Poinsettia ‘Prestige™ Red’ (*Euphorbia pulcherrima*) In Vitro Propagation

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Abstract. Slow growth rate of plantlets, few micro-shoots per explant, and slow root growth rate are restrictions of in vitro propagation of poinsettia (*Euphorbia pulcherrima* Willd. ex Koltz). The purpose of this research was to develop an efficient in vitro proliferation technique for poinsettia ‘Prestige™ Red’. Explants (apical buds and axillary buds) placed on Murashige and Skoog (MS) basal medium containing only 6-benzylaminopurine (BA) and combinations of BA and indole-3-acetic acid (IAA) mostly produced red callus, which is productive and some white and gray-green calluses at the base of plantlets after 1 month, whereas explants in a medium without plant growth regulators (PGRs) produced no callus. Addition of IAA into the rooting medium increased rooting efficiency; plantlets grown in half-strength MS salts and vitamins with 28.5 μM IAA initiated rooting 11 days earlier than the plantlets grown with no PGRs. Optimization of PGR concentrations during poinsettia micropropagation helped resolve previous restrictions of in vitro poinsettia proliferation. Chemical names used: 6-benzylaminopurine (BA); indole-3-acetic acid (IAA)

Milky sap (latex) secreted at the cut surfaces is a common feature of the family Euphorbiaceae; the genus *Euphorbia* consists of close to 2000 species (Ecke et al., 2004). *Euphorbia pulcherrima* Willd. ex Klotzsch, poinsettia, belongs to Euphorbiaceae and is the number one flowering potted plant in the United States (Clarke et al., 2008).

The cultivar Prestige™ Red (Eckespoint® Prestige™) was developed in 2002 for improved greenhouse performance (Ecke et al., 2004). Characteristics of ‘Prestige™ Red’ include strong stems, upright growth habit, resistance to stem breakage, and uniform shoot growth, which facilitate floral display and makes it a popular cultivar (Paul Ecke Ranch, 2008).

Few studies have reported in vitro organogenesis and axillary bud proliferation protocols of poinsettia (De Langhe et al., 1974; Pickens et al., 2005; Roy and Jinnah, 2001). The role of cytokinins in developing caulogenetic callus from internodal explants was first reported in poinsettia ‘Paul Mikkelsen’; higher cytokinin:auxin produced more micro-buds from existing callus (De Langhe et al., 1974). Repeated subculture of internodal explants in a medium supplemented with 6.65 μM 6-benzylaminopurine (BA),

2.32 μM kinetin (Kn), and 15% coconut milk resulted in rapid shoot proliferation; shoots were rooted on a half-strength Murashige and Skoog (MS) medium containing 4.92 μM indole-3-butyric acid and 2.85 μM indole-3-acetic acid (IAA) (Roy and Jinnah, 2001). Pickens et al. (2005) developed protocols for both axillary bud proliferation and shoot organogenesis using terminal buds and leaf tissues of poinsettia ‘Winter Rose’. The explants produced the greatest number of axillary buds on media containing between 2.2 and 8.8 μM BA.

The development of in vitro protocols for plant regeneration through either organogenesis or somatic embryogenesis is one of the main prerequisites for the potential applications of clonal propagation and genetic transformation (Vila et al., 2003). In vitro clonal propagation of poinsettia is important because conventional propagation of poinsettia by cuttings and seed has several limitations. Seed propagation of poinsettia is used less because seed lose viability on storage and plants originating from seed have genetic variability (Jasrai et al., 2003). Propagation through cuttings leads to disease transfer from the mother plant. Therefore, the objective of this research was to develop an efficient in vitro propagation technique for poinsettia for use in transformation research.

Materials and Methods

Research was conducted in the tissue culture laboratory of Dorman Hall and the greenhouse (glass-glazed greenhouse with pad and fan cooling and hot water-finned tube perimeter heating) complex at Mississippi State University from Jan. to May 2008.

Plant material and culture conditions. Apical buds and axillary buds (explants) with 1 to 2 cm of stem of *E. pulcherrima* ‘Prestige™ Red’ were excised during Jan. 2008 from healthy plants grown in the greenhouse under long-day photoperiods. Long days were created during October to Dec. 2007 using night interruption lighting with incandescent bulbs ($1.8 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) from 2200 to 0200 HR. The buds were surface-disinfected by placing them in a 1% Tween 20 solution for 5 min followed by 70% ethyl alcohol for 1 min and then 20% bleach (97% sodium hypochlorite) for 15 min (Pickens et al., 2005). Finally, buds were dipped in sterile water three times for 5 min. Basal medium (20 mL) containing MS salts ($4.4 \text{ g}\cdot\text{L}^{-1}$), myo-inositol ($0.1 \text{ g}\cdot\text{L}^{-1}$), sucrose ($30 \text{ g}\cdot\text{L}^{-1}$), and agar ($7 \text{ g}\cdot\text{L}^{-1}$) (pH 5.7 to 5.8) (Pickens et al., 2005) was poured into 100-mL baby food jars and autoclaved at a temperature of 121 °C at 15 psi for 15 min. Various concentrations of plant growth regulators (PGRs) were incorporated into MS basal medium depending on treatment type. BA was incorporated before autoclaving the liquid medium and IAA (filter-sterilized) was incorporated into the liquid medium after autoclaving. Disinfected buds were placed on the autoclaved medium solidified with agar in aseptic conditions under a laminar flow hood and incubated in a growth chamber with a 16-h photoperiod ($125 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ illumination with fluorescent bulbs) at 25 °C day and 22 °C night temperatures. Effects of PGRs on callus induction and rooting were evaluated in two studies.

Callus study. The callus study was composed of two experiments. For the first experiment, BA was incorporated into the basal medium at concentrations of 0 μM , 4 μM , 6 μM , 8 μM , 10 μM , and 12 μM ; and for the second experiment, 4 μM , 6 μM , 8 μM , 10 μM , and 12 μM of BA were incorporated in combination with 4 μM of IAA. An experimental control with 0 μM of BA and 0 μM of IAA was also incorporated in the second experiment. After 1 month, explants were visually evaluated for percentage of explants having red callus at the base and callus color. Furthermore, the number of micro-buds and micro-shoots was examined. Explants were subcultured four times at monthly intervals onto a new medium with the same ingredients.

Rooting study. Four-month-old in vitro-grown poinsettias were transferred onto a rooting media consisting of one of the treatments: MS with 28.5 μM IAA (Roy and Jinnah, 2001), half-strength MS salts and vitamins with 28.5 μM IAA, or MS with 4 μM BA. An experimental control with MS and no PGRs was also included. The plants were evaluated for average number of days for root initiation and the number of roots initiated per shoot. The average number of days for root initiation was calculated as the total number of days taken for root initiation (number of days from shoots sticking into the medium through root initiation) divided by the total number of plantlets per treatment. The experiment was ended after 5 months.

Statistical analysis. Each experiment had five explants per treatment (a vessel with an

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explant is an experimental unit) and each experiment was repeated once within each subculture cycle. All treatments were arranged in a randomized complete block design and data were analyzed by the General Linear Model using Statistical Analysis System (SAS Version 9.1.2; SAS Institute, 2004). Treatment means were separated by the least significant difference method at $\alpha = 0.05$.

Results and Discussion

Explant disinfection, plant material, and growth conditions. Surface disinfection of explants was difficult as a result of latex secreted at the cut surfaces of explants. At least 15% to 25% of the explants were contaminated per each micropropagation cycle during a preliminary study. The contaminations were greater (40%) without using 1% Tween 20 solution during the disinfection process of explants. Thus, Pickens et al. (2005) disinfection protocol was used to minimize contaminations encountered. Explants (apical and axillary buds) of poinsettia 'Prestige™ Red' responded to the PGR combinations (IAA and BA) by developing calluses at the base of the explant before organogenesis. Monthly repeated subculture onto a new medium was necessary to achieve organogenesis and plantlets.

Callus study. Primarily two types of calluses were identified in poinsettia micropropagation: red callus (Fig. 1A) and white callus (Fig. 1B). With continuous subculturing in a medium with BA (4 to 12 μ M), red callus produced more micro-buds, micro-

shoots (data not shown), and healthy plants, whereas white callus remained unchanged. White callus is a result of recalcitrant cells, which are unproductive. Red callus is the most important in poinsettia proliferation; it proliferates fast and produces into micro-buds and micro-shoots. The results observed were consistent with other experiments of poinsettia such as De Langhe et al. (1974) and Pickens et al. (2005) in which red callus produced plantlets efficiently. Apart from red and white calluses, gray-green callus was also observed with low BA levels (2 to 4 μ M). This was consistent with the study of De Langhe et al. (1974), in which the dominant callus color was red with low IAA:Kn. The higher the ratio, the more grayish green callus produced.

Explants grown in media supplemented with BA produced red callus at the base of the explant, whereas explants in media without BA did not give rise to any callus. There is a significant ($\alpha = 0.05$) difference between plants that are treated with BA and without BA (Table 1). Therefore, use of BA in poinsettia micropropagation is effective and increased red callus production. BA can be used to generate red callus (Table 1), micro-buds (Fig. 1C), and micro-shoots (Fig. 1D) in poinsettia micropropagation. The impact of cytokinins on poinsettia in vitro bud development was reported previously by De Langhe et al. (1974) who observed faster bud development with higher levels of the cytokinin 2-isopentenyl adenine, which is a cytokinin. This finding was similar to our research in which cytokinin (BA) helped produce additional micro-buds.

Explants grown in media supplemented with both BA and IAA produced red callus at the base of the explant showing a significant ($\alpha = 0.05$) difference among treatments; treatments with BA greater than 8 μ M produced more callus than treatments with 8 μ M BA or less (Table 2). Continuous growth in a medium supplemented with BA (4 to 12 μ M) alone resulted in a greater number of micro-buds and micro-shoots compared with medium supplemented with both IAA (4 μ M) and BA (4 to 12 μ M) (data not shown). Uchida et al. (2004) reported the impact of cytokinins on adventitious bud formation of *E. tirucalli* L.; adventitious buds were efficiently induced when the medium was supplemented with thidiazuron (TDZ) compared with a medium with both TDZ and IAA. We observed similar results in which BA alone induced micro-buds efficiently compared with a medium with both BA and IAA. Incorporation of IAA and BA into the medium generated additional red callus, but the BA:IAA ratio is critical in generating red callus. The greater the BA:IAA, the greater the potential for red callus production when the medium was provided with both BA and IAA. Equimolar concentrations of cytokinins to auxins are generally used to sustain callus, whereas higher cytokinin:auxin stimulates shoot development (Pickens et al., 2005).

Rooting study. Four-month-old poinsettia (40 micro-shoots) transferred into rooting media rooted irrespective of IAA concentrations. Incorporation of BA had an inhibitory

Fig. 1. Poinsettia 'Prestige Red' explants (apical buds and axillary buds) producing red and white calluses, micro-buds, and micro-shoots in poinsettia micropropagation. (A) Red callus on a medium with 10 μ M 6-benzylaminopurine (BA) (1 month old); (B) white callus on a medium with 4 μ M indole-3-acetic acid (IAA) and 10 μ M BA (1 month old); (C) micro-buds on a medium with 12 μ M BA (2 months old); (D) micro-shoots on a medium with 10 μ M BA (3 months old).

Table 1. Red callus initiation from 1-month-old poinsettia 'Prestige Red' explants (apical buds and axillary buds) on Murashige and Skoog (MS) medium supplemented with 6-benzylaminopurine (BA).

BA conc (μ M)	Percent of explants with a red callus at the base
0	0 b ^z
4	100 a
6	100 a
8	100 a
10	100 a
12	100 a

^zTreatments with the same letter are not significantly different. Means were separated using least significant difference at $P_{\alpha} = 0.05$.

Table 2. Red callus initiation from one mo. old poinsettia 'Prestige Red' explants (apical buds and axillary buds) on Murashige and Skoog (MS) medium supplemented with indole-3-acetic acid (IAA) + 6-benzylaminopurine (BA).

PGR conc (μ M)		Percent of explants with a red callus at the base
IAA	BA	
0	0	0 a ^z
4	4	83.7 b
4	6	83.7 b
4	8	100 c
4	10	100 c
4	12	100 c

^zTreatments with the same letter are not significantly different. Means were separated using least significant difference at $P_{\alpha} = 0.05$.

PGR = plant growth regulator.

Table 3. Root initiation from four mo. old poinsettia ‘Prestige Red’ micro-shoots (4 months old) on Murashige and Skoog (MS) medium supplemented with plant growth regulators (PGRs).

PGR conc (μM)	Avg no. of days for root initiation	No. of roots initiated
No PGRs	35 a ^z	2 a ^z
4 μM BA	0 d	0 c
Half-strength MS with 28.5 μM IAA	24 c	3 b
Full MS with 28.5 μM IAA	28 d	3 b

^zTreatments with different letters are significantly different. Means were separated using least significant difference at $P_{\alpha} = 0.05$.

effect on rooting; plants treated with BA developed callus at the base of the plant instead of rooting. In our study, the best medium for poinsettia in vitro rooting was half-strength MS salts and vitamins with 28.5 μM IAA because it increased rooting efficiency (Table 3). Higher IAA concentrations had a negative impact on rooting leading to callus development at the base of the plant (data not shown).

In summary, BA (4 to 12 μM) can be used effectively to generate red callus, micro-buds, and micro-shoots from apical and axillary buds of poinsettia ‘Prestige™ Red’. Incorporation of IAA and BA into the medium generates red callus but BA:IAA is critical in generating red callus; the greater the BA:IAA ratio, the greater potential for red callus production. Red callus is more

important for poinsettia proliferation than white callus. The best medium for in vitro rooting of poinsettia was half-strength MS salts and vitamins with 28.5 μM IAA for rooting efficiency. Results from this research can be used as an in vitro micropropagation and proliferation protocol for poinsettia ‘Prestige™ Red’. This protocol can be used in both poinsettia in vitro proliferation and as the first step in transgenic poinsettia production. Further research is necessary to determine how to minimize development of white callus during in vitro poinsettia culture.

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