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In Vitro Propagation, Regeneration, Attempted Tetraploid Induction, and Agrobacterium-mediated Transformation of Euphorbia pulchurrima 'Winter Rose'™

Kimberly Ann Pickens
University of Tennessee - Knoxville

To the Graduate Council:

I am submitting herewith a dissertation written by Kimberly Ann Pickens entitled "In Vitro Propagation, Regeneration, Attempted Tetraploid Induction, and Agrobacterium-mediated Transformation of Euphorbia pulchurrima 'Winter Rose'™." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Plant Sciences.

Zong-Ming (Max) Cheng, Major Professor

We have read this dissertation and recommend its acceptance:

Stephen Garton, Stephen Kania, Vince Pantalone

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Stephen Garton

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Vince Pantalone

Acceptance for the Council:

Anne Mayhew
Vice Chancellor and Dean of Graduate Studies

(Original signatures on file with official student records)

IN VITRO PROPAGATION, REGENERATION, ATTEMPTED TETRAPLOID INDUCTION, AND AGROBACTERIUM-MEDIATED TRANSFORMATION OF *EUPHORBIA PULCHURRIMA* 'WINTER ROSE'™.

A Dissertation

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Kimberly Ann Pickens

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ABSTRACT

Poinsettia, *Euphorbia pulchurrima,* is the number one potted flowering plant in the United States. 'Winter Rose'™ is a very popular cultivar with more than one million plants sold each year. To further improve this cultivar, particularly for larger flower heads and free branching, this research aimed at establishing some *in vitro* systems for application of biotechnology to poinsettia genetic improvement.

A protocol was established for *in vitro* axillary bud proliferation using greenhouse grown terminal buds. Buds were placed on Murashige-Skoog (MS) basal medium supplemented with benzlyaminopurine (BA). Explants produced the greatest number of axillary buds on medium containing between 2.2-8.8 μM BA. The number of explants that produced axillary buds increased with increasing BA concentration. An organogenesis system was also established using *in vitro* grown leaf tissues. The greatest amount of callus and shoots were produced from leaf midvein sections placed on MS medium containing 8.8-13.3 μM benzylaminopurine (BA) and 17.1μM indole-3-acetic acid (IAA) for one month and then transferred to medium containing only BA. Adventitious buds were produced only from red-pigmented callus, and explants that produced callus continued to produce adventitious shoots in the presence of IAA. Five-month-old shoots derived from shoot culture or organogeneses rooted readily in artificial soil without treatment with IBA or treated with 50 or 100 mg/l IBA and were acclimated in the greenhouse.

The effects of colchicine and oryzalin on callus production and adventitious shoot formation and their ability to induce tetraploid formation of *Euphorbia pulchurrima*

'Winter Rose'™ were evaluated. *In vitro* grown leaf midvein sections were placed in/on various forms (liquid or solid) of medium supplemented with either colchicine or oryzalin. A range of duration times between 1-4 days was also evaluated. Colchicine was less damaging to leaf tissues at concentrations of 0.25 µM or 250.4 µM. A large amount of callus was produced as well as a few adventitious shoots. Oryzalin inhibited plant regeneration from leaf tissues at all concentrations tested, and caused severe necrosis. Tissues produced callus, but no shoots were formed. A protocol was established for using flow cytometry to determine the ploidy level in poinsettia. Sample calluses and regenerated shoots from colchicine treatments were evaluated using the flow cytometer and were found to be diploid. Callus from explants exposed to oryzalin-containing medium was also tested using the flow cytometer and no tetraploid tissue was found. Since colchicine showed generally less inhibitory effect than oryzalin, colchicine is considered to be a better mitotic inhibitor chemical for tetraploid formation in poinsettia than oryzalin.

The factors influencing Agrobacterium-mediated transformation of *Euphorbia pulchurrima* 'Winter Rose'™ were also evaluated. Kanamycin at 50 mg/L was sufficient to inhibit poinsettia callus and shoot formation, and appeared to be a suitable selectable antibiotic for selecting transformed cells in poinsettia. Variables evaluated in these studies included plasmid type (pBI121, pMON690), with or without addition of the antibiotics CCK (cefotaxime, carbenicillin, and kanamycin) or acetosyringone. Since all tissues infected with agrobacterium (co-cultivation) died in 1-2 months, it appeared that poinsettia is highly sensitive to agrobacterium infection. Because of this premature death of infected tissues, other variables such as acetosyringone, and CCK could not be

evaluated effectively as to their effect on the transformation of ‘Winter Rose’™. Further study into antinecrosis chemicals or a change in explant type is needed in order to establish a protocol for Agrobacterium-mediated transformation of *E. pulchurrima* ‘Winter Rose’™.

This research established foundation studies on which to build a biotechnological improvement program for *E. pulchurrima* ‘Winter Rose’™.

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PART 1

INTRODUCTION

Euphorbia pulchurrima, poinsettia, is an important winter holiday symbol in many parts of the world including the United States and Europe. Flowering plants sold in pots are the second largest floral crop after bedding plants, and poinsettia is the biggest selling of the potted flowering plants. More than 67 million pots worth approximately $246 million dollars were sold in 2001 alone (USDA 2002) with the wholesale value estimated at $256 million dollars in 2003 (USDA 2002). There are more than 75 varieties available and over 100 cultivars grown commercially (Hartley 1992), which may attribute to the popularity of poinsettia.

The 'Winter Rose' ™ family of poinsettias was bred by Paul Ecke Farms in Encinitas, California and was first introduced by Fruehwirth after 30 years of breeding and trials (Poinsettias.com). Part of the popularity of the 'Winter Rose'™ family may be due to its characteristic dark green incurved foliage, incurved bracts, and rose-shaped flower heads (bracts with true flowers). It comes in a variety of colors including traditional red, white, and pink. Because of its incurved bracts it can be placed at tighter spacing and requires less water than normal cultivars. The blooms initiate around the 25th of September and can last three to four months and therefore perfectly fit into the Christmas season (Ecke.com 2002).

Since the 1970's, biotechnological approaches have been widely used in crop improvement and basic biological research. For example, *in vitro* multiplication of plants has allowed many novel plants to be mass propagated and become quickly available to consumers. Available plant regeneration systems have enabled us to generate mutants such as tetraploids and introduce new genes such as those for resistance or beneficial traits into target plants.

Although ‘Winter Rose’™ is a very popular cultivar, there are traits which can be further improved. Recent cultivar evaluations have shown that ‘Winter Rose’™ is a minimal branching cultivar and thus produces a low number of inflorescences (Harkess 1997). In particular, it produces a low number of small inflorescences. *In vitro* chromosome number manipulation may be used for increasing inflorescence and bract size. Mutagenic chemicals such as colchicine and oryzalin have been used to increase the size of inflorescences successfully in many species including Gerbera Daisies (Tosca et al. 1995), rhododendron (Väinölä 2000), and roses (Kermani et al. 2003).

‘Winter Rose’™ is also low branching cultivar, which contributes to the minimal number of inflorescences present on each plant, and often requires multiple pinches to increase branching. Genetic transformation with novel genes, such as *iaa-L*, may increase branching and thus the number of flower buds produced. These methods may lead to further improved ‘Winter Rose’™ poinsettia that have an increased number of larger inflorescences.

Very little research, however, has been done with *Euphorbia pulchurrima*. Only a few reports were found in the 1970’s on seed germination and callus induction (Nataraja et al. 1973; 1975) or shoot differentiation using internode and petiole explants (de Langhe et al. 1974). Therefore the objectives of this study were 1) to develop a protocol for in vitro propagation of *Euphorbia pulchurrima* cv. ‘Winter Rose’™, which is a prerequisite for applying most *in vitro* genetic techniques for poinsettia improvement, 2) to evaluate the effect of the mitotic-inhibiting mutagenic chemicals colchicine and oryzalin on tetraploid formation in ‘Winter Rose’™, and 3) to investigate the possibility of developing a protocol for genetic transformation of ‘Winter Rose’™ poinsettia.

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PART 2

LITERATURE REVIEW

Poinsettia

History of the Poinsettia

First cultivated by the Aztecs, poinsettia is native to present day Taxco, Mexico. Joel Robert Poinsett brought poinsettia to the United States in 1825 (Ecke et al. 1990). In 1909 the Ecke family started specializing in the growth of poinsettia including the varieties 'St. Louis', 'Early Red' and 'Hollywood' (Ecke et al. 1990). Most commercially important cultivars from 1923 to 1960 were derived from the 'Oak Leaf' seedling introduced by Ecke (Hartley 1992). New cultivars such as 'Indiana', introduced in 1931, produced shorter growing plants. Ecke Farms also introduced 'Henriette Ecke', a double–bracted cultivar in 1927 (Ecke et al. 1990).

It wasn't until the 1950's that poinsettia breeding started to grow in popularity. During this time breeding projects were begun by institutions such as Pennsylvania State, University of Maryland and the USDA (Ecke et al. 1990). In 1963, a new cultivar, 'Paul Mikkelsen' was introduced that had more stable foliage retention and allowed poinsettia to be sold by cuttings (Hartley 1992).

In the early 1960's, European markets became interested in poinsettias. In 1964 'Annette Hegg Red' was introduced as a free-branching cultivar in Norway. This new cultivar sported several new cultivars that were multi-flowered and could produce 5-8 branches from a pinch (Ecke et al. 1990). Several other cultivars that came from European breeding include the Gutbier poinsettias that have a characteristic pink or marbled appearance and can be forced to flower at lower temperatures (Hartley 1992).

At present poinsettia breeders are focusing on a number of different characteristics. Colors such as a golden yellow such as 'Eckespoint Lemon Drop' and 'Pink Peppermint' have been introduced. Free-branching cultivars of old favorites such as 'Eckespoint Celebrate 2' are also being introduced (Ecke et al. 1990).

Other characteristics that breeders are looking for include cuttings that root rapidly and uniformly, earlier flowering under normal conditions, and disease and insect resistant plants. New cultivars also need to be resilient in order to handle the stresses of shipping and possible poor conditions of post-production including improper watering (Ecke et al. 1990).

Consumers are looking for plants and flowers that are long lasting with very little maintenance. A choice of color is also favorable to consumers and they want the product available when needed, usually around the holidays.

Botanical Characteristics and Economic Importance

Poinsettia, *Euphorbia pulchurrima*, is a member of the Euphorbiaceae, or spurge, family. This family contains about 283 genera and 7,300 species and contains species of various forms. Plants in this family grow as herbs, shrubs, trees or other forms. The characteristic of this family of plants is that species have milky, white latex that is exuded upon injury. Many species of this family are economically important. In addition to containing many ornamental species, this family consists of plants that produce rubber, edible roots and fruits, and are important medicinally (Bailey et al. 2000). *Hevea brasiliensis*, the Para Rubber Tree, is a very important source of natural rubber. *Manihot esculenta*, cassava, is an edible root species. *Ricinus communis*, Castor Bean, is a

powerful poison (Armstrong 2001). The distribution of this family is widespread with the main distribution centering in the tropics and into the temperate zone.

The genus of poinsettia, Euphorbia, is the largest member of the Euphorbiaceae family, and contains over 1,660 species with variable forms and a wide distribution. The leaves of this genus may be in an alternate, opposite, or whorled arrangement and may be entire or toothed, sessile or petioled. Flowers are in cyathia or simple. These cyathia may be terminal, axillary, clusters in the axils of leaves, or arranged in simple umbel-like, paniculate, or whorled cymes (Bailey et al. 2000). The juice of this genus is milky latex that can be irritating or toxic, causing a severe dermatitis in some people, akin to poison ivy exposure. Some species of Euphorbia are toxic and may be fatal to fish (Bailey et al. 2000). Many species of Euphorbia are viable as floriculture crops including *E. splendens*, crown of thorns, and *E. marginata*, snow-on-the-mountain. Other species in this genus are commercially important for their waxy exudates. Some of these include *E. antisyphilitica*, Candelilla, which is used for candles, cosmetics, adhesives and coatings, and *E. lagacae,* used for plastics and protective coatings (Janick 2002).

Euphorbia pulchurrima, poinsettia, is the best known of the Euphorbia genus. Poinsettia is a perennial, winter-flowering shrub that can reach heights up to 8 feet. The leaves of poinsettia are ovate-elliptic to lanceolate, 4-7 inches long and can be entire or toothed with long petioles. The cymes are umbel-like, terminal, and surrounded by many vibrant, colorful bracts. The cyathia have an involucre with one yellow gland. Bracts can be seen in white, pink and various shades of red (Bailey et al. 2000). Poinsettia is an important Christmas symbol in many parts of the world including the United States and

Europe. More than 67 million pots worth approximately $246 million dollars were sold in 2001 alone (USDA 2002).

Branching Habit of Euphorbia Pulchurrima

Native poinsettias of southern Mexico, which are the ancestors of modern poinsettias, grow into straight, tall trees (Lee 2000) that can reach up to 8 feet high (Suszkiw 1998). Once introduced to the United States, numerous growers such as Ecke Farms in California bred many new cultivars including shorter, free-branching cultivars (Lee 2000; Vandaveer 2001). It was first assumed that the new free branching cultivars were a byproduct of being infected with Poinsettia Mosaic Virus (PnMV). This was deducted because all of the free-branching cultivars were infected with this virus. It was also found that when the virus was eliminated using heat shock, cultivars reverted back to the native restricted-branching form (Dole et al. 1993), thus it was believed that infection with the virus was causative of the free-branching form. There was, however, an inconsistency in this theory in that a few of the restricted-branching cultivars were infected with PnMV as well. Later, in 1997, Lee et al. discovered that the free-branching form was in fact caused by a phytoplasma found in the phloem tissue of poinsettia (Lee et. al. 1997). Phytoplasma are uncultivable *in vitro* and can only be transferred via vector or grafting, thus making it difficult to induce free-branching in restricted-branching cultivars (Lee et al. 1997).

Restricted-branching and minimal free-branching cultivars can be induced to produce multiple flowers in the greenhouse by pinching the primary shoot tips. Most of the plants produced in commercial markets are pinched plants. Pinching serves a dual

purpose to commercial growers: providing multi-branched plants as well as controlling plant height (Hartley 1992). It is recommended that removal of the shoot tip be early enough for sufficient growth of the branch to desired length (Ecke et al. 1990). The nearer a plant is pinched to flower initiation date the shorter the plant (Hartley 1992). It has also been recommended that a chemical growth regulator such as Florel® be applied after pinching to further optimize branching (Ecke 1999). The disadvantage to manual pinching is that it can be time-consuming, therefore incurring heavy labor costs.

Euphorbia pulchurrima cv. 'Winter Rose'™

The Winter Rose ™ Family of poinsettias was bred by Paul Ecke Farms in Encinitas, California and was first introduced by Fruehwirth, the head breeder at Ecke Ranch, after 30 years of breeding and trials (Poinsettias.com 2002). These cultivars are characterized by having dark green incurved foliage, incurved bracts, and globe-shaped flower heads. It comes in a variety of colors including traditional red, white, and pink. Because of their incurved bracts they can be grown at tighter spacing and require less water than their normal counterparts. The blooms initiate around the 25th of September and can last three to four months (Ecke.com 2002). 'Winter Rose' ™ poinsettias have become a very popular cultivar. Recent cultivar evaluations have shown that Winter Rose™ poinsettias are a minimal branching cultivar and thus produce a low number of inflorescences (Harkess 1997). This can be a problem for both consumer and growers alike. Growers might find it hard to ship taller plants without breakage, and consumers may not buy poinsettias with few inflorescences.

In Vitro Propagation of Other Euphorbiaceae

Many members of the Euphorbiaceae family have been successfully propagated *in vitro*. A large study by Tideman in 1981 evaluated *in vitro* propagation of several latex producing plants including several members of the Euphorbia genus. This study evaluated multiple types of explants, along with several types of medium formulations and hormone concentrations. It was found that it was possible to propagate these latex-producing plants *in vitro*, but that each species had individual requirements of explant type, medium, and hormone concentration. *E. peplus* produced numerous shoots using apical shoot tips as explants on Murashige and Skoog (MS) (Murashige and Skoog 1962) medium containing a high concentration of cytokinin and a low amount of auxin. *E. tannensis* responded best with stem node segments on ½ MS with high cytokinin concentrations and low auxin content. *E lathyris* was also evaluated, and it was found that stem nodes would produce a minimal amount of shoots on MS medium containing 10μM 6-benzylaminopurine (BA) (Tideman et al. 1981). *E. lathyris,* an important invasive species, was also studied for propagation using apical shoot tips. It was found that a modified MS medium containing 0.1 mg/L N^6- [2-isopentenyl] adenine (2iP) produced numerous shoots that were excised and rooted in soil (Lee et al. 1982). *E. fulgens*, Scarlet Plume, a popular ornamental Euphorbia species was also cultured *in vitro* by using nodal sections on a medium containing 9.1 μM Zeatin. Explants produced 40 transplantable shoots within 12 weeks of culture, and show that this method could be a valuable means of producing cuttings in a short amount of time (Zhang et al. 1987).

Other genuses of the Euphorbiaceae family have also been propagated *in vitro*. *Excoecaria agallocha*, a mangrove species that has many medicinal uses, was cultivated *in vitro* using a modified low mineral content medium supplemented with 13.3 μM BA, 4.65 μM Zeatin, and 1.23 μM indolebutyric Acid (IBA). Nodal sections sprouted numerous axillary buds, and repeated subculturing produced 10-12 shoots per explant in 3 months (Rao et al. 1998). *Phyllanthus uniraria*, an important medicinal plant was also cultured *in vitro* using single node explants. The largest amount of multiplication of 16-20 shoots per explant was obtained using either MS or AR (Anderson Rhododendron) medium supplemented 5.0 μM Kinetin. The shoots were rooted on basal medium and regenerated plants were outplanted with a 91% survival rate (Catapan 2002). Catapan et al. (2000) also developed a protocol for the medicinally important Euphorbiaceae *Phyllanthus caroliniensis*. Nodal segments produced the highest number of shoots (21-21 shoots per explant) using MS or AR medium supplemented with 5 μM BA, 1.25-5.0 μM Kinetin, or 2.5-5.0 μM 2iP. Shoots were successfully rooted in MS containing 1.1μM naphthaleneacetic Acid (NAA) (Catapan 2000).

In Vitro Propagation of Euphorbia pulchurrima

Studies have been done on the culture of poinsettia *in vitro*. The first studies were done in 1973. Seeds of *E. pulchurrima* were sterilized and cultured on modified White's basal medium (BM) with or without plant growth regulators. Results found that the basal BM medium was the most successful in germinating seeds with a 90% germination rate. Growth regulators such as BA, 2iP, and 2,4-dichlorophenoxyacetic acid (2,4-D) reduced germination and produced callus (Nataraja et al. 1973). Further experiments with seeds of

poinsettia on BM medium containing various levels and combinations of coconut milk, gibberellic acid (GA), indole-3-acetic acid (IAA), kinetin, GA and 2,4-D produced callus and several of the cultures developed embryoids that later developed into plantlets (Nataraja 1975). Experiments on callus induction and differentiation into shoots were also done using internode and petiole explants. These experiments found that in petiole explants, high ratios of 2iP to NAA produced numerous shoots, the higher the 2iP level the quicker the buds appeared. With internode explants, it was found that low IAA levels coupled with higher levels of kinetin produced a type of red callus that was inductive to shoot regeneration (de Langhe et al. 1974). Shoots developed on medium were easily rooted in BM. Embryogenic cultures have also been established using hypocotyls tissues on an auxin-containing medium for induction and an auxin-free medium for germination (Osternack et al. 1999). A system for mass propagation using an embryogenic bioreactor culture was also established using shoot tips. Callus was initiated on liquid MS containing 2.0 mg/L IAA and 0.2 mg/L BA, after callus was formed cultures were transferred to liquid MS containing 0.2 mg/L *p*-chlorophenoxyacetic acid (CPA) and 0.2 mg/L BA. Embryos were successfully germinated and planted in the greenhouse with a survival rate of over 90% (Preil et al. 1991).

Tetraploid Formation

Natural Tetraploid Formation

The formation of polyploids, or those with duplicate numbers of chromosomes, is fairly common in plants. It is also considered as an important vehicle for evolution and

diversification of plant species. The tetraploid formation happens in plants when gametes pass through the cell cycle undergoing mitosis without cytokinesis, thus producing a gamete with 2n number of chromosomes. When the gametes fuse, numbers of chromosomes doubled in the resulting offspring.

The formation of natural polyploids can be traced in modern crops by comparison of species and chromosome number. Coffee species have been found containing 22, 44, 66, and 88 chromosomes. This suggests that the ancestor to these species was a plant containing a basic set chromosome number of 11 that evolved to different polyploid descendants. Many species are natural polyploids including corn, banana, and peanut.

There are two types of tetraploids, allotetraploids and autotetraploids. An allopolyploid is an individual that has two or more genomes from different species. An autotetraploid is an individual that has two or more genomes from the same species. Combining breeding techniques can develop tetraploid plants. Two species can be bred to produce a hybrid; chromosomes of the hybrid are doubled to produce an allotetraploid. This procedure is often used when a hybrid has very low fertility or when it is unable to be backcrossed to the parent (Fehr 1993). The tetraploid can increase yield as well as provide a genetic bridge between species. An example of a successful allopolyploid is triticale. This tetraploid (4x) is a cross between rye and perennial wheat and acts as a bridge to introduce genes between the two species (Fehr 1993). Autotetraploidy is the production of a tetraploid using the same species. Some examples of successful autotetraploid species include sugar beets and forage red clover (Fehr 1993). There are advantages to producing tetraploid cultivars. Tetraploids have abnormally large organs, including thickened leaves, larger fruit and larger sepals and petals. Induced tetraploids

have reduced fertility and are slow to grow and flower so they are better used on plants where vegetative material is harvested. This is beneficial to ornamental plant growers as well as those producing forage and tuber crops.

Colchicine

Colchicine is a chemical derived from a species of saffron named *Colchicum autumnale*. The use of colchicine can be dated back to ancient Egypt where it was used medicinally to treat rheumatism and gout. Research done to evaluate its medical applications and mode of action discovered that the compound acted by disrupting mitotic activity within cells (Eigsti et al. 1955). Colchicine works by inhibiting the spindle fiber mechanism in cells. This prevents the migration of the daughter chromosomes to the poles during anaphase. Cells exposed to colchicine undergo normal mitosis until the end of prophase. The nuclear membrane then breaks down but no spindle mechanism is formed. The chromatid arms separate and the centromeres divide producing duplicate sets of chromosomes. However, the replicated chromosomes are not pulled to the separate ends. A nuclear envelope is then formed around all of the chromosomes, thus doubling the chromosome number in the resultant cells (Dawson 1962). A mitotic inhibitor such as colchicine is applied to the meristematic portions of the plant such as apical meristems, but it can also be applied to germinating seeds, seedlings, roots, and developing meristems.

The technique for using colchicine for induction of polyploids was introduced in 1937. Blakeslee et al. (1934) first reported the use of colchicine in polyploid formation on *Datura stramonium*. They found that a 4-day immersion in a 0.4% solution of

colchicine produced 100% tetraploid plants (Blakeslee et al. 1934). The determination of a tetraploid was done by visual cues and by proportional size of pollen grains. Blakeslee et al. (1934) also successfully developed tetraploid Cosmos seedlings as well as *Portulaca grandiflora* by treating seeds. Experiments were also successful using explants such as stem sections and terminal buds as well as with different application techniques including agar application, spraying and drop methods. All methods were successful in developing some tetraploids, however methods such as immersion and agar application produced the highest percentage of tetraploid formation (Blakeslee et al. 1934). Since those first experiments with *Datura*, many new crop and ornamental cultivars have been developed by tetraploid and polyploid formation (Blakeslee et al. 1934).

Seed and embryo treatments are often handled by employing immersion in a colchicine solution of 0.02-0.1% for 2-48 hours. Germinating seedlings are generally treated with the same concentration for a longer period of time ranging from 12-48 hours (Sharma et al. 1980). Seeds of *Portulaca grandiflora* were exposed to 0.1% colchicine in liquid MS medium for 48 hours then planted. Recovered seedlings showed a high percentage of polystomy from 2C to 64C. The characteristics of the tetraploid plants included additional petals that were rearranged from the stamens and more spherical than diploid petals. Flowers also had enlarged pollen grains and decreased pollen fertility and seed set (Mishiba et al. 2000).

Colchicine has also been used for diploid formation. Haploid ovules of *Beta vulgaris* L. were cultured in 0.4% colchicine for 3-5 hours and produced 60% diploid plants (Hansen et al. 1995). Chromosome numbers of ovule cultures were also doubled

in *Gerbera jamesonii* using 0.05% colchicine exposure for 2-6 days in vitro (Tosca et al. 1995). Anther culture has also been used to develop doubled-haploid plants using colchicine. In wheat, callus derived from anther cultures was exposed to colchicine, from 313 μM to 626 μM, and oryzalin at 5 and 10 μ M for 72 hours. The callus was then exposed to hormones and produced somatic embryos that developed into plants. Results showed that colchicine was the most effective in producing double haploids with 89% of the plantlets derived from callus (Hassawi et al. 1991). Many other double-haploids have been induced by anther culture and treatment with colchicine including *Brassica juncea* (Lionneton et al. 2001), barley (Finch et al. 1979), and *Zea Mays* L. (Saisingtong et al. 1996). These experiments allow for self-fertilization in sterile species such as hybrids that cannot self or cannot breed normally. The doubled haploid plants also have been used extensively to study the genetic control of traits that would otherwise be difficult to determine.

Many plants have been induced into tetraploid formation by treating meristematic regions such as buds or nodes with colchicine. *Ex vitro* seedlings can be treated by using soaked cotton plugs placed over the growing tip for 2-4 hours or by applying the colchicine in the form of a paste mixed with glycerin or lanoline (Sharma et al. 1980). This method has been used to induce tetraploid formation of poinsettia lateral meristems. A 1% colchicine lanolin emulsion was applied to the buds *ex vivo*. Several of the resulted plants have thicker leaves, shorter and broader leaves, larger bracts and larger inflorescences. Of these plants, nine out of ten were tetraploids when chromosome counts were done (Stewart 1950). Supplementing agarose medium with colchicine can also induce *in vitro* chromosome doubling. An effective method for doubling

chromosome numbers in clover, *Trifolium spp*., was developed by using axillary meristems on proliferation medium containing 0.1% colchicine for 48-72 hours. Chromosome doubling frequencies were 81% for initial root tips and 44% for mature shoots. Chromosome doubled plants showed larger, more rounded leaves and a change in pollen shape (Anderson et al. 1990). A treatment of 0.05% colchicine on MS medium to shoot apices and axillary shoot buds for 48 hours produced over 20% tetraploid plants in hop, *Humulus lupulus* L. (Roy et al. 2001). Nodal explants have also been used to restore fertility in lilac hybrids. A hybrid of *Syringa vulgaris* and *S. pinnatifolia* was developed to create a large-flowered lilac with pinnate leaves. In order to create a fertile hybrid of these species, experiments were done using colchicine to induce tetraploid formation. Optimal tetraploid formation occurred with nodal explants placed in 0.05-0.25 mM colchicine for a period of 1-3 days. The tetraploids produced were distinguished by having slower growth, darker green leaves and shorter internodes (Rose et al. 2000). Rose et al. also have induced tetraploids in *Buddleia globosa* using the same methods (Rose et al. 2000).

Oryzalin

Oryzalin is a selective pre-emergence herbicide sold under many trade names including Dirimal, Ryzelan, and Surflan. It is used for control of annual grasses and broadleaf weeds in orchards, vineyards and established bermuda grass and ornamental landscapes. It is a surface-applied herbicide that inhibits the growth of germinating weed seeds by blocking cell division in the meristems (Extension Toxicology Network 1996). The mode of action for Oryzalin is that it disrupts mitosis by inhibiting the formation of

microtubules. In studies with potato suspension cells, oryzalin was shown to increase the percentage of cells arrested in metaphase by 14.7% while colchicine only increased by 8.1% (Ramulu et al. 1991). Oryzalin also specifically inhibits plant tubulin assembly and has no effect on the assembly of animal tubulin at the concentrations that inhibit plant tubulin. Since the discovery of its potential for polyploid formation, oryzalin has been used to induce polyploids in many crop and ornamental species. Oryzalin is also preferable to colchicine, which has an affinity to animal tubulin over plant tubulin (Wan et al. 1991).

Oryzalin has been used to restore fertility to sterile hybrids. Interspecific hybrids of *Lilium longiflorum* x Whilito and *L. henryi* x *L. candidum* were induced to form polyploids using various levels of oryzalin. Concentrations ranging from 0.005-0.01% induced several levels of ploidy in embryo culture (van Tuyl et al. 1992). Oryzalin was also noted to have a less inhibiting effect on cell growth than colchicine and tetraploid plants came into flower and showed restored fertility. (van Tuyl et al. 1992). In the same experiment, bulblets of *Nerine bowdenii* 'Albivetta' were induced to form polyploids by treatment with 0.01% oryzalin while treatments with colchicine proved lethal. Sixteen of 41 bulblets treated formed polyploids including 13 tetraploids (van Tuyl et al. 1992). Similar results were attained in gynogenetic haploids of *Gerbera* where 120-480 μM produced a maximum number of 59-61% di-haploid nuclei without the phytotoxicity found in colchicine cultures (Tosca et al. 1995).

Oryzalin has also been successful in producing double haploids from callus derived from anther microspores. Breeders use this technique to accelerate development of desirable cultivars (Hassawi et al. 1991). The induction of double haploids via

microspore callus treatment with oryzalin has been investigated in several crop species. Although oryzalin was effective in inducing chromosome doubling in maize microspore culture it had an inhibitory effect on growth and regeneration of callus and plant regeneration (Wan et al. 1991). On studies done with wheat, oryzalin was found to produce healthy double-haploid plants with viable seed, however, it was less effective than colchicine (Hassawi et al. 1991).

In vitro grown apple shoots have also been induced to produce polyploids using oryzalin. In *Malus domestica* shoots of two cultivars produced *in vitro* were subjected to 5, 15, and 30 μM oryzalin in agarose medium. Survival rate of explants was very high (93.8-100%) and efficiency of polyploidation ranged from 75-100% with efficiency decreasing with increasing oryzalin concentration (Bouvier et al. 1994).

Leaf tissue has also been utilized as an explant for polyploid formation using oryzalin. Leaves of kiwifruit, *Actinidia deliciosa*, trihaploids were treated with agar solution containing oryzalin or colchicine for 30 days. Colchicine, at all concentrations tested, proved toxic to leaf segments, while leaves treated with oryzalin as high as 30μM had a survival rate of up to 81.6% with increasing mortality with increasing concentration. None of the concentrations of oryzalin prevented adventitious shoot formation. Many polyploids were obtained from oryzalin-treated leaf tissues also including 42% hexaploids (Chalak et al. 1996).

Larger Inflorescences and Bracts and Polyploid Formation

Many characteristics have been investigated in plant breeding programs involving the mutagenic chemicals colchicine and oryzalin. Many improved traits involving color,

habit, disease resistance, and durability have successfully been bred into new cultivars. Success with breeding larger inflorescences with traditional techniques has been minimal at best, perhaps due to the large number of genes involved in inflorescence size. Blakeslee has used mutagenic chemicals such as colchicine and oryzalin to increase ploidy number and increase inflorescence size since the discovery of colchicine's properties in 1937. The first tetraploid daylilies bloomed in California in 1947. Now over 50, 000 selections of daylily have been developed with much of the crop improvement being done by using tetraploid selections (UACES 2004). Numerous patents have been given to floral crops made by chemical mutation using colchicine and Oryzalin. Balrufpurp™, a tetraploid petunia developed by the Ball Floral Plant Company (USPTO PP 11 928) is a tetraploid petunia cultivar with large red purple double flowers (Strope 2001). A tetraploid rhododendron, 'Northern Starburst' (USPTO PP 10, 388) was produced by Briggs Nursery Inc. using colchicine. This species produced large, vivid blossoms of varying shades of lavender/ pink, and was twice the size of the diploid form (McCulloch 1998).

Determination of Ploidy Level

Determination of chromosome number can be a very important tool in obtaining information about a species. The chromosome number can reveal several aspects about a species evolution and taxonomy. By comparing the chromosome number of closely related species, it can be determined whether polyploid or chimeric changes lead to the speciation. Since chromosome numbers are often highly conserved within genera, certain assumptions can be made about speciation events. Also induced polyploid formation

using mitotic inhibitors such as colchicine or oryzalin can be assayed and confirmed by chromosome number.

Chromosome Counting

The technique of manual chromosome counting using a light microscope has been used for many years to determine the chromosome number and ploidy of species. In order to examine chromosomes, cells must be in metaphase where the DNA is supercoiled into visible chromosomes. Highly mitotic root tip cells are harvested to ensure that many of the cells are in metaphase and the chromosomes are visible.

In this technique, root tip cells are harvested under the root cap, then fixed with an acetic acid-based fixative and stained. The stains used are a DNA specific stain such as Acetocarmine or Acetic-Orcein. Cells are squashed and observed under a light microscope. Cells in metaphase will have visible chromosomes and can be counted to evaluate chromosome number.

This type of ploidy evaluation has been used on numerous species and is still used today, both by itself to determine chromosome number and as a verification tool in use with other methods such as flow cytometry. It has been used to confirm ploidy in plants derived from somatic embryos to determine whether chimeras or polyploids had been formed during the process. Chromosome counting has been used to evaluate several populations of species to determine breeding strategies and genetic studies. Several natural populations of orchardgrass were evaluated for ploidy level and found to be tetraploid, thus indicating that diploid orchardgrass plants were rare or absent in the natural populations of that region (Tuna et al. 2004). Manual chromosome counting has

also been used to evaluate plants that have been induced to produce tetraploids by use of a mitotic inhibitor. In order to ascertain the effects of tetraploid formation on yellow-flowered cyclamens, tubers of the species were exposed to colchicine. Chromosome counting was used to evaluate ploidy level of the shoots from the exposed tubers (Takamura et al. 1996).

Chromosome counting is a very inexpensive and non-technical way to evaluate the chromosome number and ploidy level of species. Breeders and taxonomists use it extensively as a way to assess species. It is however, time consuming and tedious and has therefore become a secondary means of verifying ploidy levels in species which have been evaluated by other means such as flow cytometry.

Flow Cytometry

Flow cytometry is a method that quantifies components or structural features of cells by optical means. Flow cytometers were invented in the early 1970's and are often used for complete blood counts in clinical laboratories (University of Massachusetts, Amherst 2003). Over the past 10 years flow cytometers have been used widely in diagnosis and analysis of cancers, but this method may also be adapted for use with plants in cell cycle analysis to determine the capacity of cell cultures for dedifferentiation and rapid growth (UMass Amherst 2003). Flow cytometry can also be used to determine the ability of cell cycle inhibitors used to increase secondary metabolite accumulation by reducing vegetative growth. Ploidy of a plant or cell can also be determined using flow cytometry. This is important in cell cultures where polystomaty is common, and in

analyzing the results of whole plants that have been exposed to mutagenic chemicals in order to induce polyploid formation (UMass Amherst 2003).

In order for the cells or cell components to be analyzed by the flow cytometer they must first be in a single cell (component) suspension. The suspension is then brought into the flow cytometer and focused into a single particle stream. The stream is then passed across the focus of a laser. The particles in the stream scatter the light or, if a fluorescent dye is added to the suspension, fluoresce at a certain wavelength. The scattered light or fluorescence is collected by an optical lens and passed through optical filters, which will pick up the fluorescence wavelength or scattered light particles to be examined and filter out the remainder of the light. Detectors will convert the light pulses to electric current pulses, amplify the signal and display it as a histogram. The data displayed on the histogram is a relative content that can be compared to a standard DNA content or ploidy level in order to determine sample content or ploidy (UMass Amherst 2003).

Flow cytometry has been used for many purposes in plants and has potential for many future uses. One of the most obvious uses of flow cytometry in plants is evaluation of the 2C nuclear DNA content. Comparing the position of the DNA histogram peak of the sample to a reference standard such as chicken red blood cells or plant nuclei of a ploidy level size helps determine DNA content. The DNA content of a plant cell can be useful in determining both phylogeny, and genome structure, particularly the degree of repetitive sequences. Genome size analysis of several varieties of *Agave tequilana* revealed a conserved genome with a loss of certain DNA sequences after polyploidization. This information would be useful in taxonomy, determining breeding

techniques and biotechnological applications to this genus (Palomino et al. 2003). Flow cytometry can also be useful in determining whether genome size has been altered due to somaclonal variation in dedifferentiated plant cell cultures. Somaclonal variation is very common in cultured plant cells and may be due to many influences such as altered nutrient levels or light quality (Yanpaisan et al. 1999). Wilhelm et al. reported the use of flow cytometry to monitor the mutability of somatic cell lines of various *Quercus* species and found relatively stable culture lines with tetraploid formation only in older culture lines (Wilhelm 2000).

Flow cytometry is most commonly used as a tool for confirming polyploid plant formation through breeding. Polyploid plants produced by breeding can be assayed according to their nuclear DNA content using the flow cytometer. Sexually derived polyploids of hop were verified using flow cytometry in order to ascertain which plants were tetraploid. These tetraploids were used by breeders to form seedless, triploid hop plants by crossing with diploid species (Beatson et al. 2003).

Flow cytometry has also become a significant tool in evaluating species derived from artificial polyploid induction. Flow cytometry is used to evaluate doubled haploid cultures derived from cultured seeds or anther cultures exposed to colchicine. *Malus x domestica*, apple, does not inbreed well because of self-incompatibility and spontaneous doubling very rarely takes place. Bouvier et al. used mitotic inhibitors to artificially double chromosome number in order to produce species of doubled-haploids. Haploid apple shoots resulting from immature seed culture obtained by pollination with gamma irradiated pollen were exposed to mitotic inhibitors by dipping them in dilute solutions for certain lengths of time. Results were determined by flow cytometry as well as by

chromosome counting. A protocol for chromosome doubling of these haploid shoots with minimal chimeric frequency using oryzalin at a concentration of 0.01 μM (Bouvier et al. 1994) was developed. Species of *Buddleia globosa* were induced to form tetraploids using colchicine. Using flow cytometry, these plants were confirmed to be tetraploid and mixoploid (Rose et al. 2000).

Flow cytometry is increasingly becoming a valuable tool for breeders and taxonomists alike. This technique allows for quick cytological determination of both DNA content and ploidy with minimal preparation time and a small amount of tissue.

Transformation of Euphorbia pulchurrima

Agrobacterium-mediated Transformation

The basic principle behind Agrobacterium-mediated transformation is the use of the bacterium *Agrobacterium tumefaciens* and its natural transformation process to insert foreign DNA into plant tissue. *Agrobacterium tumefaciens* is a Gram-negative soil bacterium that causes crown gall disease in many plants. A list of host plants was compiled in 1976 and included 596 dicotyledons, 42 gymnosperms and 5 monocotyledons (de Cleene et al. 1976). The bacterium infects the plant through a wound in the stem or roots and inserts a portion of its own Ti (Tumor inducing) plasmid DNA into the nuclear DNA of the plant. The bacterial DNA transferred, called the T-DNA, is stably maintained in the genome of the transformed cells. Most of the genes that are needed for the transfer, integration, and maintenance of bacterial infection are contained in the Ti plasmid. Included in the Ti plasmid is a region of DNA called the T-DNA

region that is specifically transferred into the plant cells and inserts itself into the plant genome. The T-DNA carries genes that are responsible for tumor formation as well as genes that produce opines, which are used by the bacterium as a carbon and nitrogen source. Only the border regions of the T-DNA region is necessary to transfer foreign DNA into the plant genome, therefore the T-DNA regions can be "disarmed" by removing the tumor formation genes and all but the border regions. This "disarmed" plasmid can then be used as a vector for inserting desired foreign genes into plants (Zambryski et al. 1983).

The first transgenic tobacco plants produced by Agrobacterium-mediated transformation were created in 1983 in Belgium (Herrera-Estrella 1983). This was a crucial step in developing a protocol for inserting foreign DNA into plants. However many of the most important cereal crops were unable to be transformed in this method until the 1990's (Hiei et al. 1994; Cheng et al. 1998). Today Agrobacterium-mediated transformation has been used to transform over 120 species including economic crops, vegetables, fruit and forest trees, ornamental plants, and medicinal crops (Birch 1997). Numerous genes influencing agronomic and horticultural traits have been introduced into crops using Agrobacterium-mediated transformation. Flower color was changed as well as color pattern in torenia (*Torenia fournieri)* by transferring either the chalcone snythase (CHS) or dihydroflavonol-4-reductase (DFR) gene (Aida 2000). Resistance to powdery mildew was conferred to roses (*Rosa hybrida* cv. Carefree Beauty) by inserting the ACE-AMP1 antimicrobial protein gene (Li 2003). Delayed flower senescence was achieved in petunia by inserting the IPT gene under the control of the SAG12 senescence-associated gene (Chang 2003).

Particle Bombardment for Genetic Transformation of Plants

The basic methods of particle bombardment involve coating microscopic metal particles with genetically engineered DNA and shooting these particles into living plant cells with the hope of stably integrating the engineered DNA into the plant genome. Sanford first demonstrated the use of high velocity projectile bombardment to introduce foreign materials into plants in 1987 using onion epidermal cells. A modified bullet gun was used and transient expression of CAT activity was verified after 3 days (Sanford et al. 1987). The first stable transformations were reported using immature soybean embryos (Christou et al. 1988, 1989). The tissue was bombarded using an electrical discharge apparatus and protoplasts were isolated from the tissue. These tissues proved to be transgenic although no plants were regenerated from the tissue (Christou et al. 1988, 1989). Soon after, stable transgenic soybeans plants were recovered from intact meristems (McCabe et al. 1988). After transformed tobacco (Klein et al. 1988a) and maize (Klein et al. 1988b) callus were recovered, the system was validated as being applicable to other species.

There are several variables that are to be considered in order to achieve stable transformation using this method. The first is the type of metal to be used as a particle for bombardment. The particle must be of large enough mass to reach high velocity and penetrate cellular tissue. The particle must also be inert so that it will not interact with the engineered DNA or the target cell. Some of the metals that can be considered for this method include gold, tungsten, palladium, rhodium, platinum and iridium. The type of DNA used as well as the concentration is also very important. Both single-stranded and

double-stranded DNA have been successful and concentration of DNA to be applied is very species dependent. The type of tissue to be bombarded also has to be considered. These cells not only have to be capable of transformation, but also have to be able to be regenerated into whole plants. Equally important to tissue viability is depth of penetration into the cells. Too shallow a penetration may not transform tissues, while too deep a penetration might lead to irreparable tissue damage. Other considerations for particle bombardment include environmental factors such as temperature photoperiod and humidity and biological factors such as culture conditions that affect plant regeneration from the bombarded tissues or cells (Christou et al. 1996).

There are many varieties of instruments for bombarding plant cells. The first instrument used was a gunpowder device (Sanford et al. 1987), there have also been instruments using electrical discharge (Christou et al. 1990), and most the most common commercially made gun, a compressed helium version (Christou et al. 1996). Each of these instruments has its own pros and cons to its use, of which it is left to the scientist to choose.

Applications of Plant Transformation in Euphorbia sp.

There are many different applications and incentives for genetic manipulation of plants through Agrobacterium-mediated transformation or particle bombardment. The first application is the transfer of novel genes or traits into plants. Transformation has allowed scientists to introduce traits from one plant to another in species that would otherwise be unable to reproduce through breeding. For example, Agrobacterium-mediated transformation was used to transform *Oncidium* orchid plants with sweet

pepper ferredoxin-like protein (*pflp*) cDNA. This protein is thought to function as a natural defense in against microbial infection. This protein may confer resistance to soft rot disease, caused by *E. carotovora*, in Orchids. Transformation with *pflp* cDNA showed enhanced resistance to *E. carotovora*, even when the entire plant was challenged with the pathogen (Liau et al. 2003).

Agrobacterium-mediated and biolistic transformation methods have also allowed scientists to insert novel traits and genes into plants from other sources such as virus and bacterial DNA. The bacterium *Bacillus thuringiensis* produces a protein that is very toxic to certain moths and butterflies. This gene has been inserted into many species to confer insect resistance to moths (Anderson, 2003). In tobacco, the gene for a toxin produced by *B. thuringiensis* var. berliner conferred resistance to tobacco hornworm infestation (Vaek et al. 1987). Resistance to abiotic stresses can also be conferred to plants through transformation. Lee et al. produced heat-tolerant *Arabadopsis* plants by altering the expression levels of heat shock protein factor (AtHSF) (Lee et al. 1995).

Transformation has also been utilized to make improvements in quality and yield. Exploration of the metabolic pathways of plants using transformation has allowed a better understanding of synthesis of valuable compounds. Using this information, scientists have been able to manipulate these pathways to improve product quality and yield. Calgene, USA inserted the antisense RNA against the polygalacturonase (PG) enzyme-encoding gene into tomato to produce a product with a longer shelf life named "Flavr Savr" (Chawla 2002). Many experiments are underway to improve crop yield and quality including greater oil yield in soybean as well as increased iron content.

Through genetic transformation plants can also be engineered to be production systems for pharmaceutical products. Mason et al. transformed tobacco with the hepatitis B surface antigen (HbsAg), which showed a low level of expression (Mason et al. 1992). An edible plant vaccine for diarrhea has been expressed in potato, and these plants are already in human trails (Thaket et al. 1998). Because the vaccine is denatured by heat, new experiments are being done to introduce these types of genes into bananas (Chawla 2002). With the new technology of transformation, plants can be made to produce many vaccines and pharmaceutical drugs, as well as overproduce natural products that are beneficial to mankind such as taxol.

Relatively limited experimentation has been done on transformation of *Euphorbia pulchurrima*. Vik et al. (2000) experimented with the uncommon transformation technique DNA electrophoresis of genes into the apical meristem in poinsettia. Nine of 34 shoots were transformed and showing GUS expression. Other Euphorbia species have been successfully transformed using *Agrobacterium rhizogenes*. In an effort to increase terpene production in roots, *Euphorbia lathyris* was transformed with the *Agrobacterium rhizogenes* strain ATCC 15834 (Cheetham et al. 1996). Stems were scratched with a scalpel containing *A. rhizogenes* and the wounds regenerated callus and roots in 14-28 days. Roots were excised for verification of transformation using 2D thin layer chromatography. Cultured roots were found to have produced more bioactive compounds both in quantity and variety. The transformed cultures of this *Euphorbia* were genetically stable and grow rapidly in culture on medium but failed to regenerate into whole plants (Cheetham et al. 1996). Transformation of *Euphorbia pulchurrima* was achieved using particle bombardment with the genes encoding for various Magainin and PGL classes of

antimicrobial peptides. Stable transformation was achieved and shoots showed increased resistance to Botrytis, Rhizoctonia, Phytophthora, and powdery mildew (Smith et al. 2001).

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PART 3

AXILLARY BUD PROLIFERATION AND ORGANOGENESIS OF

EUPHORBIA PULCHURRIMA 'WINTER ROSE'™.

ABSTRACT

Protocols for both axillary bud proliferation and shoot organogenesis of *Euphorbia pulchurrima* 'Winter Rose'™ were developed using terminal buds and leaf tissues. Greenhouse grown terminal buds were placed on Murashige-Skoog (MS) medium supplemented with various concentrations of either benzylaminopurine (BA) or thidiazuron (TDZ). Explants produced the greatest number of axillary buds on media containing between 2.2-8.8 μM BA. The number of explants that produced axillary buds increased with increasing BA concentration. TDZ at concentrations between 2.3-23.0μM caused vitrification of shoots and were not effective in promoting shoot proliferation. The most callus and shoots were produced from leaf midvein sections from in vitro grown plants placed on medium containing 8.8-13.3 μM BA and 17.1μM IAA for one month before transfer to medium containing only BA. Adventitious buds were produced only from red-pigmented callus, and explants that produced callus continued to produce adventitious shoots in the presence of IAA. Five-month-old shoots derived from shoot culture or organogenesis rooted readily in artificial soil with or without treatment with IBA, and were acclimated in the greenhouse.

Key Words: poinsettia, terminal buds, regeneration, organogenesis, leaf sections

INTRODUCTION

Euphorbia pulchurrima, poinsettia, is an important winter holiday symbol in many parts of the world including North America and Europe. More than 67 million pots of poinsettias, worth approximately $246 million dollars at wholesale, were sold in 2001 in the US (USDA Economics, Statistics and Market Information System http://usda.mannlib.cornell.edu/, 2002). Sales of poinsettia were rated number one among all flowering potted plants in the United States (http://www.urbanext.uiuc.edu/poinsettia/facts.html 2004). The popularity of poinsettias may be due to the diversity of color in foliage and bracts (Hartley 1992) found in the more than 100 cultivars (USDA Agricultural Research Service 2001). The 'Winter Rose'™ family of poinsettias was bred by Paul Ecke Ranch and was first introduced in 1999 by Hans Fruewirth after 30 years of breeding and trials (www.poinsettias.com 2002). This group of cultivars has characteristic rose-like 'flowers', incurved bracts, and a globe-shaped flower head with a full color series. They are also very popular due to their unusual form.

Poinsettias are propagated vegetatively by use of terminal stem cuttings that root readily in the greenhouse and yield a consistency of color and form in each cultivar (Ecke et al. 1990). Few studies have been done on the in vitro culture of poinsettia. In one study, seeds of E. pulchurrima were sterilized and cultured on modified White's basal medium (WBM) with or without plant growth regulators (Nataraja et al. 1973). Results indicated that basal medium, without plant growth regulators, was the most suitable with a 90% germination rate. Growth regulators such as 6-benzylaminopurine (BA), N^6- [2-

isopentenyl] adenine (2iP), and 2,4-dichlorophenoxyacetic acid (2,4-D) reduced germination rates or prohibited germination and produced callus (Nataraja et al. 1973). Further experiments with seeds of poinsettia on WBM medium containing various concentrations and combinations of coconut milk, gibberellic acid (GA), indole-3-acetic acid (IAA), kinetin, BA, and 2,4-D produced callus of which several developed embryoids that later developed into plantlets (Nataraja 1975). Shoot regeneration from red callus was achieved with internode explants cultured on medium containing, low IAA concentrations coupled with higher concentrations of kinetin. Shoots were easily rooted in basal WBM medium (de Langhe et al. 1974). Using internode and petiole explants from greenhouse grown plants, shoots were also produced on media containing high ratios of 2iP to naphthalene acetic acid (NAA) (de Langhe et al. 1974). These experiments revealed that in vitro seed germination and plant regeneration of poinsettia were feasible, however research results were not published describing micropropagation from terminal or axillary buds, or leaves of in vitro cultured plants.

We describe in vitro micropropagation and plant regeneration of Winter Rose™, a popular commercial poinsettia cultivar that may be used to enhance breeding programs by implementation of in vitro mutation breeding and genetic transformation.

MATERIALS AND METHODS

Plant Material and General Culture Methods

Shoot tips of *E. pulchurrima* 'Winter Rose'™ were obtained from vigorously growing plants in the greenhouse. Soft terminal buds were removed along with a portion of the stem. Surface sterilization was achieved by soaking in a 1% Tween 20 solution for five min, followed by dipping in 70% ethyl alcohol for one min, and soaking in 20% commercial bleach containing 5.25% sodium hypochlorite solution for 15 min. Plant organs were rinsed three times (5 minutes each) with sterile water. A 1-cm portion of the stem apex was excised and placed on medium containing Murashige and Skoog salts (MS) (Murashige and Skoog 1962) with the addition of 100 mg/L myo-inositol and 3.0% sucrose. The pH of the medium was adjusted to 5.7-5.8 and agar powder was added (0.7% w/v) prior to being autoclaved. One shoot was placed per baby food jar (100 ml) containing 40 ml of medium. All cultures were subcultured to fresh medium every 4 weeks. Unless otherwise specified, cultures were maintained in a growth room at 25°C under a 16 h photoperiod (16 h light/ 8 h dark) with illumination of 125 μmol mol^{-2} s^{-1}.

Effect of Plant Growth Regulators on Axillary Bud Proliferation

Surface sterilized apical buds of greenhouse-grown plants were cultured on media containing 2.8 μM IAA combined with one of four concentrations of BA (0, 2.2, 4.4, or 8.8 μM BA) for one month (Table 3.1). Five explants were included in each

treatment and the experiment was replicated once. After one month, explants were placed on media containing only BA for the remainder of the experiment. Explants were evaluated after four and eight weeks. Data on number of shoots and height, callus production, percent rooting were collected after four and eight weeks.

Apical buds were also cultured on media with 0.0, 0.4, 2.3, 4.5, and 22.7 μM TDZ. One shoot tip was transferred to each of ten replicate vessels and the experiment was replicated. Explants were evaluated after one month for number of shoots, percent producing callus, rooting percentage and height.

Plantlet Formation from Microshoots

Shoots were removed from cultured organs and rinsed thoroughly with distilled deionized water. Callus tissues and roots present at the base of the plant were removed. Shoots were then exposed to 0, 245, or 490 μM of indole butyric acid (IBA) dissolved in distilled water by dipping the basal 1-5 mm section for five sec. Shoots were placed in a sterile potting mix (Premier Pro-Mix, Premiere Horticulture LTD, Red Tail, PA) in 5-cm diameter rose pots and placed in a covered container. Explants were acclimatized to ambient conditions by progressively opening the lid and increasing size of opening over one week. Each treatment contained ten microshoots, and the experiment was conducted twice. Heights of plants were measured every four weeks. At the end of three months, plantlets were removed from the potting mix, the roots were removed from plantlets and examined for total length, surface area, volume and average diameter using a modified scanner with Win-Rhizo software (Regent Instruments Inc., CA).

Adventitious Shoot Proliferation

Leaves from in vitro grown plantlets were cut into 1 cm sections containing a portion of the midvein. Midvein sections were placed on media containing one of four treatments listed in Table 3.2. Each treatment consisted of four petri plates with eight leaf sections per plate and the experiment was repeated. After one month, leaf sections were transferred to media containing respective concentrations of BA, but without IAA. Explants were evaluated every four weeks for the presence of callus and/or shoots. Association of callus color with organogenesis was also noted. The experiment was terminated after 16 weeks.

Experiments were also conducted using leaf explants on media containing 2,4-D (0.0, 2.3, 4.5, 9.0, and 18.1 μM). Each treatment consisted of four petri plates with eight explants per plate. Explants were evaluated every four weeks for number of explants with callus, and number of shoots. The experiment was terminated after 16 weeks.

Histology

Callus tissue with shoot masses from organogenesis experiments were fixed in a 50% FAA (5 ml 37% formaldehyde, 5 ml glacial acetic acid, 90 ml of 55% ethanol) solution and then dehydrated in an isopropyl alcohol series before paraffin infiltration. Paraffin sections (10 μm) were obtained using a Histocut microtome (Reichert-Jeng) and placed on slides. A modified triple stain series consisting of Safranin O, Crystal Violet

and Fast Green (Johansen 1940) was used to stain tissue for observation under a light microscope.

Statistical analysis

The randomized block design was used for all experiments and data were analyzed by the analysis of variance (ANOVA) procedures of the Statistical Analysis System (SAS Institute 1995). Means were analyzed using Tukey's Studentized Range (HSD) at $P<0.05$. Percentage data were normalized with arcsin transformation before analyzing.

RESULTS AND DISCUSSION

Effect of Plant Growth Regulators on Axillary Bud Proliferation

White callus formed from the cut surface and roots emerged from the callus on medium containing only IAA (Figure 3.1A). Explants grown on media containing BA produced significantly more calli than those grown on medium without BA (Table 3.1). Both red and white callus tissues were produced and continued to grow regardless of the presence of IAA in the medium. In media containing BA, shoots emerged from axillary buds on the explant (Figure 3.1B-D) and continued to produce new axillary shoots along with callus. The number of explants producing axillary shoots increased with increasing concentrations of BA, however there were no significant differences in the number of shoots produced by each explant (Table 3.1). Similar results were reported with *E. peplus* where nodal segments produced large amounts of callus and shoots when grown on MS medium supplemented with 10 μM BA and 0.5 μM NAA (Tideman et al. 1981). There were no significant differences in the heights of the shoots. Only shoots grown on medium without plant growth regulators produced roots, similar to results found in *E. fulgens* (Zhang et al. 1987) where in vitro grown shoots rooted best on basal medium and the addition of NAA to the medium had a negative effect on rooting. The rapid rooting of *in vitro* grown shoots may be due to indigenous auxin in the shoots that was sufficient to induce rooting in basal medium.

Apical buds placed on TDZ containing medium did not produce any axillary buds or elongated shoots (data not shown). Explants vitrified and ceased to grow when placed

onto medium containing TDZ, whereas explants on medium without TDZ continued to develop. TDZ is very active in tissue culture at low concentrations (Beyl 2000) and high concentrations of cytokinins can cause rooting difficulty and vitrification.

Plantlet Formation from Microshoots

Shoots treated by dipping into water or into an aqueous solution of 245, 490 μM IBA produced numerous roots, and the plantlets appeared healthy. Data recorded with the modified digital scanner and Win-Rhizo software showed no significant difference in any of the variables measured (data not shown). There were no significant differences in the heights of the plants.

Adventitious Shoot Regeneration

Initial experiments with adventitious shoot induction revealed that combinations of BA from 8.8 to 13.3 μM and IAA from 5.7 to 11.4 μM induced red callus and organogenic callus masses in cultured *E. pulchurrima* explants (data not shown). In further experiments (Table 3.2) only the explants on medium containing 8.8-13.3 μM BA and 17.1 μM IAA produced organogenic masses (Fig 3.2A). The results also showed that media with 8.8-13.3 μM BA and 17.1 μM IAA most effectively induced red callus and is consistent with results reported by de Langhe et al. (1974) with petiole explants. All shoots were formed from red callus, none appeared from white callus. This is similar to results seen with shoots developed from internode explants of poinsettia (de Langhe et al. 1974). Shoots formed after transfer of masses of callus tissue to basal medium without

plant growth regulators. The regenerated shoots were removed from callus and placed on basal medium to form roots and remaining callus was subcultured to produce more plantlets in culture (Fig 3.2B). Equimolar concentrations of cytokinins to auxins are generally used to maintain callus, whereas higher cytokinin to auxin ratios tend to induce shoot development. The results observed were consistent with known physiological effects of exogenous BA application and with other experiments with *E. pulchurrima* (de Langhe et al. 1974).

Experiments were also conducted to assess the effects of 2,4-D concentrations on callus and shoot formation in explants. The presence of 2,4-D in the media caused explants to produce exclusively white, non-uniform callus regardless of subsequent treatments (data not shown). Since the red callus tissue characteristic of this species prior to organogenesis did not develop, 2,4-D at the concentrations applied was undesirable for inducing organogenic callus tissue in poinsettia.

Histological survey

Histological sectioning of the callus and bud masses revealed that buds were adventitious in origin. Regions of localized mitotic activity within the outermost layers of callus formed into globular green protrusions (Figure 3.2C), with a meristemoid-like organization. These protrusions developed on the surface of the original explant and had an epidermis contiguous with the original explant. These globular green meristemoids, when placed on basal medium, developed into buds with well-defined apical meristems

and leaf primordia (Fig 3.2D). These buds elongated into shoots on the callus and these shoots could be removed from callus mass and rooted in basal medium.

These experiments demonstrate a rapid and reproducible protocol for the in vitro propagation of *E. pulchurrima* 'Winter Rose'™ via enhancement of axillary branching. This is the first report of axillary bud proliferation from apical buds of poinsettia. A protocol for adventitious shoot induction from in vitro derived leaf tissues, containing a portion of the midvein, of poinsettia is described. Latex had little effect on growth and development of poinsettia plants in vitro. This protocol can be used to produce consistent, sterile poinsettia tissues and organs that may be applied to plant improvement or genetic manipulation. Adventitious shoot formation from leaf meristem sections can be reliably produced via organogenesis. These shoots can be elongated, removed and then transferred to soil for rooting. This organogenesis system may be used for genetic transformation or other in vitro genetic manipulations, such as *in vitro* selection and mutagenesis.

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Table 3.1: Axillary Bud Proliferation in *Euphorbia pulchurrima* 'Winter Rose'™

BA (μM)	Average Shoots per Explant	% Explants with Callus	Explants with Roots
0.0	2.0a	11.6a[x]	2.5a
2.2	2.2a	70.8b	0.0b
4.4	2.6a	84.6b	0.0b
8.8	2.7a	100.0b	0.0b

[x]Means separation in columns by Tukey's Studentized Range (HSD) at P< 0.05

Table 3.2: Effect of Plant Growth Regulators on Callus and Shoot Formation from Leaf Sections of *Euphorbia pulchurrima* 'Winter Rose'™

PGR (μM)		% Explants w/Callus	% Explants w/Shoots
BA	IAA		
0.0	0.0	0a[xy]	0a
13.3	11.4	88.2b	3.70a
13.3	17.1	88.2b	14.8a
8.9	17.1	88.2b	36.7a

[x]Means separation in columns by Tukey's Studentized Range (HSD) at P< 0.05
[y]Percentage data subjected to arcsin tranformation

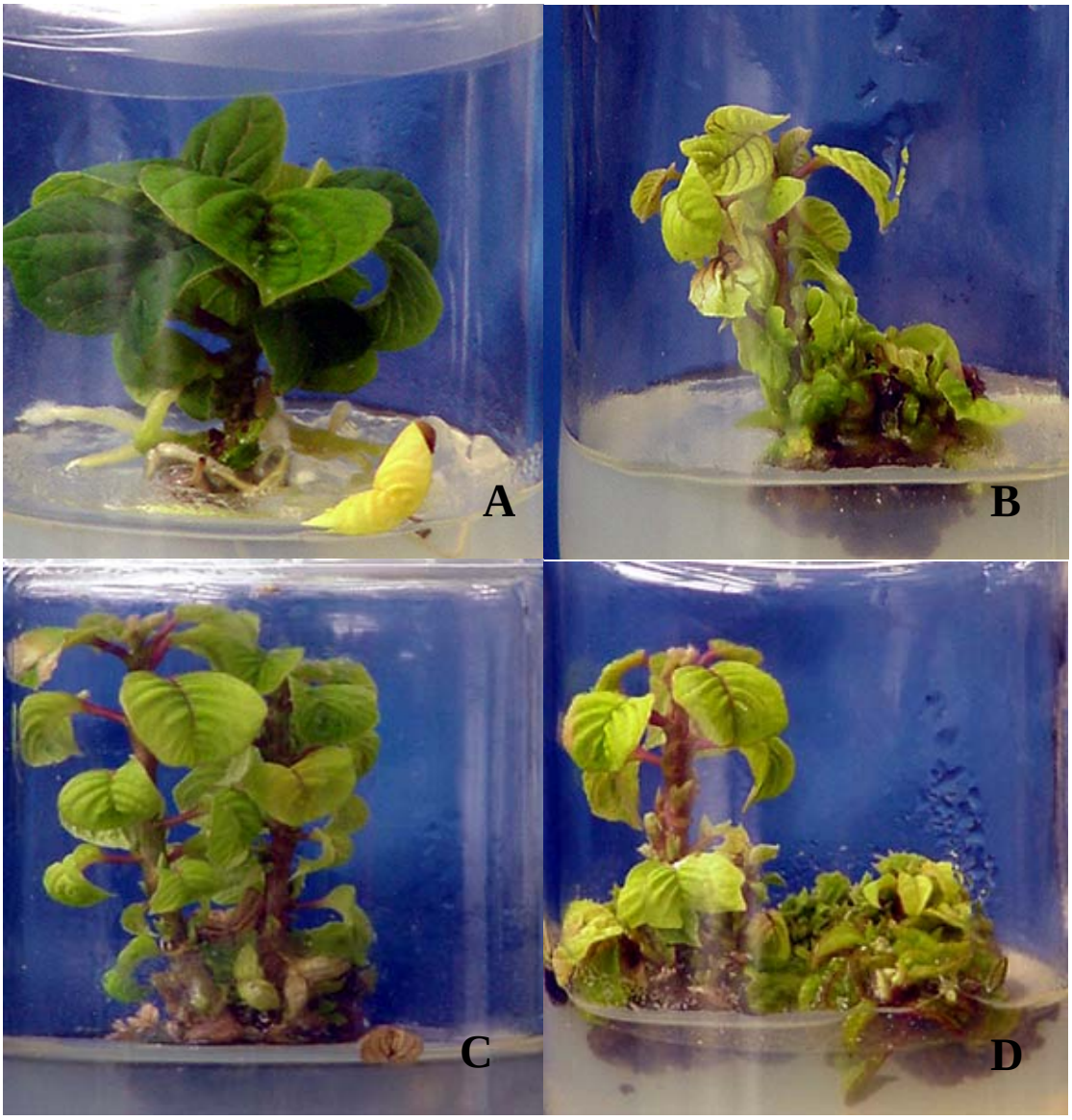

Fig. 3.1. Axillary bud proliferation of *Euphorbia pulchurrima* 'Winter Rose'™. Explants grown on medium containing no plant growth regulators (panel A), noticing single shoot and many roots. Containing 2.2 μM BA (panel B) producing axillary shoots. Containing 4.4(panel C) and 8.8 (panel D) μM BA showing more axillary shoot proliferation and no roots.

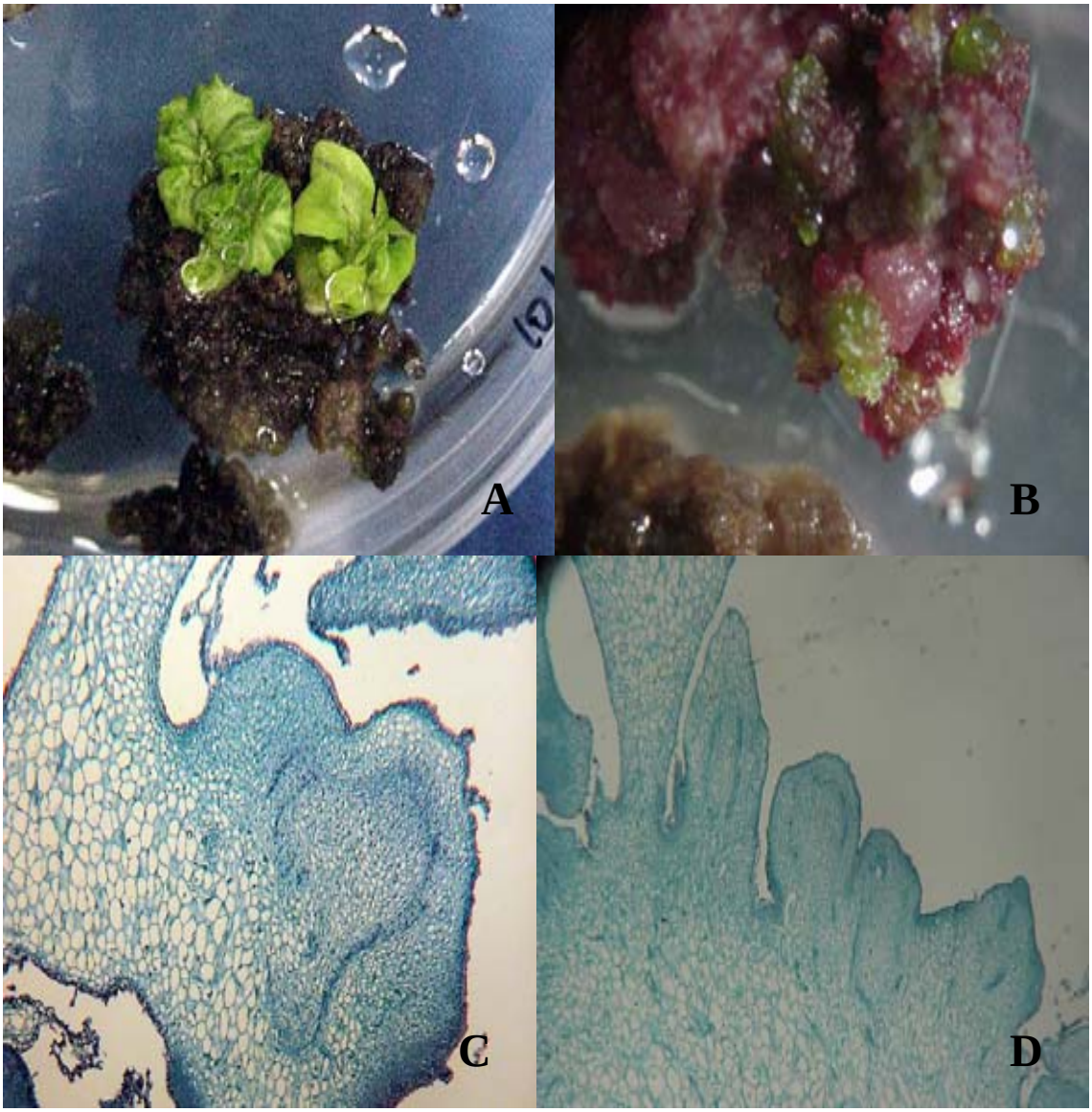

Fig. 3.2. Adventitious shoot formation from *Euphorbia pulchurrima* 'Winter Rose'™. A, Green shoot primordia arose from reddish callus; B, Elongating Shoots; C, A section of the shoot primordia showing multiple meristems; and D, Section of shoot primordia showing vascular tissue connection with original explant and origin of the shoot primordia.

PART 4

EFFECTS OF COLCHICINE AND ORYZALIN ON CALLUS AND ADVENTITIOUS SHOOT FORMATION AND TETRAPLOID FORMATION OF *EUPHORBIA PULCHURRIMA* 'WINTER ROSE'™

ABSTRACT

The mitotic inhibitors colchicine and oryzalin were evaluated for their effects on callus and adventitious shoot formation and tetraploid induction of *Euphorbia pulchurrima* 'Winter Rose'™. *In vitro* grown leaf midvein sections were placed on various media supplemented with either colchicine or oryzalin at various concentrations for 1 to 4 days. Colchicine was least damaging to leaf tissues at concentrations of 0.25 μM or 250.4 μM. A large amount of callus as well as adventitious shoots were produced. Regenerated shoots were found to be diploid determined by flow cytometry. On media with oryzalin (28.9 μM to 144 μM) leaf tissues produced callus, but not adventitious shoots. Callus was tested using the flow cytometer and was found to be diploid.

Key Words: poinsettia, diploid, regeneration, colchicine, oryzalin, tetraploid

INTRODUCTION

Poinsettia, *Euphorbia pulchurrima*, is an important holiday symbol and is the number one flowering potted plant in the United States (University of Illinois Extension Poinsettia Pages). In 2001 alone more than 67 million pots were sold in the United States worth an approximate wholesale value of $246 million dollars (USDA Economics, Statistics and Market Information System 2002). *Euphorbia pulchurrima* 'Winter Rose'™ is an unusual cultivar developed by the Paul Ecke Ranch after 30 years of breeding and trials (poinsettias.com). This cultivar is characterized by having incurved bracts along with the true flower, forming a rose-like flower head and comes in a full color series. Because of this remarkable characteristic it has become a very popular cultivar. Recent cultivar evaluations have shown that 'Winter Rose'™ is a minimal branching cultivar and thus produces a low number of flower heads without pinching (Harkess 1997). The 'flowers' are also typically smaller than those of other cultivars of poinsettia due to the incurved bracts. This can be a problem for both consumer and growers alike. Growers might find it hard to ship taller plants without breakage, and consumers may not buy poinsettias with few flower heads.

Colchicine is an alkaloid found in the autumn crocus (*Colchicum autumnale*) and is known to be a natural mitotic inhibitor by inhibiting the development of spindle fibers therefore preventing cell division (Eigsti 1955). Colchicine has been employed for the treatment of rheumatism and arthritis by ancient Greeks, and is approved by the FDA to treat gout. Blakeslee and Avery were the first to report on the use of colchicine to inhibit

spindle formation in plants, therefore inducing chromosome doubling and tetraploid formation. Blakeslee evaluated many methods for applying colchicine as well as various concentrations. The effects on species including *Datura*, *Portulaca*, *Petunia*, *Zea*, *Allium*, and *Cucurbita* were also examined. Resulting plants generally had greater inflorescences, larger fruit and pollen size, and shorter stems, however results vary according to species, methods of application, and concentration of colchicine (Blakeslee et al. 1934). Since then, colchicine has been applied both in situ and *in vitro* to induce polyploid formation in many ornamental species including *Gerbera jamesonii* (Tosca et al. 1995), and *Buddleia globosa* (Rose et al. 2001).

Oryzalin is a selective pre-emergence herbicide sold under many trade names including Dirimal, Ryzelan, and Surflan. It is used for control of annual grasses and broadleaf weeds in orchards, vineyards and established bermuda grass and ornamental plots (Atland et al. 2003). It is a surface-applied herbicide that inhibits the growth of germinating weed seeds by blocking cell division in the meristems (Extension Toxicology Network 1996). Oryzalin disrupts mitosis by inhibiting the formation of microtubules (Ramulu et al. 1991). Because colchicine has an affinity for animal tubulin over plant tubulin, oryzalin, which has no affinity for animal tubulin, is preferable to colchicine (Wan et al. 1991). Since the discovery of its ability to inhibit microtubule formation, oryzalin has been used to induce polyploids in several crop and ornamental species including *Lilium longiflorum* (van Tuyl et al. 1992) and *Gerbera* (Tosca et al. 1995).

Many characteristics have been investigated in breeding *Euphorbia pulchurrima*, including color, habit, disease resistance, and durability been bred into new cultivars.

Success with breeding larger inflorescences has been minimal at best, perhaps due to the large number of genes involved in inflorescence size. The use of chromosome doubling has been utilized in order to increase size of flowers, stems and leaves of many species, and has been used in poinsettia breeding to obtain new varieties. 'Pearl', a tetraploid sport developed by Peter Jacobsen for Ecke Ranch, was induced using a 1% in situ colchicine application to the cut surface of a vegetative stem after removal of the terminal apex (USPTO PP10, 160, 1997). This tetraploid mutant shows thicker stems, larger leaves and inflorescences as well as being self-branching and of a more compact height. Application of colchicine or oryzalin to *in* vitro *tissues* may be used to enlarge the inflorescences and reduce the height of 'Winter Rose'™ poinsettias, reduce the likelihood of chimeric tetraploids and provide a rapid means for producing many tetraploid plants.

The purpose of this research was to evaluate the effect of colchicine and oryzalin on callus and adventitious shoot formation of 'Winter Rose'™ poinsettia with *in vitro* grown leaf tissues and its potential for tetraploid induction.

MATERIALS AND METHODS

General Culture Methods for 'Winter Rose'™ poinsettia

Leaves were obtained from *Euphorbia pulchurrima* 'Winter Rose'™ in vitro shoots that were maintained in MS medium (Murashige et al. 1962) without plant growth regulators. Midvein sections, ~1cm square, were cut and placed on a callus induction medium (CI) in 15 mm petri dishes containing MS media with the addition of 555 µM myo-inositol, 3% sucrose, 8.9 µM benzylaminopurine (BA) and 17.1 µM indole-3-acetic acid (IAA). After for one month, cultures were transferred to shoot induction (SI) medium containing only BA. The medium was adjusted to a pH of 5.7-5.8 and solidified with 0.7% (w/v) agar prior to being autoclaved at 121ºC at a PSI of 2.9 Mpa for 20 min. Unless otherwise specified, cultures were maintained in a growth room at 25ºC under a 16-hour photoperiod (16h light/ 8h dark) with illumination of 125 µmol mol^{-2} s^{-1}. All cultures were subcultured to fresh medium every four weeks. The generated adventitious shoots were transferred from SI medium to baby food jars containing solidified basal MS medium for shoot growth and proliferation.

Effect of Colchicine Concentration and Duration on Callus and Shoot Formation of 'Winter Rose'™ poinsettia

Solid-solid medium. Leaf midvein sections were cultured on CI medium solidified with 0.7% agar. Colchicine was dissolved in deionized water and was added to

the medium at 0, 0.25, 2.5, 25.0, and 250.4 μM. Leaf explants were exposed to colchicine containing medium for 0, 48, and 96 hours, were then transferred to CI medium for one month from the time they were placed on colchicine-containing medium, then placed on SI medium for the remainder of the experiment. Each treatment contained seven plates, each plate contained five leaf explants and the experiment was conducted twice. Explants were evaluated for four months for callus formation, callus color, and shoot formation. After four months callus was determined for ploidy level using flow cytometry. Adventitious shoots were also grown up and leaves from shoots were determined for ploidy using flow cytometry.

Liquid-solid medium. Leaf explants were cultured on liquid CI medium containing one of five concentrations of colchicine (0, 62.6, 125.2, 187.8,and 250.4μM) for 0, 24, and 48 hours. Explants were washed with liquid basal MS medium, dried with sterile paper towels and placed on solidified CI medium for one month from the time they were placed on liquid medium, and then placed on SI medium for the remainder of the experiment. There were four replications of each treatment and eight explants per replication. The experiment was repeated once. Explants were evaluated for four months for callus formation, callus color, and shoot formation. Callus and adventitious shoots formed were evaluated for tetraploid formation after four months using flow cytometry.

Liquid-liquid medium. Leaf explants were cultured in liquid CI medium containing one of four concentrations of colchicine (0, 2.5, 25.0, 250.4 μM) for one of five duration times (0, 2, 4, 8, 16 days). The explants were then washed in basal medium and placed in liquid CI medium. After one month from the time they were placed on liquid medium, explants were placed in SI medium for the remainder of the experiment.

There were two replications per treatment and 8 sections per flask. Explants were evaluated for three months for callus formation, callus color, and shoot formation.

Effect of Oryzalin Concentration and Duration on Callus and Adventitious Shoot Formation of 'Winter Rose'™ poinsettia

Solid-solid medium. Leaf midvein sections were cultured on solid CI containing 0, 2.9, 14.4, and 28.9 μM of oryzalin, respectively. Oryzalin was dissolved in dimethyl sulfoxide, DMSO, before it was added to the medium. Leaf explants were exposed to oryzalin containing medium for 0, 2, and 4 days and were then transferred onto CI medium for one month from the time they were placed on oryzalin-containing medium, then placed on SI medium for the remainder of the experiment. Each treatment contained four petri plates, each plate contained eight leaf explants, and the experiment was repeated once. Explants were evaluated for four months for callus formation, callus color, and shoot formation. Callus was evaluated for ploidy level after four months using flow cytometry.

Liquid-solid medium. Leaf midvein sections were cultured on liquid CI medium in 125 ml Erlenmeyer flasks containing one of five concentrations of oryzalin (0, 28.8, 72.2, 115.5, and 144.3 μM). The flasks were placed on a shaker and shook at 100 rpm for 0, 24, and 48 hours. Explants were washed with liquid basal MS medium, dried with sterile paper towels and placed on solidified CI medium for one month from the time they were placed on liquid medium, and then placed on SI medium for the remainder of the experiment. There were four replications of each treatment and eight explants per

replication. The experiment was repeated once. Explants were evaluated for four months for callus formation, callus color, and shoot formation. Callus was evaluated for ploidy level after four months using flow cytometry.

Flow Cytometry to Evaluate Ploidy Level of 'Winter Rose'™ poinsettia

Leaf and callus preparation. Leaves and callus samples were prepared and stained using the CyStain® PI Absolute P staining kit (Partec GmbH., Műnster, Germany). Samples were prepared by using approximately 0.5 cm^2 of tissue and chopping with a double-sided razor in Extraction Buffer and allowed to incubate for ~1 min. Samples were strained through Falcon Tubes with Cell Strainer Caps (Fisher Scientific International) with 35 μm nylon mesh filters, and then stained with a staining solution containing RNAse, propidium iodide. After staining buffer was added, samples were incubated for one hour, then run through the flow cytometer. Samples were run on a Beckman Coulter Epics XL flow cytometer with a 488nm argon laser. Controls for this experiment were diploid 'Winter Rose'™ samples as well as two confirmed tetraploids 'Barbara Ecke Supreme' and 'Supjibi', obtained from Ruth Kobayashi, head breeder at Ecke Farms, Encinitas, CA.

Statistical analyses

Randomized block design was used for all experiments and data were analyzed by the analysis of variance (ANOVA) procedures of the Statistical Analysis System (SAS

Institute 1995). Means were analyzed using Tukey's Studentized Range (HSD) at $P<0.05$. Percentage data were normalized using arcsin transformation before analyzing.

RESULTS AND DISCUSSION

Effect of Colchicine Concentration and Duration on Callus Formation and Adventitious Shoot Formation of 'Winter Rose'™ Poinsettia

Explants cultured on agar-solidified medium, produced a large amount of white or brown callus. Significantly more explants produced callus when placed on medium containing 0 μM, 0.25 μM for 48 hours, and 2.5 μM of colchicine for 48 and 96 hours than those placed on medium containing 25.0 μM colchicine for 96 hours. There was no significant difference in number of explants with callus among other treatments (Table 4.1). There was no correlation between concentration of colchicine or duration of colchicine application and number of explants producing callus. Only on three media that contained colchicine did explants produce shoots. Explants cultured on media containing 0.25 μM or on media with 250.4μM colchicine (duration of 2 or 4 days) produced significantly more shoots than explants cultured on media with other concentrations of colchicine. Explants first produced the red callus indicative of adventitious shoot formation (Fig 4.1A). Green globulous organs with clear shoot and leaf primordia were observed after ~ 2 months of culture. Shoot primordia developed into adventitious shoots after 4 months of culture (Fig 4.1B).

Explants that were first cultured on liquid medium containing colchicine, then transferred to solid CI medium produced a large amount of white or brown callus. Significantly more explants cultured on media containing 250.4 μM colchicine for 2 days

produced callus than explants cultured on a medium with a lower colchicine concentration, 125.2 μM. There was no significant difference in the number of explants producing callus among other concentrations or durations of culture. Only explants exposed to colchicine at 62.6 μM for 24 or 48 hours and at 187.8μM for 24 hours produced red callus from which developed into red or green globulous organs and adventitious shoots developed (Table 4.2). There was no correlation between concentration or duration of exposure to colchicine and number of explants producing callus or those producing adventitious shoots.

Explants that were cultured exclusively on liquid medium enlarged when placed in liquid medium, but later turned brown. No callus was produced from either colchicine-containing cultures or from control cultures.

Colchicine is a highly toxic chemical and its dosage and exposure can be associated with high mortality rates and growth inhibition. Explants that were exposed to the lowest concentration of colchicine produced the greatest abundance of shoots in both liquid and solid mediums. The lower the dose of colchicine, regardless of the exposure time, the lower the toxicity of the chemical. These results are consistent with many studies including those with *Rhododendron* where higher concentrations of colchicine killed explants (Väinölä 2000).

The induction of shoots by explants exposed to high concentrations of colchicine (187.8 - 250.4μM) with no tetraploid induction was unexpected, because the concentrations have been reported to be high enough to induce tetraploids in many species such as potato (Teparkum 1998). However reports have also shown that some species may tolerate high levels of colchicine and may require very high concentrations

to induce tetraploid formation. In a study to optimize the *in vitro* chromosome-doubling procedure in wheat anther culture, Redha et al. (1998) found that exposure to 100 or 1000mg/l of colchicine for 1-3 days were needed to produce the greatest number of autotetraploid plants.

There have been other studies reporting problems with inducing tetraploid formation using colchicine. In studies with potato anther culture, explants were exposed to a range of colchicine concentrations (0-200 mg/l) produced no chromosome doubling. They indicated that this response may be due to secondary embryogenesis from inner tissue that may not have been exposed to colchicine (Teparkum 1998). Our results were consistent between experiments regardless of the type of medium used. Diploid shoots resulting from these explants may be due to regeneration of cells within the interior of the explant that were protected from the effects of the colchicine. This may also be due to a defense mechanism initiated by the plant when under stress conditions. Poinsettia is a plant that thrives under high stress conditions, and may have developed mechanisms for adapting to stress by protecting sensitive vascular layers of the plant.

Effect of Oryzalin Concentration and Duration on Callus Formation of 'Winter Rose'™ poinsettia

When explants were cultured on agar-solidified medium continuously, they produced a significantly reduced amount of callus with increased exposure time to oryzalin. Explants that were exposed to oryzalin produced significantly less callus than

those that were not exposed to oryzalin (Table 4.3). A negative correlation was found between oryzalin concentration and callus production by leaf sections cultured on solid medium containing oryzalin (Fig 4.2). Callus produced was white, appeared undifferentiated and later turned brown. No red callus was produced and none of the callus resulted in shoot primordia or shoots. Many of the explants exposed to concentrations higher than 115.5μM for two days produced no callus, later deteriorated, and died (Fig 4.1C).

Explants that were first cultured on liquid medium containing oryzalin, then transferred to solid CI medium produced progressively less callus with increase in concentration and duration of oryzalin (Table 4.4). Explant mortality also increased progressively. Explants cultured without oryzalin exhibited a significantly lower death rate than explants exposed to oryzalin (Table 4.4). Many explants did not produce any callus and died quickly after exposure to oryzalin, as did the explants cultured on solid medium.

Oryzalin is known to be less toxic to plant tissues than colchicine in several species. Lilium and Nerine showed less inhibition in regeneration of shoots when exposed to oryzalin than when exposed to colchicine. Oryzalin treatment also produced tetraploids at a lower concentration of oryzalin than at those needed for colchicine treatment (van Tuyl et al. 1992). In the case of 'Winter Rose'™, oryzalin appears unsuitable for tetraploid formation because it caused high mortality of explants even at lower concentration (28.9μM), significantly inhibited callus production, and prohibited shoot formation. Negative results have also been observed in a few other species.

Rhododendron species show a high mortality to oryzalin at higher concentrations regardless of duration of treatment (Eeckhaut et al. 2004).

Flow Cytometry of Colchicine-Induced Callus and Shoots and Oryzalin-Induced Callus of 'Winter Rose'™

Flow cytometry was used to evaluate ploidy level of samples of callus and/or shoots exposed to colchicine or oryzalin. These samples were compared to known samples of diploid and tetraploid poinsettia (Table 4.5). Control samples, diploid samples of 'Winter Rose'™ leaves (Fig 4.3C), and the known tetraploid species 'Barbara Ecke Supreme' (Fig 4.3D) and 'Supjibi' (Fig 4.3E), showed expected histograms of diploid and tetraploid species. Red callus samples generated from explants exposed to colchicine on solid medium were tested for the presence of tetraploid cells. Flow cytometry histograms revealed no tetraploid cells in the callus. Leaves of adventitious shoots were also tested for the presence of tetraploid cells and were found to be diploid (Fig 4.3A). Callus from explants exposed to colchicine in liquid medium were also tested for ploidy level, and found to be diploid (data not shown).

Flow cytometry was also used to evaluate the ploidy level of explants exposed to oryzalin either by means of liquid or solid medium. Callus samples from each treatment were evaluated. Flow cytometry histograms revealed no tetraploid cells (Fig 4.3B).

The lack of tetraploid cells in callus or shoots was not due to the flow cytometry testing procedures because the control diploid and tetraploid poinsettias showed expected histograms (Fig 4.3D-E). A histogram of an internal control containing both the diploid

'Winter Rose'™ and the tetraploid 'Supjibi' shows both a diploid and tetraploid peak (Fig 4.3F). A protocol for nuclei extraction and flow cytometric evaluation of ploidy level in *Euphorbia pulchurrima* has been established.

Responses to both colchicine and oryzalin have been reported to be highly species specific. In studies with two species of *Acacia*, colchicine was reported to give differential response in tetraploid formation of *A. dealbata* and *A. mangium* (Blakesley et al. 2002). Studies with oryzalin have showed differential response according to genotype in *Miscanthus sinensis* (Peterson et al., 2003). The studies in this research indicate that oryzalin is not a suitable reagent for tetraploid formation in 'Winter Rose'™ and that colchicine is less toxic for regeneration of adventitious shoots after exposure. This baseline information along with the flow cytometry protocol established will allow us or other researchers to further improve the protocol of *in vitro* tetraploid induction in poinsettia

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Table 4.1: Effects of Colchicine on Callus and Adventitious Shoot Formation of *Euphorbia pulchurrima* 'Winter Rose'™ - Solid-Solid Treatments

Colchicine (μM)	Duration (Days)	# of Explants Producing Callus	# of Explants Producing Shoots
0.0	0	4.3a[x]	0.0b
0.25	2	3.9a	0.0b
0.25	4	3.4ab	0.2ab
2.5	2	3.6a	0.9a
2.5	4	4.0a	0.0b
25.0	2	3.0ab	0.0b
25.0	4	2.1b	0.0b
250.4	2	2.8ab	0.0b
250.4	4	3.1ab	0.2ab

[x]Means separation in columns by Tukey's Studentized Range (HSD) at $P < 0.05$

Table 4.2: Effects of Colchicine on Callus and Adventitious Shoot Formation of *Euphorbia pulchurrima* 'Winter Rose'™ - Liquid-Solid Treatments

Colchicine (μM)	Duration (Days)	# of Explants Producing Callus	# of Explants Producing Shoots
0.0	0	3.4ab[x]	0.0a
0.0	1	2.6ab	0.38a
0.0	2	3.1ab	0.0a
62.6	1	2.3ab	0.5a
62.6	2	2.9ab	0.63a
125.2	1	2.0ab	0.0a
125.2	2	1.6b	0.0a
187.8	1	2.6ab	0.25a
187.8	2	1.8ab	0.0a
250.4	1	2.4ab	0.0a
250.4	2	3.6a	0.0a

[x]Means separation in columns by Tukey's Studentized Range (HSD) at P< 0.05

Table 4.3: Effects of Oryzalin Concentration on Callus Formation of *Euphorbia pulchurrima* 'Winter Rose'™ - Solid-Solid Treatments

Oryzalin (μM)	Duration (Days)	# of Explants Producing Callus
0	0	7.9a[x]
0	2	7.8a
0	4	7.9a
0	6	7.5a
2.9	2	7.8a
2.9	4	7.6a
2.9	6	7.6a
14.4	2	6.3a
14.4	4	4.4b
14.4	6	3.3b
28.9	2	1c
28.9	4	1c
28.9	6	0.3c

[x]Means separation in columns by Tukey's Studentized Range (HSD) at P< 0.05

Table 4.4: Effects of Oryzalin Concentration on Callus Formation of *Euphorbia pulchurrima* 'Winter Rose'™ - Liquid-Solid Treatments

Oryzalin (μM)	Duration (Days)	# of Explants Producing Callus	# of Dead Explants per Plate
0	0	3.5a[x]	0.3c
0	1	3.1ab	0.3c
0	2	2.5b	1c
28.9	1	0.5cd	2.9ab
28.9	2	0d	3.8a
72.2	1	1.1c	2.3b
72.2	2	0.6cd	3.6a
115.5	1	0.5cd	3.4ab
115.5	2	0.4cd	3.6a
144.3	1	0.5cd	3.5ab
144.3	2	0.63cd	3ab

[x]Means separation in columns by Tukey's Studentized Range (HSD) at $P < 0.05$

Table 4.5: Flow Cytometry Evaluation of Ploidy Level of 'Winter Rose'™ Poinsettias

Materials	Source of Materials	# of Samples	Diploid	Tetraploid
Callus	Colchicine S-S	10	X	
Shoots	Colchicine S-S	5	X	
Callus	Colchicine L-S	5	X	
Callus	Oryzalin S-S	5	X	
Leaves	Diploid Poinsettia 'Winter Rose'™	2	X	
Leaves	Tetraploid Poinsettia 'Barbara Ecke Supreme'	2		X
Leaves	Tetraploid Poinsettia 'Supjibi'	2		X

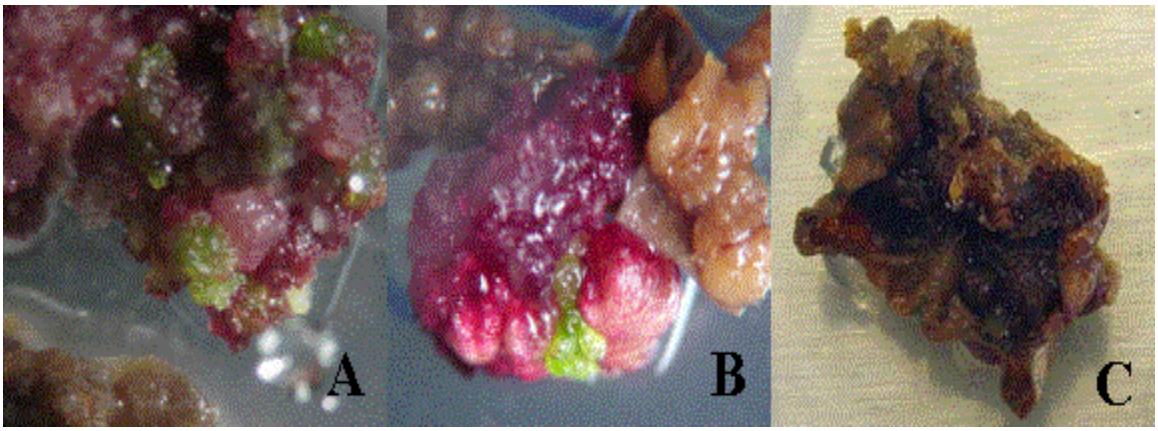

Fig 4.1: Callus proliferation from explants exposed to Colchicine and Oryzalin
A) Red callus indicative of axillary shoot proliferation from explants exposed to 62.6 μM colchicine in liquid medium for 2 days, B) Axillary shoot buds from explants exposed to 2.5 μM colchicine in solid medium for 2 days, C) Effects of exposure to 28.9 μM oryzalin in liquid medium for 1 day on leaf explants.

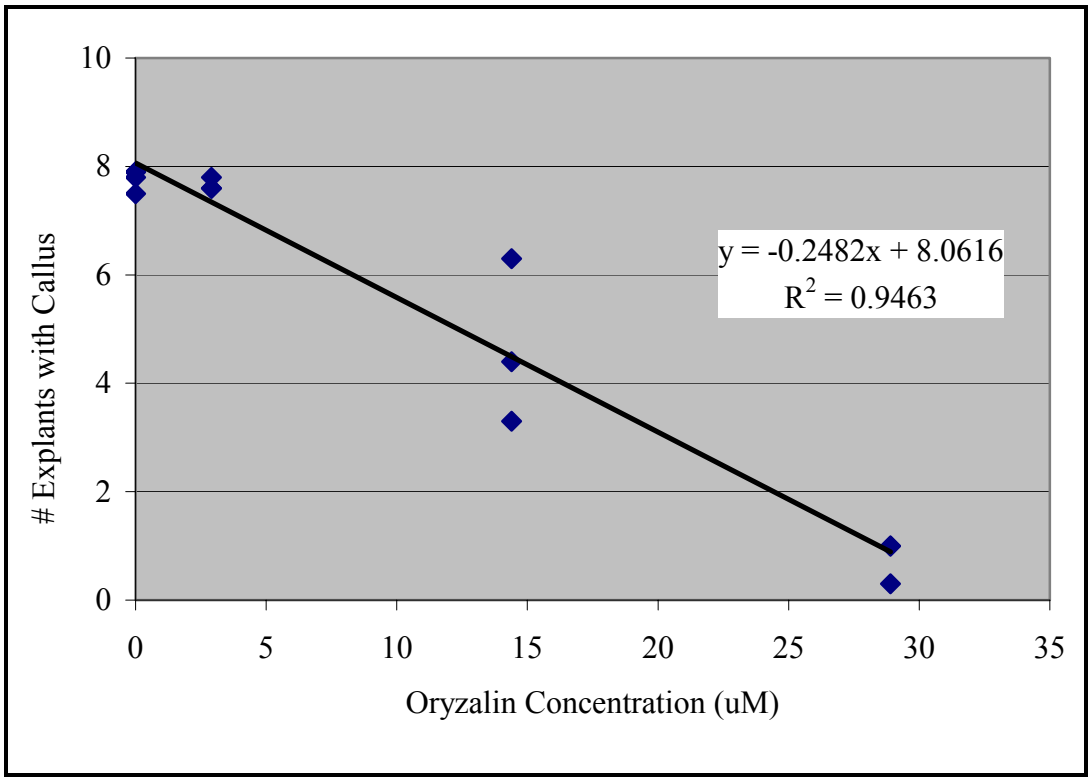

Fig. 4.2: Effects of Oryzalin on Callus Growth and Proliferation of Euphorbia pulcurrima 'Winter Rose'™

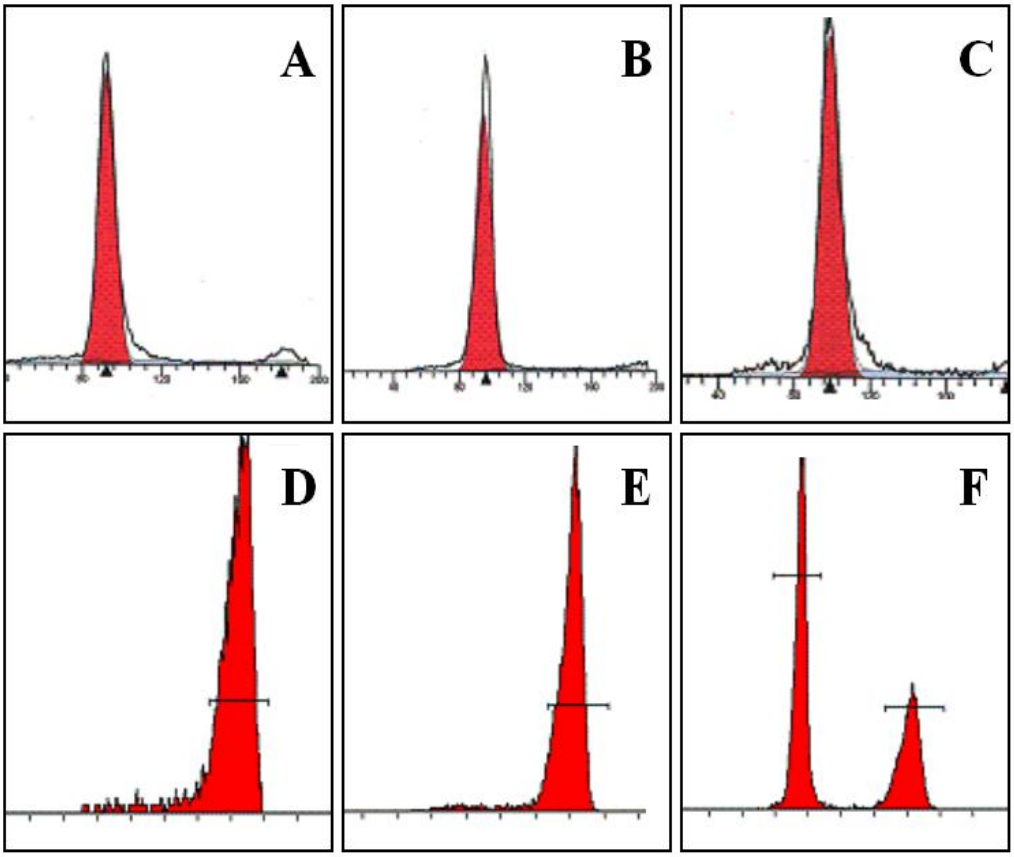

Fig 4.3: Flow Cytometry Histographs of Callus from Explants Exposed to Colchicine and Oryzalin. A) Callus exposed to colchicine in solid medium showing diploid peak, B) Diploid callus exposed to oryzalin on solid medium showing diploid peak, C) Diploid control leaf of 'Winter Rose'™ poinsettia, D) Tetraploid leaf 'Barbara Ecke Supreme, E) Tetraploid leaf Supjibi, F) Histogram of mixed leaf samples of Diploid leaf 'Winter Rose'™ + Tetraploid 'Supjibi'

PART 5

FACTORS INFLUENCING AGROBACTERIUM-MEDIATED TRANSFORMATION OF *EUPHORBIA PULCHURRIMA* 'WINTER ROSE'™

ABSTRACT

This paper examined the factors influencing Agrobacterium-mediated transformation of *Euphorbia pulchurrima* 'Winter Rose'™, a popular cultivar characterized by having incurved bracts and leaves and by low branching. Kanamycin at 50 mg/L inhibited callus formation of 'Winter Rose'™ leaf tissues, suggesting that it may be used as a selective marker gene for poinsettia transformation. After co-cultivation, agrobacterium can be eliminated by washing the leaf tissues infected by agrobacterium in liquid MS media with CCK for 20 min on a shaker at 120 RPM. Transfer of the co-cultivated tissues to solid MS medium with CCK was not effective in eliminating the agrobacterium. Leaf tissue infected with agrobacterium harboring two different plasmids (pBI121, pMON690), with or without the inducer acetosyringone, did not produce callus in the presence of CCK (cefotaxime, carbenicillin, and kanamycin), and died after 1-2 months. The presence of agrobacterium produced necrosis in all explants exposed to it. These results suggest that poinsettia may be very recalcitrant to Agrobacterium-mediated transformation, and further study into antinecrosis chemicals may be needed in order to establish a protocol for Agrobacterium-mediated transformation of *E. pulchurrima* 'Winter Rose'™.

Key Words: poinsettia, agrobacterium, *iaa-L*, transformation

Euphorbia pulchurrima, poinsettia, was first introduced to the United States by Joel Robert Poinsett in 1825 (Ecke et al. 1990). Poinsettia has become the number one flowering potted plant in the United States (USDA Agricultural Research Service, 2002). In 2001 more than 67 million pots were sold in the United States worth an approximate wholesale value of $246 million dollars (USDA Economics, Statistics and Market Information System, 2002). There are over 100 cultivars of poinsettia available today (http://www.urbanext.uiuc.edu/poinsettia/facts.html, 2004) with many different colors, forms, heights, and blooming periods.

E. pulchurrima 'Winter Rose'™ is a family of cultivars bred by Paul Ecke Ranch and was first introduced by Hans Fruewirth in 1999, after 30 years of effort (Poinsettias.com, 2002). Now, over one million plants are sold each year (Ruth Kobayashi, personal communication). This group of cultivars has characteristic rose-like 'flowers', incurved bracts, and a globe-shaped flower head, with a full color series. 'Winter Rose'™ poinsettias are a minimal branching cultivar and thus produce a low number of inflorescences (Harkess 1997), which is less desirable for both consumer and growers. Growers might find it hard to ship taller plants without breakage, and consumers may not buy poinsettias with few inflorescences.

Minimal branching is a common characteristic of poinsettias. Native poinsettias of southern Mexico, which are the ancestors of modern poinsettias, grow into straight, tall trees (Lee 2000) that can reach up to 8 feet high (Suszkiw 1998). Restricted-branching and minimal free-branching cultivars can be induced to produce multiple flowers in the greenhouse by pinching the primary shoot tips. Most of the plants produced in commercial markets are pinched plants. A chemical growth regulator such as Florel® can

also be applied after pinching to further optimize branching (Ecke 1999). The disadvantage to manual pinching is that it can be time-consuming, therefore incurring heavy labor costs.

Genetic transformation has been widely used in crop improvement in the past 20 years. One of the greatest advantages of genetic engineering is that it can introduce a single or a few traits into an established cultivar without altering the overall genetic makeup (Stewart 2003). Unfortunately, relatively limited experimentation has been done on transformation of *Euphorbia pulchurrima*. Vik et al. (2000) experimented with the uncommon transformation technique, DNA electrophoresis, to introduce genes into the apical meristem in poinsettia. Nine of the 34 shoots recovered showed GUS expression. In an effort to increase terpene production in roots, *Euphorbia lathyris* was transformed with the *Agrobacterium rhizogenes* strain ATCC 15834 (Cheetham et al.1996). The transformed roots of this *Euphorbia* were genetically stable and grow rapidly in culture on medium but failed to regenerate into whole plants (Cheetham et al.1996). Transformed roots were also found to produce more bioactive compounds both in quantity and variety. These experiments suggest that this latex-producing *Euphorbia* is susceptible to infection and may be successful in other members of the *Euphorbiaceae* family. However, no *Agrobacterium tumefaciens* mediated transformation has been reported.

The objectives of this research were to: a) Examine the factors which influence Agrobacterium-mediated transformation of *E. pulchurrima* 'Winter Rose'™ and b) determine the possibility of using Agrobacterium-mediated transformation for the insertion of the *iaaL*-synthetase gene into 'Winter Rose'™ to stimulate branching.

MATERIALS AND METHODS

Plant Material and General Culture Methods for 'Winter Rose'™ poinsettia

Stock plants of *Euphorbia pulchurrima* were maintained in baby food jars containing solid medium. The medium was composed of MS salts (Murashige et al. 1962) with the addition of 100 mg/L myo-inositol and 3% sucrose. No plant growth regulators were added. The medium was adjusted to a pH of 5.7-5.8 and solidified with 0.7% (w/v) agar prior to being autoclaved at 121ºC for 20 min and a PSI of 2.9 Mpa. Unless otherwise specified, cultures were maintained in a growth room at 25ºC under a 16-hour photoperiod (16h light/ 8h dark) with illumination of 125 $\mu mol \bullet mol^{-2} \bullet s^{-1}$.

Agrobacterium tumefaciens culture. Agrobacterium cultures were maintained in liquid Luria Bertani (LB) medium adjusted to a pH of 7.0 and supplemented with 50mg/L kanamycin and stored in 4% glycerol stock at -80ºC. Fresh culture was initiated by streaking this stock on LB medium solidified with 1.5 % (w/v) bactoagar. Individual colonies were selected and grown up in 3 ml of liquid LB supplemented with kanamycin and rifampicin for 16 hours at 28ºC with 200 rpm shaking.

Two types of plasmids were used for this experiment and both were harbored in the *Agrobacterium tumefaciens* strain EHA105 (Hood et al. 1993). The first plasmid was the binary Ti plasmid pBI121 (Clontech, Palo Alto, CA). This plasmid contained the *npt*II gene that codes for neomycin phosphotransferase II (*npt*II) and is driven by the nopaline synthetase (NOS) promoter. It also contains the *uid*A gene coding for β-glucuronidase (GUS) (Jefferson et al. 1987), driven by the cauliflower mosaic virus

(CaMV) 35S promoter. The second plasmid was the pMON690 (Romano et al. 1991) obtained from Dr. Li (University of Connecticut). The plasmid contained the *iaaL*-synthetase gene driven by a CaMV 35S promoter. The *iaaL* synthetase gene from *Pseudomonas savastanoi* encodes for an enzyme that metabolizes IAA into a less-active conjugate, IAA-Lysine (Roberto et al.1991). When the gene was inserted into tobacco plants under the control of the constitutive 35S promoter, plants exhibited reduced apical dominance, reduced rooting, and increased axillary bud growth indicative of an anti-auxin gene (Romano 1991). These plants produced 19-fold less auxin in the juvenile stage and showed increased branching at the aerial nodes (Napoli et al. 1993). The plasmid also contains the *npt*II gene that codes for neomycin phosphotransferase II, driven by the nopaline synthetase (NOS) promoter.

Sensitivity of 'Winter Rose'™ to Kanamycin

Since both plasmids contained the *npt*II gene as the selectable marker gene, the sensitivity of leaf tissues to kanamycin was tested. Leaf midvein sections of *in vitro* grown plantlets were placed on callus induction (CI) medium containing one of four concentrations of kanamycin (0, 50, 100, and 250 mg/L). There were two replications per treatment consisting of eight explants per plate and the experiment was repeated once. Explants were evaluated for two months for callus formation and mortality rate.

Agrobacterium-Mediated Transformation of *Euphorbia pulchurrima* 'Winter Rose'™

The basic protocol of Agrobacterium-mediated transformation was based on an aspen transformation protocol (Dai et al., 2003). *A. tumefaciens* strains of EHA105 were grown in liquid Luria Betani (LB) medium with 50 mg/l kanamycin on a shaker at 28 ^{o}C for 20 hours. *A. tumefaciens* cells were collected and resuspended in 50 ml LB medium with 20 μM acetosyringone (AS), the inducer of the *vir* genes in agrobacterium, and grown for another 6 hours. Bacteria were diluted to a final density of 1.0 Abs at OD_{600} with fresh LB medium.

Leaves were obtained from *in vitro* stock *Euphorbia pulchurrima* 'Winter Rose'™ specimens. Young leaves were cut into 0.5 x 0.5 cm segments and gently shaken in the bacterial suspensions for 30 minutes, and then blotted dry with a sterile paper towel. Infected leaf segments were placed onto CI medium containing MS salts with 2 mg/l benzylaminopurine (BA) and 3 mg/l indole-3-acetic acid (IAA) for co-cultivation with agrobacterium at 24ºC in the dark. After 72 hours, leaf explants were disinfected with sterile water containing CCK [cefotaxime (250 mg/l), carbenicillin (500 mg/l), and kanamycin (50 mg/L)], and were transferred to CI medium with CCK for one month and then to the shoot induction (SI) medium containing only BA with CCK for the remainder of the experiment. All cultures were placed in 15 mm petri dishes and were subcultured to fresh medium every four weeks for 8 weeks.

Using these basic protocols several variables including two types of plasmids containing the GUS and *iaaL* genes respectively with or without the addition of acetosyringone, and CCK (Table 5.2) were evaluated. There were eight plates per treatment and five leaf midvein sections per plate. The experiment was repeated once.

Explants were evaluated every four weeks for callus and adventitious shoot formation, and mortality rate. The experiments were terminated after eight weeks.

RESULTS AND DISCUSSION

Sensitivity of 'Winter Rose'™ to Kanamycin

Explants grown on medium without kanamycin, or the control treatments, showed no mortality and produced a large amount of callus (Table 5.1) while explants exposed to the lowest concentration of kanamycin (50 mg/L) showed mortality in all explants within one month. Explants turned brown and produced no callus after two months of observation. The explants exposed to 100 mg/L or more Kanamycin showed no callus growth and died within the first month (Table 5.1). These results indicate that 'Winter Rose'™ is sensitive to kanamycin, and kanamycin at 50 mg/L is sufficient for selection of transformed 'Winter Rose"™ cells after Agrobacterium-mediated transformation.

Agrobacterium-mediated transformation of *E. pulchurrima* 'Winter Rose'™

The first experiment involving both plasmids, agrobacterium overgrew after co-cultivation, and leaf explants died. Successive treatments to eliminate the agrobacterium from the explants by reducing co-cultivation time from 72 hours to 48 hours, a wash after co-cultivation in basal MS medium with CCK for 20 minutes, and increased cefotaxime (500 mg/L) and carbenicillin (750 mg/L) concentrations in the wash were successful in eliminating agrobacterium, but explants died.

Further experiments included a wash after co-cultivation in basal MS medium with CCK fro 20 minutes, which was effective in eliminating agrobacterium, however

explants continued to turn brown and die before two months into the experiment and no callus was produced by the explants.

Acetosyringone, a signal molecule produced by plant cells when they are wounded, was evaluated for its effect on induction of *vir* gene expression. In the medium containing 0 or 20 μM acetosyringone and agrobacterium (Treatments V, VI, VII and VIII, Table 5.2), all explants turned brown and died within the first month. Treatments that did not contain agrobacterium remained green, and produced callus and adventitious buds regardless of the presence of Acetosyringone (Treatments I and IV, Table 5.2). Acetosyringone has been shown to increase the transformation frequency in many species such including wheat (Wu et al. 2003), white spruce (Le et al. 2001), and sugarbeet (Jacq et al. 1993). Because of the death of explants, the effect of acetosyringone was difficult to determine.

CCK is a combination of the antibiotics cefotaxime, carbenicillin, and kanamycin (Dai et al., 2002) for selecting transformed plant cells by kanamycin and for eliminating *A. tumefaciens* by cefotaxime and carbenicillin. The effect of antibiotics in culture was tested because antibiotic-sensitive species can exhibit reduced regeneration or even death depending on which type of antibiotic is used. Certain *Fragaria* species have been shown to be highly susceptible to kanamycin during transformation, therefore a regime requiring incremental increases over time of kanamycin concentration was needed until the desired selection concentration was achieved (Alsheikh et al. 2002). Explants without exposure to CCK produced callus and appeared to suffer no ill effects (Treatments I and IV, Table 5.2) and explants that were exposed to CCK without transformation died (Treatments I and III, Table 5.2). Explants that were infected by

agrobacterium and exposed to CCK died as well (Treatments V-VIII, Table 5.2), but it is unclear as to whether to death was due to Agrobacterium infection or CCK.

These experiments revealed that all treatments were infected by agrobacterium, regardless of type of agrobacterium, died. Treatments that were infected by agrobacterium, both in the absence or presence of acetosyringone, produced no callus and died (Treatments V, VI, VII, and VIII, Table 5.2).

The failure to transform 'Winter Rose'™ poinsettia may have been caused by several factors. In this experiment, we evaluated kanamycin at 0, 50, and 100 mg/L and found that 50 mg/L was effective in killing non-transformed tissues, but this concentration may not be the lowest concentration of kanamycin that is still effective. Too high concentrations of kanamycin may kill the non-transformed cells to quickly and inhibit the growth of transformed cells. This can be due to inhibition of growth due to high antibiotic concentrations. In walnut transformation experiments, the agrobacterium strain C58C1 ATHV Rif^R was found to be resistant to carbenicillin and cefotaxime at high concentrations, and was only eliminated by a high dose of timentin. It has also been found that high doses of antibiotics used to remove the agrobacterium stunted growth and regeneration of the explants (Tang et al. 2000).

The results in this experiment showing that leaf tissues infected by agrobacterium quickly died, may suggest a hypersensitive response. This is true in many monocot species including sugar cane, where inoculation with agrobacterium was traumatic to the cells and produced necrosis in the explants (de la Riva et al. 1998). This problem was remedied by treating the explants (meristems) with anti-necrotic compounds such as ascorbic acid, cystein and silver nitrate before co-cultivation with agrobacterium

(Enríquez-Obregón et al. 1998, 1999). This phenomenon of extreme stress response has also been seen in some dicots. Suspension cell culture lines of periwinkle had a severe necrotic stress response when co-cultured with agrobacterium cultures. This was remedied by using a different type of explant (cotyledons) (Garnier et al. 1996). Another dicot species that suffers necrotic response to Agrobacterium-mediated transformation is grape. This response is a cultivar specific and Ti-plasmid related. Their results found that this necrotic response was exacerbated by exogenous auxin application and relieved by exogenous kinetin application (Pu et al. 1992).

It is clear that the severe necrosis suffered in explants is in fact a response to agrobacterium co-cultivation. Further modifications in procedure such as culture conditions, medium addendums (anti-oxidants or exogenous hormones), and agrobacterium strain selection in the future may yield a functional protocol for Agrobacterium-mediated transformation of *Euphorbia pulchurrima* 'Winter Rose'™.

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Table 5.1: Effects of Kanamycin Concentration on Callus Proliferation and Mortality of Leaf Sections of 'Winter Rose'™ after four weeks

Kanamycin	% of Explants Producing Callus	% of Dead Explants
I.0 mg/L	100	0
II.50 mg/L	0	100
III.100 mg/L	0	100
IV.250 mg/L	0	100

Table 5.2: Variables Evaluated for Agrobacterium-Mediated Transformation of *Euphorbia pulchurrima* 'Winter Rose'™ with GUS and *iaaL* genes.

Treatment	Results
I. No Agro - AS - CCK	Explant produced callus and shoots
II. No Agro + AS + CCK	Explants died after ~1 month, no shoots, no callus
III. No Agro - AS + CCK	Explants died after ~1 month, no callus, no shoots
IV. No Agro + AS - CCK	Explants produced callus and shoots
V. Agro (GUS) + AS + CCK	Explants turned brown and died before 1 month, no callus, no shoots
VI. Agro (GUS) - AS + CCK	Explants turned brown and died before 1 month, no callus, no shoots
VII. Agro (*iaaL*) + AS + CCK	Explants gradually turned brown and died after ~1 month, no callus, no shoots
VIII. Agro (*iaaL*) - AS + CCK	Explants gradually turned brown and died after ~1 month, no callus, no shoots

Agro = *Agrobacterium tumefaciens*
AS = Acetosyringone
CCK = Cefotaxime, Carbenecillin, and Kanamycin
GUS = *A. tumefaciens* containing the pBI121, GUS gene, plasmid
IaaL = *A. tumefaciens* containing the pMON690, *iaaL* gene, plasmid

PART 6

CONCLUSION AND FUTURE PERSPECTIVES

Poinsettia, *Euphorbia pulchurrima*, is a well-known plant throughout the world, and is associated, almost without thought, with Christmas holidays. It is the number one flowering potted plant in the United States and it brings in over 200 million dollars every year. It is staggering to consider that this plant is almost exclusively sold within a 3-month window, October through December, generating approximately $2 million dollars per day for wholesale growers.

Surprisingly, little biotechnological research has been performed on this species. Traditional breeding techniques have produced approximately 100 new cultivars of poinsettia since its entry into the United States in 1825. The purpose of this dissertation research was to develop some basic biotechnology tools for manipulation of 'Winter Rose'™ poinsettia. Our first objective was to develop a protocol for *in vitro* propagation of 'Winter Rose'™ through axillary bud proliferation and organogenesis from leaf tissues. Our second objective was to evaluate the effects of colchicine and oryzalin on callus and adventitious shoot formation, and possibly tetraploid formation of 'Winter Rose'™. Our final objective was to evaluate some factors influencing Agrobacterium-mediated transformation of 'Winter Rose'™.

The first objective of axillary bud proliferation and organogenesis was successful. *In vitro* shoot cultures were established using terminal buds from greenhouse plants. Explants were induced to produce axillary buds on MS medium with the cytokinin BA added. The proliferated shoots were grown up on basal MS medium and removed from the primary shoot. Plantlets easily rooted both on basal medium and in soil without auxin treatments and were outplanted into soil successfully. In the beginning of the research with poinsettia we had concern that latex may be a significant obstacle to establishing in

vitro poinsettia culture as suggested by some messages on the Net Tissue Culture List group message board. Surprisingly we had no problems with shoot culture. The successful establishment of two additional tetraploid cultivars leads us to believe that this problem is not cultivar specific. Therefore the latex problems associated with poinsettia in vitro culture may be more fear than fact. Poinsettias are vegetatively propagated and cuttings root easily. Although this in vitro propagation method is unlikely to be used for commercial propagation, it can be used to clean up pathogens or contaminated germplasms in order to facilitate shipping of the germplasms freely without concerns for spreading the pathogen. This system may also be used to select somoclonal variants and to induce mutants if mutagens are used.

Organogenesis using leaf tissues sections was also successful. Adventitious shoots were obtained and verified using histological techniques. Adventitious shoots were only produced on red callus, explants that produced white, or brown callus produced no shoots. Although the frequency of adventitious shoot formation was relatively low (~4%), explants that produced adventitious shoots continued to produce shoots if the explant remained on auxin-containing medium. If early selection was made and only red callus were kept, the efficiency could be greatly improved. Shoots that were produced from adventitious shoot formation rooted easily. These explants exhibited no unusual growth pattern and appeared to be identical to those cultured from greenhouse-grown plants. Further optimization of the protocol can also be made to enhance adventitious shoot production. Optimization could be done by examining the duration of IAA treatment, leaf age, or other culture conditions. The association of red callus with

shoot organogenesis is useful and may be used to identify the genes expressed or related to organogenesis.

The second objective of this research was to evaluate the effects of colchicine and oryzalin on callus and shoot formation and attempt to induce tetraploid formation. The experiment revealed that caused less severe necrosis in this particular species at any concentration or is less toxic than oryzalin. Leaf explants produced callus and shoots even after exposure to 75-100 mg/L of colchicine. However, none of the explants produced tetraploid shoots or callus. Explants that had been exposed to oryzalin only produced callus but no shoots. None of the callus or shoots produced were tetraploid. At this point it is hard to correlate which mitotic inhibitor is more suitable for inducing tetraploids. If we continue this research to try and produce tetraploid plants, we will focus on using colchicine. This is because even if oryzalin can produce tetraploid callus, it will remain useless because the callus cannot be regenerated into shoots. From this aspect, colchicine may be a more suitable mitotic inhibitor for inducing tetraploid poinsettia.

This particular situation may be remedied in many ways. The first may be to change explant type, and anti-necrotic chemicals may be used to reduce necrotic response. If the problem is that the tissues are not being reached by the colchicine, wounding or reducing the cuticle layer with soap may enhance the absorption of the colchicine.

The final objective of this research was to evaluate factors influencing the transformation of this poinsettia via *Agrobacterium tumefaciens* Ti plasmid vector. The overall experiments were unsuccessful because infection by agrobacterium caused

necrosis of leaf tissues. Other factors such as acetosyringone and plasmid type and CCK could not be easily evaluated. Development of a successful genetic transformation system for a particular species is a combination of science, art, and luck. Despite the fact that many species and particular genotypes have been successfully transformed, still many more have been unsuccessful. This has been the subject of numerous reviews and books, so it is not unique to poinsettia. If the research is to be continued, we need to focus on measures to prevent the necrosis, such as using antioxidant compounds like silver nitrate and ascorbic acid, or perhaps changing the type of explant, or adding certain plant growth regulators. After the necrotic response is eliminated, other factors can be studied to optimize the transformation system.

Regardless of the problems with these experiments, much has been learned from them. A protocol for the *in vitro* propagation and shoot organogenesis of 'Winter Rose'™ has been established. This is the first step for applying all other in vitro biotechnological approaches for poinsettia genetic improvement, such as in vitro tetraploid induction and transformation. The results of effects of colchicine and oryzalin will offer information for future research to induce tetraploids, however these results have revealed that much research is needed to overcome necrotic response after co-cultivation.

The preliminary data with *Agrobacterium*-mediated transformation suggests that poinsettia may be very recalcitrant to *Agrobacterium*-mediated transformation. Since poinsettia has been transformed via particle bombardment, this may be a better choice in short term. If a transformation system is available, many genes that are available for

transformation may be introduced such as those for disease resistance or to alter growth habit.

Overall, relatively limited biotechnology research has been done in poinsettia, and as a matter of fact, other ornamental plants in general. Poinsettia, however, offers a unique opportunity for biotechnology from a business perspective because 70% of poinsettias sold are red cultivars, and 80% of the red poinsettias sold are 'Freedom Red' and a few other cultivars such as 'Winter Rose'™, it is wise to focus on these few cultivars in order to receive a tremendous investment return. We hope more research will be devoted to poinsettia in order to develop new and unique poinsettias that will both help growers and delight consumers.

VITA

Kimberly Pickens was born in Toccoa, GA in 1972. She was raised in Toccoa and got her BS in Botany in 1997 from the University of Georgia in Athens, GA as well as her MS in Horticulture in 2001. She received her PhD in Plant Sciences from the University of Tennessee, Knoxville, TN in 2004.

Currently she is working at Macon State College in Macon, GA as an Assistant Professor of Biology.