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Effects of Colchicine and Oryzalin on Callus and Adventitious Shoot Formation of *Euphorbia pulchurrima* 'Winter Rose'

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Abstract. **The mitotic inhibitors, colchicine and oryzalin, were evaluated for their effects on callus, adventitious shoot formation, and tetraploid induction of *Euphorbia pulchurrima* 'Winter Rose'. In vitro grown leaf sections were placed on various media supplemented with either colchicine or oryzalin at various concentrations for 1 to 4 days. Colchicine was less damaging to leaf tissues than oryzalin. On various colchicine-containing media, prolific calluses were produced and adventitious shoot formation was observed. Regenerated shoots were found to be diploid as determined by flow cytometry. On media supplemented with oryzalin (28.9 μM to 144 μM), leaf tissues produced callus but failed to form adventitious shoots. Samples of calluses produced on oryzalin-containing media were subject to analysis using flow cytometry and were found to be diploid. These results suggest that the colchicine is less toxic on poinsettia tissues and shoot induction than oryzalin. Additional experiments are needed to establish a protocol for in vitro induction of poinsettia tetraploid with colchicine and oryzalin.**

Introduction

Colchicine is an alkaloid found in the autumn crocus (*Colchicum autumnale*) and is known to be a natural mitotic inhibitor by blocking the development of spindle fibers and preventing cell division (Eigsti and Dustin, 1955). Colchicine-treated *Datura*, *Portulaca*, *Petunia*, *Zea*, *Allium*, and *Cucurbita* generally produced larger inflorescences, fruit, and pollen grains and had shorter stems. Specific effects were dependent on plant species, methods of application, and concentration of colchicine (Blakeslee and Avery, 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., 2000 and reference therein).

Oryzalin, sold under trade names such as Surflan, Dirimal, and Ryzelan, is a selective preemergence herbicide for controlling annual grasses and broadleaf weeds in orchards, vineyards, and floral plots (Atland et al., 2003). It is a surface-applied herbicide that inhibits the growth of germinating weed seeds by blocking cell division in primary meristems (Extension Toxicology Network, 1996). Oryzalin disrupts mitosis by inhibiting the formation of microtubules (Ramulu et al., 1991). Unlike colchicine, oryzalin has no affinity to animal tubulin; therefore, it poses low toxicity to humans and 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 such as *Lilium longiflorum* (van Tuyl et al., 1992) and *Gerbera* (Tosca et al., 1995).

Poinsettia, *Euphorbia pulchurrima*, is an important holiday symbol and is the number one selling potted flowering plant in the United States. In 2001 alone, more than 67 million pots were sold in the United States worth an approximate wholesale value of $246 million (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 (www.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' produces a low number of flower heads without pinching (Harkess, 1997). The "flowers" (true flower organs and bracts) are also typically smaller than those of other cultivars of poinsettia as a result of the incurved bracts.

Many characteristics, including color, growth habit, disease resistance, and durability, have been investigated in breeding *E. pulchurrima* and have been bred into new cultivars. The technique of chromosome doubling has been used to increase size of flowers, stems, and leaves of many species and has been used in poinsettia breeding to obtain new cultivars. 'Pearl', a tetraploid sport developed by Peter Jacobsen for Ecke Ranch, was induced using in situ application of 1% colchicine to the cut surface of a vegetative stem after removal of the terminal apex (USPTO PP10160, 1997). This tetraploid mutant exhibits thicker stems, larger leaves, and inflorescences as well as being self-branching and of a more compact height than the diploid parent. Application of colchicine or oryzalin to in vitro tissues may be used to induce polyploids, which may exhibit larger inflorescences and bracts and reduce height compared with diploid 'Winter Rose' poinsettias.

The purposes of this research were 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 the potential for induction of polyploid tissues and organs and to compare the effect of colchicine and oryzalin in liquid with solid media on callus and shoot induction of 'Winter Rose' poinsettia.

Materials and Methods

General culture methods for 'Winter Rose' poinsettia. Leaves were excised from in vitro shoot cultures of *E. pulchurrima* 'Winter Rose' that were maintained in MS medium (Murashige and Skoog, 1962) without plant growth regulators. Middle sections, ≈1 cm^2, were cut and placed on a callus induction medium (CIM) in 15-mm Petri plates containing MS media with 555 μM myo-inositol, 3% sucrose, 8.9 μM benzylaminopurine (BA), and 17.1 μM indole-3-acetic acid (IAA). After 1 month, leaf segments were transferred to shoot induction medium (SIM) containing only BA (8.9 μM). The medium was adjusted to pH 5.7 to 5.8 and solidified with 0.7% (w/v) agar before being autoclaved at 121 °C at 2.9 Mpa for 20 min. 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 $\mu mol \cdot m^{-2} \cdot s^{-1}$. All cultures were subcultured to fresh medium every month. The regenerated adventitious shoots were transferred from SIM to baby food jars containing solidified MS medium for shoot growth and proliferation. The basic tissue culture and regeneration protocol was described earlier (Pickens and Cheng, 2005).

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Effect of colchicine concentration and duration on callus and shoot formation of 'Winter Rose' poinsettia

Solid-solid medium. Leaf sections were cultured on CIM solidified with 0.7% agar. Colchicine was dissolved in deionized water, sterilized through a 0.2 μmeter filter (catalog # 431,215, Corning, N.Y.), and added to the medium at 0, 0.25, 2.5, 25.0, or 250.4 μM after the medium was autoclaved. Leaf explants were cultured on colchicine-containing media for 0, 2, or 4 d, then transferred to CIM for 1 month from the time they were placed on colchicine-containing medium, and then placed on SIM for the remainder of the experiment. Each medium treatment consisted of seven Petri plates, each plate contained five leaf explants, and the experiment was conducted twice. Explants were evaluated for 4 months for callus formation, callus color, and shoot formation. After 4 months, callus samples were analyzed for ploidy level using flow cytometry. Adventitious shoots were also grown up for 3 months, and leaves were determined for ploidy using flow cytometry.

Liquid-solid medium. Leaf explants were cultured in liquid CIM containing one of five concentrations of colchicine (0, 62.6, 125.2, 187.8, or 250.4 μM) for 0, 1, or 2 d. The higher concentrations within the upper limit were tested because no obvious differences were observed between treatments of lower concentrations and the control treatment. Explants were washed with liquid basal MS medium, blotted with sterile paper towels, and placed on solidified CIM for 1 month from the time they were placed on liquid medium, and then placed on SIM for the remainder time of the experiment. Each treatment consisted of four Petri plates, and Petri plates contained eight explants. The experiment was conducted twice. Explants were evaluated every month for 4 months for callus formation, callus color, and shoot formation. The data of the final observation are presented. Callus and adventitious shoots formed were evaluated for ploidy after 4 months using flow cytometry.

Liquid-liquid medium. Leaf explants were cultured in liquid CIM (25 mL in a 125-mL flask) containing one of four concentrations of colchicine (0, 2.5, 25.0, or 250.4 μM) for one of five durations (0, 2, 4, 8, or 16 d). The explants were washed in basal medium and then placed in liquid CIM. After 1 month from the time they were placed on liquid medium, explants were placed in SIM for the remainder of the experiment. Each treatment had two replications, and each flask contained eight leaf sections. Explants were evaluated every month for 3 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 sections were cultured on solid CIM containing 0, 2.9, 14.4, or 28.9 μM of oryzalin. Oryzalin was dissolved in dimethyl sulfoxide (DMSO), filter-sterilized through a 0.2 μMeter filter (catalog # 431,215, Corning, N.Y.), then added to the autoclaved medium. Leaf explants were cultured on medium containing oryzalin for 0, 2, or 4 d and were transferred onto CIM for 1 month from the time they were placed on oryzalin-containing medium and then placed on SIM for the remainder of the experiment. Each treatment consisted of four Petri plates, each plate contained eight leaf explants, and the experiment was repeated once. Explants were evaluated every month for callus formation, callus color, and shoot formation. After 4 months, sample callus tissues were analyzed for ploidy using flow cytometry.

Liquid-solid medium. Leaf sections were cultured in liquid CIM in 125-mL Erlenmeyer flasks containing 0, 28.8, 72.2, 115.5, or 144.3 μM of oryzalin. The higher concentrations were used because no tetraploids were found in the earlier solid-solid media. The flasks were placed on a shaker and shaken at 100 rpm for 0, 1, or 2 d. Explants

Table 1. Effects of colchicine on mean numbers of explants producing calluses and adventitious shoot formation on *Euphorbia pulchurrima* 'Winter Rose': solid-solid treatments[z].

Colchicine (μM)	Duration (d)	Mean number of explants producing callus	Mean number of explants producing shoots
0.0	0	4.3 a[y]	0.0 b
0.25	2	3.9 a	0.0 b
0.25	4	3.4 ab	0.2 ab
2.5	2	3.6 a	0.9 a
2.5	4	4.0 a	0.0 b
25.0	2	3.0 ab	0.0 b
25.0	4	2.1 b	0.0 b
250.4	2	2.8 ab	0.0 b
250.4	4	3.1 ab	0.2 ab

[z]Each treatment consisted seven Petri plates and each plate contained five leaf explants. The experiments were done twice.

[y]Means separation in columns by Tukey's studentized range (HSD) at $P < 0.05$. The data were averages of two experiments.

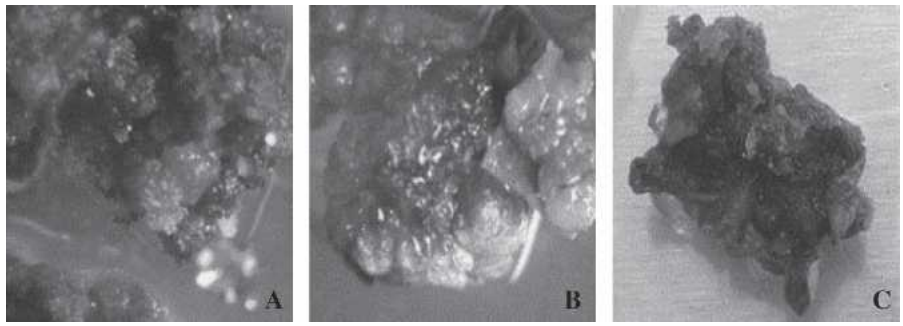

Fig. 1. Callus proliferation from explants exposed to colchicine and oryzalin. (**A** and **B**) Leaf explants of *Euphorbia pulchurrima* 'Winter Rose' exposed to 62.6 μM colchicine in liquid medium for 2 d and exposed to 2.5 μM colchicine in solid medium for 2 d, respectively. Red callus indicative of adventitious shoot formation (green buds). (**C**) Leaf explants were exposed to 28.9 μM oryzalin in liquid medium for 1 day showing nonregenerating callus.

Table 2. Effects of colchicine on mean numbers of explants producing calluses and adventitious shoots on *Euphorbia pulchurrima* 'Winter Rose': Liquid-solid treatments[z].

Colchicine (μM)	Duration (d)	Mean number of explants producing callus	Mean number of explants producing shoots
0.0	0	3.4 ab[y]	0.0 a
0.0	1	2.6 ab	0.4 a
0.0	2	3.1 ab	0.0 a
62.6	1	2.3 ab	0.5 a
62.6	2	2.9 ab	0.6 a
125.2	1	2.0 ab	0.0 a
125.2	2	1.6 b	0.0 a
187.8	1	2.6 ab	0.3 a
187.8	2	1.8 ab	0.0 a
250.4	1	2.4 ab	0.0 a
250.4	2	3.6 a	0.0 a

[z]Each treatment consisted four Petri plates and each plate contained eight leaf explants. The experiments were done twice.

[y]Means separation in columns by Tukey's studentized range (HSD) at $P < 0.05$. The data were averages of two experiments.

Table 3. Effects of oryzalin concentration on mean numbers of explants producing calluses on *Euphorbia pulchurrima* 'Winter Rose': solid-solid treatments[z].

Oryzalin (µM)	Duration (days)	mean number of explants producing calluses
0	0	7.9 a[y]
0	2	7.8 a
0	4	7.9 a
0	6	7.5 a
2.9	2	7.8 a
2.9	4	7.6 a
2.9	6	7.6 a
14.4	2	6.3 a
14.4	4	4.4 b
14.4	6	3.3 b
28.9	2	1.0 c
28.9	4	1.0 c
28.9	6	0.3 c

[z]Each treatment consisted four Petri plates and each plate contained eight leaf explants. The experiments were done twice.
[y]Means separation in columns by Tukey's studentized range (HSD) at $P < 0.05$. The data were averages of two experiments.

were washed with liquid basal MS medium, blotted with sterile paper towels, placed on solidified CIM for 1 month from the time they were placed on liquid medium, and then placed on SIM for the remainder of the experiment. Four replications were used for each treatment and eight explants per replication. The experiment was repeated once. Explants were evaluated every month for callus formation, callus color, and shoot formation. After 4 months, sample callus tissues were evaluated for ploidy using flow cytometry.

Flow cytometry to evaluate ploidy of poinsettia. About 0.5 cm^2 of callus or leaf tissue was excised and chopped with a double-sided razor in Nuclei Extraction Buffer (CyStain PI Absolute P staining kit, order number 05-5022; Partec GmbH, Műnster, Germany) and allowed to incubate for ≈1 min. Samples were strained through Falcon Tubes with Cell Strainer Caps (Fisher Scientific International, Hampton, N.H) with 35-µm nylon mesh filters and then stained with a staining solution containing RNAse and propidium iodide (CyStain PI Absolute P staining kit, order number 05-5022; Partec GmbH). After staining buffer was added, samples were incubated for 1 h and then run through the flow cytometer (Beckman Coulter Epics XL, Beckman Coulter, Fullerton, Calif.) with a 488-nm argon laser. Controls included diploid 'Winter Rose' samples and two confirmed tetraploid poinsettia cultivars 'Barbara Ecke Supreme' and 'Supjibi', obtained from Dr. Ruth Kobayashi, Head Breeder at Ecke Farms, Encinitas, Calif.

Statistical analyses. A 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 separated using Tukey's studentized range (HSD) at $P < 0.05$. Percentage data were normalized using arcsin transformation before analyzing.

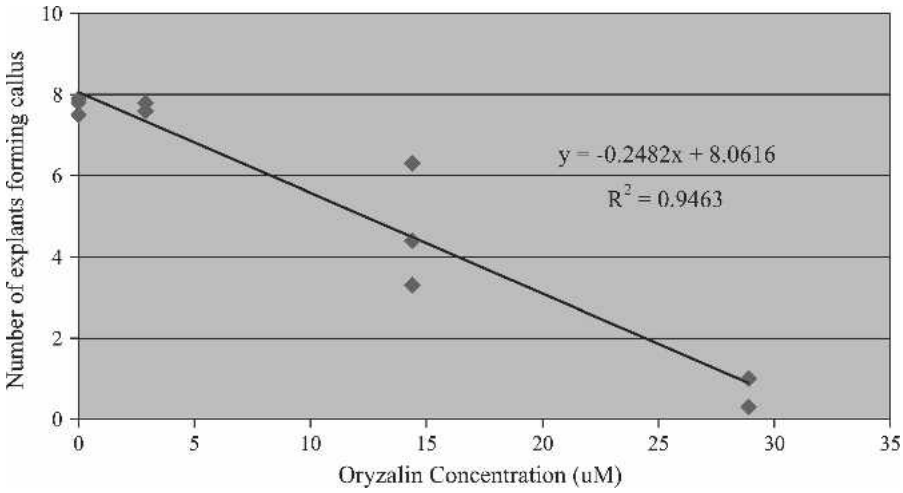

Fig. 2. Effects of oryzalin on frequency of explants of Euphorbia pulchurrima 'Winter Rose' producing callus.

Table 4. Effects of oryzalin concentration on mean numbers of explants producing calluses on *Euphorbia pulchurrima* 'Winter Rose': liquid-solid treatments[z].

Oryzalin (µM)	Duration (d)	Mean number of explants producing callus	Mean number of dead explants per plate
0	0	3.5 a[y]	0.3 c
0	1	3.1 ab	0.3 c
0	2	2.5b	1.0 c
28.9	1	0.5 cd	2.9 ab
28.9	2	0 d	3.8 a
72.2	1	1.1 c	2.3 b
72.2	2	0.6 cd	3.6 a
115.5	1	0.5 cd	3.4 ab
115.5	2	0.4 cd	3.6 a
144.3	1	0.5 cd	3.5 ab
144.3	2	0.63 cd	3 ab

[z]Each treatment consisted four Petri plates and each plate contained eight leaf explants. The experiments were done twice.
[y]Means separation in columns by Tukey's studentized range (HSD) at $P < 0.05$. The data were averages of two experiments.

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 brownish callus. Significantly more explants produced callus when placed on medium containing 0 µM, 0.25 µM of colchicine for 2 d, or 2.5 µM of colchicine for 2 and 4 d than those placed on medium containing 25.0 µM colchicine for 4 d (Table 1). Explants in other treatments produced similar amount of calluses. The concentration of or duration of colchicine treatment was unrelated to number of explants producing callus. Adventitious shoots were produced on three media that contained colchicine. Explants cultured on media containing 2.5 µM for 2 d produced significantly more shoots than explants cultured on media with other concentrations of colchicine. Explants that first produced red callus were indicative of adventitious shoot formation (Fig. 1A). Green globular 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. 1B).

Explants that were first cultured on liquid medium containing colchicine and then transferred to solid CIM produced a large amount of white or brownish callus. Significantly more explants cultured on media containing 250.4 µM colchicine for 2 d produced calluses than explants cultured on the medium with 125.2 µM of colchicine for 2 d (Table 2). Explants on other concentrations or durations of colchicine treatments produced similar amounts of calluses. Only explants exposed to colchicine at 62.6 µM for 1 or 2 d or at 187.8 µM for 1 d produced red callus from which red or green globulus organs and adventitious shoots developed (Table 2). The concentration or duration of exposure to colchicine was not correlated to number of explants producing calluses or those producing adventitious shoots.

Table 5. Poinsettia materials evaluated by flow cytometry for ploidy levels.

Materials	Source of materials	No. 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

Explants that were cultured exclusively on liquid medium enlarged when placed in liquid medium but later turned brown. Callus failed to form on any leaf explants treated in either colchicine-containing media or from control cultures.

Colchicine is a toxic chemical and its dosage and duration of treatment can be associated with mortality and growth inhibition. Explants that were exposed to the lowest concentration of colchicine produced the greatest abundance of shoots in both liquid and solid media. The lower the concentration of colchicine, regardless of the exposure time, the less chemical damage to leaf tissues. These results were consistent with many studies, including those with *Rhododendron* in which higher concentrations of colchicine killed explants (Väinölä, 2000).

The induction of shoots by explants exposed to high concentrations of colchicine (187.8 to 250.4 μM) without tetraploid induction was unexpected because the concentrations have been reported to be high enough to induce tetraploids in other species such as potato (Teparkum and Veilleux, 1998). However, reports have also shown that some species may tolerate high concentrations of colchicine and may require high concentrations to induce polyploid formation. For example, in a study to optimize the in vitro chromosome-doubling protocol in wheat anther culture, Redha et al. (1998) found that exposure to 350 μM or 3500 μM of colchicine for 1 to 3 d were needed to produce the greatest number of autotetraploid plants.

Other studies have reported ineffectiveness of colchicine for inducing chromosome doubling. Colchicine up to 700 μM failed to induce chromosome doubling in potato anther culture, and this response might have been the result of secondary embryogenesis from inner tissue that may not have been exposed to colchicine (Teparkum and Veilleux, 1998). Our results were consistent between two experiments regardless of the type of medium used. Diploid shoots resulting from these explants may be the result of regeneration from cells in the interior of the explants that were protected from colchicine.

Effect of oryzalin concentration and duration on callus formation of '*Winter Rose*' *poinsettia.* Less than 1% explants that were continuously cultured on agar-solidified medium containing 28.9 μM oryzalin produced calluses, whereas nearly 8% control explants produced calluses (Table 3). White calluses were produced, appeared disorganized, and later turned brown. Red callus was absent, and none of the callus tissues formed shoots. Many of the explants exposed to concentrations higher than 115.5 μM for 2 d failed to produce callus and later died (Fig. 1C). Frequency of callus-producing explants was negatively correlated with oryzalin concentration (Fig. 2).

Progressively fewer explants that were first cultured on oryzalin-containing liquid medium then transferred to solid CIM produced callus and showed higher mortality rates as concentration and duration of oryzalin increased (Table 4). Many explants failed to produce any callus and died quickly after exposure to oryzalin.

Oryzalin is known to be less toxic to plant tissues than colchicine (Väinöolä, 2000). For Lilium (van Tuyl et al., 1992) and Nerine (Tosca et al., 1995), shoot regeneration was less inhibited by oryzalin than by colchicine. Tetraploid Lilium was also induced by a lower concentration of oryzalin than by colchicine (van Tuyl et al., 1992). However, in our study with 'Winter Rose', oryzalin was found to be unsuitable for inducing tetraploid formation because it caused high mortality of explants even at lower concentration (28.9 μM), significantly inhibited callus production, and prohibited shoot formation. This negative result has also been observed for *Rhododendron* species that showed 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 effective for determining ploidy of samples of poinsettia callus and shoots exposed to colchicine or oryzalin and control samples (Table 5). Diploid 'Winter Rose' leaves (Fig. 3A) and the known tetraploid cultivars 'Barbara Ecke Supreme' (Fig. 3B) and 'Supjibi' (Fig. 3C) showed expected histograms of diploid and tetraploid. A histogram of an internal control containing the diploid 'Winter Rose' and the tetraploid 'Supjibi' shows both diploid and tetraploid peaks (Fig. 3D). All red callus samples generated from explants exposed to colchicine and cultured on solid medium lacked tetraploid cells. All leaf samples of adventitious shoots were also found to be diploid (Fig. 3E). Callus from explants exposed to colchicine in liquid medium were also analyzed for ploidy and were found to be diploid (data not shown).

Flow cytometry was also used to evaluate the ploidy level of explants exposed to oryzalin either in liquid or solid medium. Flow cytometry histograms revealed that all

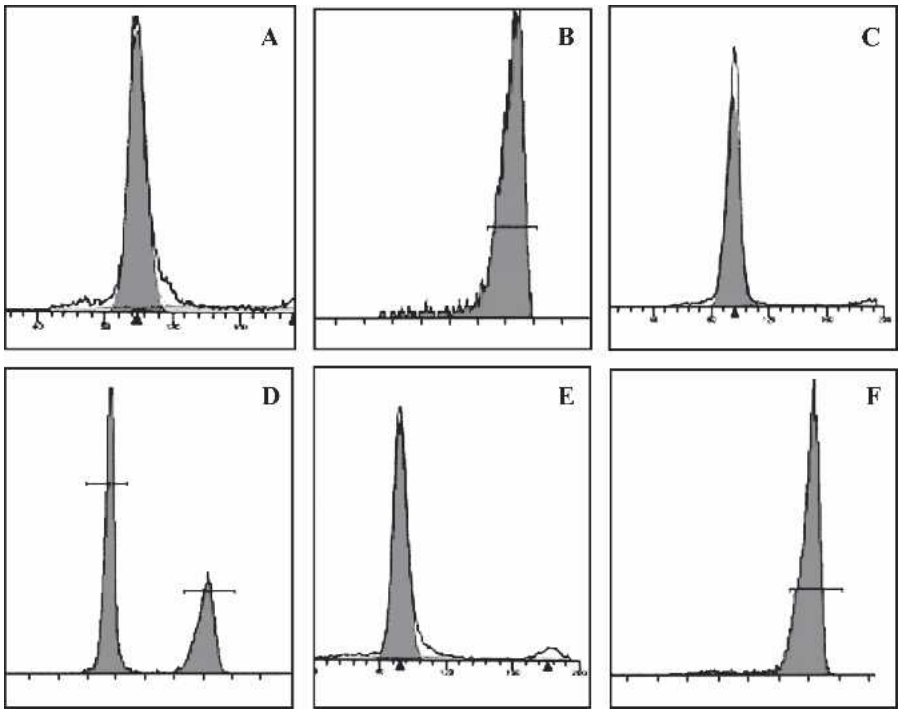

Fig. 3. Flow cytometry histographs of leaf and callus from control and explants exposed to colchicine and oryzalin. (**A**) Diploid control leaf of 'Winter Rose' poinsettia. (**B**) Tetraploid leaf 'Barbara Ecke Supreme. (**C**) Tetraploid leaf Supjibi. (**D**) Histogram of mixed leaf samples of Diploid leaf 'Winter Rose' + tetraploid 'Supjibi'. (**E**) Callus exposed to colchicine in solid medium showing diploid peak. (**F**) Diploid callus exposed to oryzalin on solid medium showing diploid peak.

cells in tested callus samples were diploid (Fig. 3F).

The flow cytometry analytical procedures seemed to work well because the control diploid and tetraploid poinsettia used showed expected histograms (Fig. 3E, F). A protocol for nuclei extraction and flow cytometric evaluation of ploidy level in *Euphorbia pulchurrima* was established.

Responses to both colchicine and oryzalin were reported to be highly species-specific (Blakesley et al., 2002; Peterson et al., 2003; Tosca et al., 1995; van Tuyl et al., 1992). 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 shown differential response according to genotype in *Miscanthus sinensis* (Peterson et al., 2003). Our research indicated that oryzalin is an unsuitable reagent for inducing tetraploid formation in 'Winter Rose' using methods described here and that colchicine is less inhibitory for adventitious shoot regeneration. This baseline information along with the flow cytometry protocol established will allow researchers to further improve the protocol of in vitro tetraploid induction in poinsettia.

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