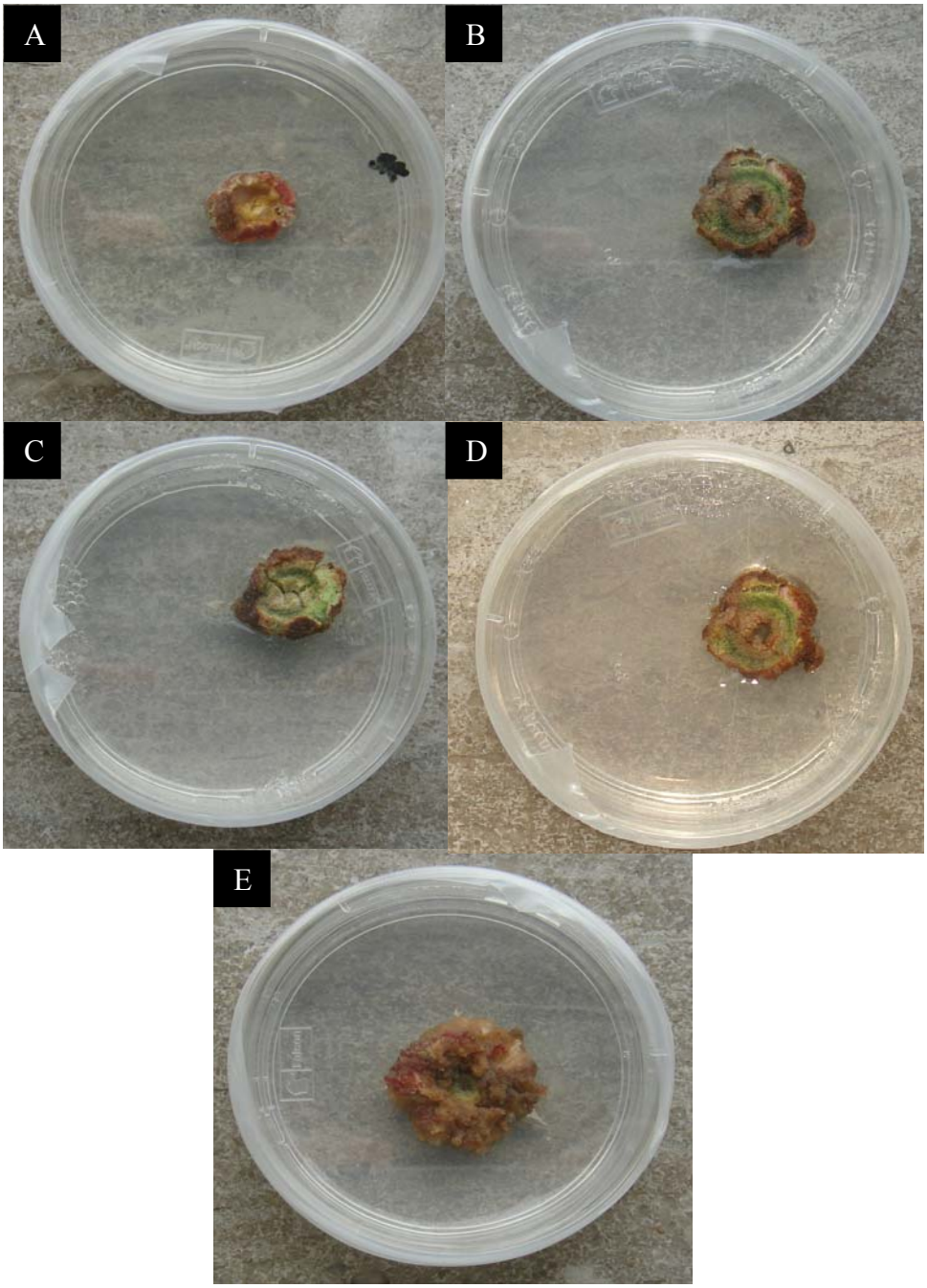


Fig. 4.4 Responsiveness of stem disks (four weeks old) of five poinsettia cultivars to somatic embryo induction (SEI) medium. A, 'Prestige Red'; B, 'Novia Red'; C, 'Elf Red'; D, 'Eckespoint Freedom Early Red'; E, 'Eckespoint Pollys Pink'

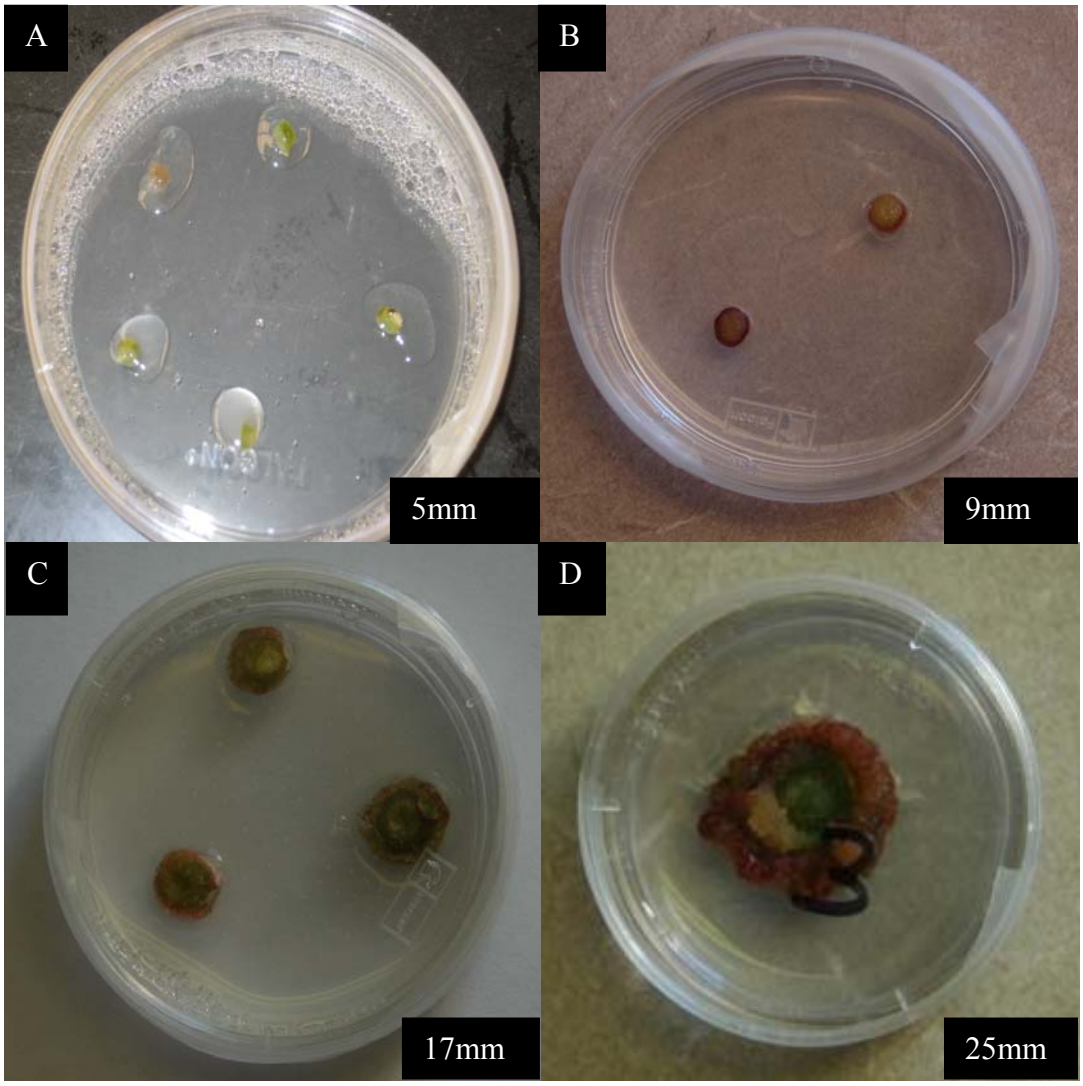


Fig. 4.5 Growth of stem disks of poinsettia 'Eckespoint Pollys Pink'.
A, At the beginning (diameter=5mm); B, 10 days old stem disks in callus induction (CI) medium (diameter=9mm); C, 3 weeks old stem disks in somatic embryo induction (SEI) medium (diameter=17mm); D, 5 weeks old pro-embryogenic masses(PEMs) on somatic embryo induction (SEI) medium (diameter=25mm)

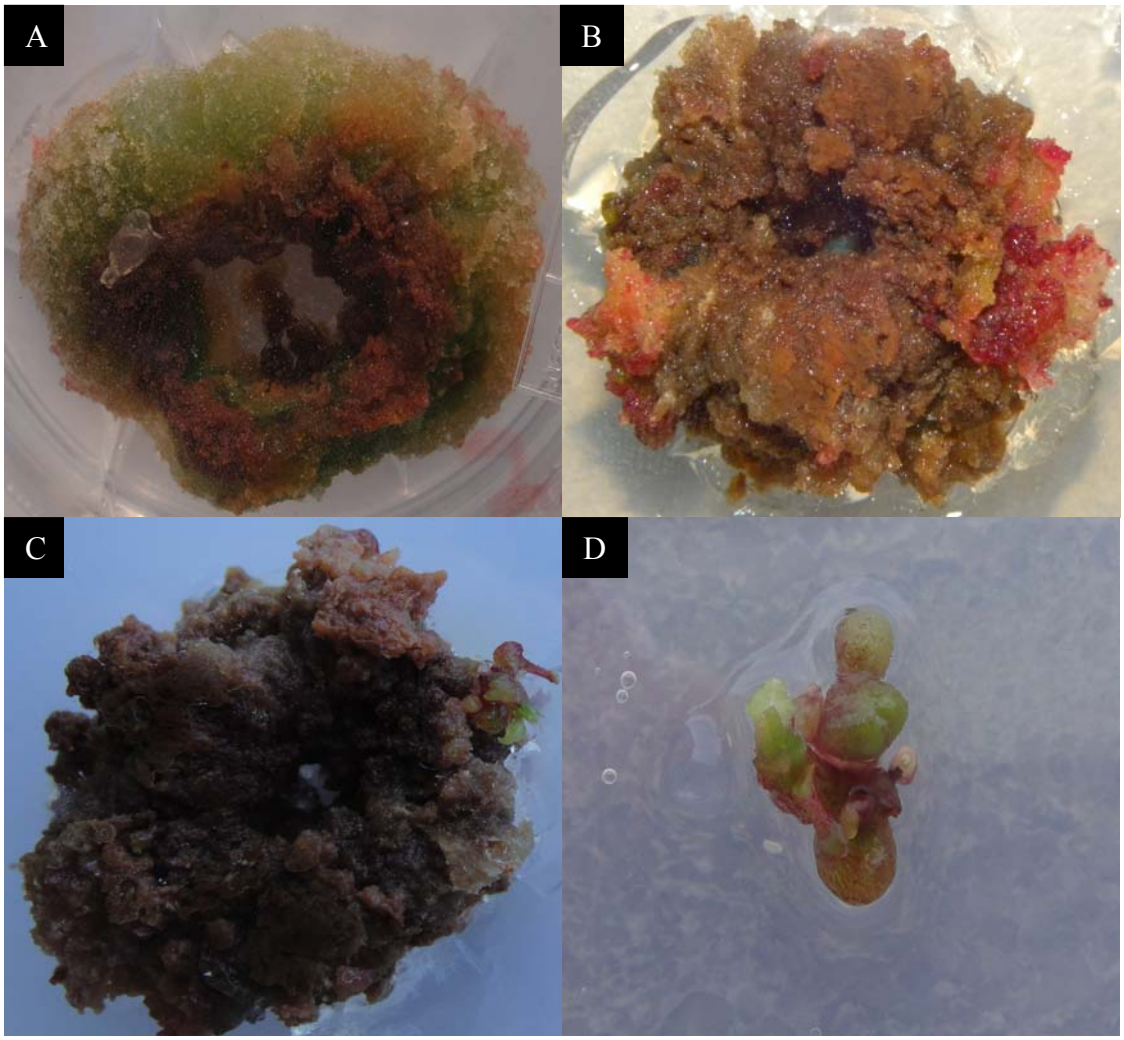


Fig. 4.6 Growth of pro-embryogenic masses (PEMs) of 'Eckespoint Pollys Pink' at different stages of somatic embryogenesis. A, Five weeks old PEMs in somatic embryo induction (SEI) medium; B, PEMs senescing in SEI medium; C, Somatic embryo appearance from mostly senesced PEMs in SEI medium; D, Maturation of somatic embryos in somatic embryo maturation (SEM) medium

LITERATURE CITED

CAMBIA. 2008. Agrobacterium-mediated transformation–Overview. 2 March 2009. http://www.patentlens.net/daisy/AgroTran/835.html

Cheetham, R., E. Follansbee and P. Weathers. 1996. Transformation of Euphorbia lathyris by Agrobacterium rhizogenes. Acta Hort. 426:511-518.

Clarke, J.L., C. Spetz and S. Haugslien. 2008. Agrobacterium tumefaciens-mediated transformation of poinsettia, *Euphorbia pulcherrima*, with virus-derived hairpin RNA constructs confers resistance to *Poinsettia mosaic virus*. Plant Cell Rep. 27(6): 1027-1038.

Clarke, J.L., S.S. Klemsdal, E. Floistad, A.K. Hvoslef-Eide, S. Haugslien, R. Moe and D.R. Blystad. 2006. Genetic engineering of poinsettia with the aim of enhancing its resistance to poinsettia mosaic virus. Acta Hort. 722: 321-325.

Cold Spring Harb. Protoc.; 2006; doi:10.1101/pdb.prot4665. 23 July 2008. http://cshprotocols.cshlp.org/cgi/content/full/2006/30/pdb.prot4665

Gartland K.M.A. and R.D. Michael. 1995. *Agrobacterium* protocols. Humana Press, Totowa, NJ.

Gelvin, S.B. 2003. *Agrobacterium*- mediated plant transformation: the biology behind the "gene-jockeying" tool. Microbiol. Mol. Biol. R.:16-37.

George E.F., M.A. Hall and G.J. De Klerk. 2008. Plant Propagation by Tissue Culture. Springer, NY.

Hongwei, X., X. XZhou, J. Lu, J. Wang and X. Wang. 2006. Hairy roots induced by *Agrobacterium rhizogenes* and production of regenerative plants in hairy root cultures in maize. Sci. China Ser. C 49(4):305-310.

Pickens, K.A., Z.M. Cheng and R.N. Trigiano. 2005. Axillary bud proliferation of Euphorbia pulcherrima Winter Rose™. In vitro Cell Dev. Biol.-Plant 41:770-774.

Pickens, K.A. 2004. *In vitro* propagation, regeneration, attempted tetraploid induction, and Agrobacterium-mediated transformation of *Euphorbia pulchurrima* 'Winter Rose'(TM). Ph.D. dissertation, The University of Tennessee, United States-Tennessee. Retrieved May 21, 2008, from Dissertations & Theses: Full Text database. (Publication No. AAT 3156536).

Sanford, J., A.D. Blowers, F. Smith and J. Van Eck. 1999. Transgenic plants using the TDC gene for crop improvement. World Intellectual property Organization. 20 December 2008. http://www.wipo.int/pctdb/en/wo.jsp?WO=1999%2F06581&IA=WO1999%2F06581&DISPLAY=DESC

Shah P.V. 2005. T-DNA. 14 March 2009. http://arabidopsis.info/students/paaras/t_dna.htm

Smith, F., T.S. Chou, R. Eisenreich, J. Sanford, A. Blowers and J. Van Eck . 2006. Production of transgenic poinsettia. United States Patent 7119262. 23 December 2007. http://www.patentstorm.us/ patents/7119262.html

Songstad, D.D., V. De Luca, N. Brisson, W.G.W. Kurz and C.L. Nessler. 1990. High levels of tryptamine accumulation in transgenic tobacco expressing tryptophan decarboxylase. Plant Physiol. 94:1410-1413.

Sumaryono, I. Riyadi and J.S. Tahardi. 2001. Morpgological variations during the development of somatic embryos of tea (*Camellia sinensis* L.) *in vitro*. Menara Perkebunan 69(2):46-57.

Thomas, J.C., D.G. Adams, C.L. Nessler, J.K. Brown, and H.J. Bohnert. 1995. Tryptophan decarboxylase, tryptamine, and reproduction of the whitefly. Plant Physiol. 109:717-720.

Vik, N.I., A.K. Hvoslef-Eide, H. Gjerde and K. Bakke. 2001. Stable transformation of poinsettia via electrophoresis. Acta Hort. 560:101-103.

Weathers, P.J., R.D. Cheetham, E. Follansbee, and K. Teoh. 1994. Artemisinin production by transformed roots of *Artemisia annua*. Biotechnol. Lett. 16:1281-1286.

Yao, K., D. Vincenzo and B. Normand. 1995. Creation of a metabolic sink for tryptophan alters the phenylpropanoid pathway and the susceptibility of potato to *phytophthora infestans*. The Plant Cell 7:1787-1799.

CHAPTER V

CONCLUSIONS

The broad objective of this research was to develop transgenic poinsettia expressing tryptophan decarboxylase (TDC) capable of protecting poinsettia against whitefly. The development of *in vitro* protocols for plant regeneration is one of the main prerequisites for the potential development of a transgenic poinsettia.

An effective and efficient *in vitro* micro propagation and proliferation technique of poinsettia 'Prestige Red' was successfully developed in this study. Optimization of PGR concentrations during poinsettia micropropagation helped resolve previous restrictions of poinsettia *in vitro* proliferation. Incorporation of various concentrations of BA and IAA into poinsettia micropropagation media at different stages of *in vitro* plantlet development enhanced rapid callus formation and accelerated shoot and root growth of poinsettia. Addition of BA (4 μM-12 μM) into the medium generated more red calluses, micro buds and micro shoots from apical and axillary buds. Incorporation of IAA (4 μM) and BA (4 μM-12 μM) into the medium also helps to generate red callus but BA:IAA is critical in generating red callus: the greater the BA:IAA, the greater the red callus production. Red callus is the most important in poinsettia *in vitro* proliferation compared to white callus. The best medium for *in vitro* rooting of poinsettia was ½ MS with 28.5 μM IAA for rooting efficiency. This protocol may be used for the potential development of transgenic poinsettia and poinsettia *in vitro* proliferation for clonal propagation.

Development of a successful *Agrobacterium*-mediated transformation technique for poinsettia was the second objective of this research prior to transformation of poinsettia with TDC using *Agrobacterium*. Poinsettia 'Prestige Red' can be successfully infected by *A. rhizogenes* when prior wounded stem nodes were injected with a bacterial suspension producing hairy roots at the site of infection. Various concentrations of IAA and BA may not be effective enough to produce callus from hairy roots. Further studies into more effective PGR concentrations are necessary in order to develop transgenic plants through hairy roots resulting from *A. rhizogenes* infection of poinsettia.

'Eckespoint Pollys Pink' can be used for the development of PEMs prior to somatic embryogenesis in response to Clarkes' protocol compared to the other four cultivars tested. However, Clarkes' protocol needs to be further modified by changing PGR concentrations for effective development of somatic embryos from the PEMs of 'Eckespoint Pollys Pink'. Since brownish phenomenon is common in both *A. tumefaciens* transformed and non-transformed PEMs, addition of some antinecrosis chemicals in to the SEI medium is necessary. Further, antibiotic concentrations need to be modified since *A. tumefaciens* transformed PEMs show delay in development compared to non-transformed PEMs. A particular *Agrobacterium*-mediated transformation protocol is cultivar specific: depending on the poinsettia cultivar, PGR concentrations and antibiotic concentrations need to be modified. Additional research into more effective PGR combinations, antibiotic concentrations and antinecrosis chemicals is required in order to develop transgenic poinsettia harboring TDC through somatic embryogenesis using *A. tumefaciens*.