Micropropagation of *Epilobium canum garretti* (Firechalice) by Axillary Shoot Culture

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Abstract. *Epilobium canum* subsp. *garrettii* (firechalice) is an herbaceous wildflower with landscape potential, but its seeds are difficult to germinate because of dormancy requirements. The objective of this study was to develop a complete micropropagation procedure for a selected accession of firechalice. Single-node stem explants from the plant were examined for their ability to establish on Murashige and Skoog (MS) medium or Woody Plant Medium (WPM). Shoot explants on MS medium supplemented with 4.4 μM benzyladenine (BA) produced more than double the number of axillary shoots compared to explants on WPM (12.6 vs. 4.9 shoots, $P = 0.0001$). Benzyladenine, kinetin (kin), 6-(γ,γ -dimethylallylamino)purine (2iP), thidiazuron (TDZ), and meta-topolin (mT) at concentrations of 0, 1.1, 2.2, 4.4, or 8.8 μM were evaluated for shoot proliferation efficacy. Stem explants treated with 8.8 μM of BA or mT produced the most shoots, 11 or 15, respectively. Benzyladenine, 2iP, and kin failed to affect shoot height even at the highest concentrations used, but 4.4 or 8.8 μM TDZ reduced shoot height to less than half of the heights of control shoots (3.1 vs. 1.2 cm, $P = 0.0001$). Firechalice shoots formed three to four roots easily without auxin added to the medium, but four to six roots formed per shoot when using up to 9 μM of indole-3-butyric acid (IBA). In contrast, 9 μM naphthaleneacetic acid (NAA) prevented root formation. When using 0–9 μM IBA for rooting, 82.5% of the rooted shoots survived transplanting. Based on these results, optimum micropropagation of firechalice may be achieved with shoots established on MS medium plus 4.4 μM BA, a concentration of 4.4 or 8.8 μM BA or mT used for shoot proliferation, and use of up to 6 μM IBA during root induction should result in >80% shoot survival after transplanting.

Many species of native plants have unique traits, including adaptability and drought tolerance, and provide value in managed home and commercial landscapes. Potentially

useful native plants have been used sparingly in the landscape trade because of seed-based propagation difficulties. Vegetative propagation could provide an alternate tool for commercial production of these plants. Asexual plant propagation may be a viable option for producing plants with low seed germination rates and slow seedling growth (Unander et al., 1995) or for obtaining genetic uniformity.

Epilobium canum (Greene) P.H. Raven *garrettii* (A. Nelson) P.H. Raven (syn. *Zauschneria garrettii*), common name firechalice or hummingbird flower, is in the Onagraceae family. This species is native to the Intermountain West, and it grows naturally in all western U.S. states except Washington, Montana, and Colorado. Firechalice spreads as a groundcover, and the flowers are bright orange-red and attractive to hummingbirds (Love et al., 2009). Plants are relatively small, usually 30–45 cm tall and 30–60 cm wide with some older plants being much wider than tall. Leaves are dark green, and lance-shaped foliage is slightly pubescent. Stems grow upright and are weakly spreading with thin branches that tend to arch. Bloom starts in June and extends

into October. Plants bloom the first year and increase in width and number of flowering stems as they age. Plants bloom when their stems are $\approx$ 30–45 cm tall (Love et al., 2009) and grow best in full sun but can tolerate part shade. This herbaceous perennial plant dies back in fall and regrows from rhizomes each spring (Winger, 1996).

Firechalice is a good choice for rock gardens and is drought tolerant, although it requires some supplemental irrigation to be attractive throughout the season and may grow better with light fertilization (Robson et al., 2008). The species tolerates most types of soil over a wide pH range (5.5–8.5). Firechalice is an outstanding xeric landscape plant that deserves more attention in the nursery trade.

Firechalice can be propagated by using various techniques. Seeds of this species are difficult to harvest, and seeds from some ecotypes germinate poorly, meaning asexual propagation would be an appropriate alternative. Close to 100% of the stem cuttings taken from *E. canum* can root if the stock plants are held at 4 to 10 °C (Anderson and Rupp, 2012). Root cuttings can also be successful (Love et al., 2009). With firechalice, stem cutting techniques are somewhat limited with respect to number of cuttings obtained from each parent plant, rooting success rate, and overall propagation efficiency.

Tissue culture propagation could provide a viable alternative for rapid propagation of firechalice with a limited amount of stems available to propagate. For plants in general, axillary shoot proliferation is the best tissue culture technique for true-to-type reproduction (Kane, 2011). Several related species in Onagraceae, including *Ludwigia repens* (Öztürk et al., 2004), *Lopezia racemosa* (Salinas et al., 2014), *Fuchsia magellanica* (Parveen and Rasheed, 2013), and several *Oenothera* species (Hafez et al., 2015; Skrzypczak et al., 1994; Taniguchi et al., 2006), have been reproduced by tissue culture. Most of these species have been micropropagated via adventitious shoot regeneration from excised seedling tissues (roots, stems, leaves, etc.), with the exception of *L. repens* and *F. magellanica* that can be reproduced by axillary buds. Three *Epilobium* species—*Epilobium parviflorum* (Deliu et al., 2007), *Epilobium hirsutum* (Tamaş et al., 2009), and *Epilobium angustifolium* (syn. *Chamaenerion angustifolium*) (Deliu et al., 2013; Dreger et al., 2016) have been micropropagated mainly by adventitious shoot regeneration from seedling tissues. Dreger et al. (2016) used shoot tips to reproduce *E. angustifolium* but found that they were less successful for producing new shoots compared with using adventitious shoot regeneration.

The goal of this research was to develop a micropropagation procedure that used axillary shoot proliferation for rapid production of a selected firechalice plant. We demonstrate that firechalice can multiply quickly in the first two stages of micropropagation along with high percentages of rooting and transplant survival resulting in high

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throughput propagation of a selected accession. Having a rapid method to multiply plant numbers will allow a superior selection of a native plant to be introduced more quickly into the landscape trade than by using stem cuttings.

Material and Methods

A selected firechalice accession (*E. canum garrettii*) used for micropropagation was obtained from Dr. Stephen Love, University of Idaho. The selection was made from a wild collection in 2008 near Tony Grove Lake, Cache County, UT. This plant was selected for upright growth habit, long bloom period, intense red flower color, and limited rhizomatous spreading compared with the species norm. Single-node stem explants were taken from rooted stem cuttings of the selected plant. Leaves were removed from stems, and ≈ 2 -cm stem explants that contained a single node were surface sterilized for 20 min in 0.6% (v/v) sodium hypochlorite containing two drops of Tween® 20.

Two media were evaluated for establishing firechalice shoots in culture. Single-node explants were placed on MS medium (Murashige and Skoog, 1962) or WPM (Lloyd and McCown, 1980). Murashige and Skoog medium (product M524; PhytoTechnology Laboratories, Shawnee Mission, KS) contained $4.3 \text{ g}\cdot\text{L}^{-1}$ mineral salts, vitamins, glycine, and myo-inositol concentrations used by Murashige and Skoog (1962), $2 \text{ mg}\cdot\text{L}^{-1}$ thiamin, and $30 \text{ g}\cdot\text{L}^{-1}$ sucrose, solidified with $7 \text{ g}\cdot\text{L}^{-1}$ agar (product A111; PhytoTechnology Laboratories), and had a pH 5.7. The medium included $3.5 \mu\text{M}$ BA in the first subculture, which was increased to $4.4 \mu\text{M}$ in the second subculture. Woody plant medium (product L449; PhytoTechnology Laboratories) contained $2.3 \text{ g}\cdot\text{L}^{-1}$ salts; the same concentrations of vitamins, glycine, and myo-inositol as the MS medium (described previously); and $20 \text{ g}\cdot\text{L}^{-1}$ sucrose. It was solidified with $7 \text{ g}\cdot\text{L}^{-1}$ agar and the pH was adjusted to 5.2 and included $3.5 \mu\text{M}$ BA. The media were dispensed into GA7 vessels (Magenta Corp. Ltd., Chicago, IL) and autoclaved at 120°C for 20 min. After inoculation, stem cultures were incubated in a SG 30S germinator (Hoffman Manufacturing Inc., Albany, OR) at $25 \pm 1^\circ\text{C}$ under a 16-h photoperiod (cool-white fluorescent lamps), with $38 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ photosynthetic photon flux (PPF). After 4 weeks, shoot cultures were subcultured and placed on fresh MS medium or WPM two times before axillary shoot proliferation was started. Because stem explants grew best on the MS salt formulation, this medium supplemented with $4.4 \mu\text{M}$ BA was used in subsequent experiments for producing shoots used for optimizing shoot proliferation and root formation.

Studies were conducted to optimize axillary shoot proliferation in preparation for later stages of micropropagation. The selected cytokinins, BA, kin, 2iP, TDZ, and mT, were examined mainly for their effects

on the number of axillary shoots produced on stem explants. These cytokinins were used at concentrations of 0, 1.1, 2.2, 4.4, or $8.8 \mu\text{M}$ and were added to the culture medium before autoclaving. After 4 weeks, shoot growth parameters were determined (described in the following paragraphs).

Several treatments were used to root shoot explants. Combinations of BA and IBA were evaluated for their ability to maintain or improve shoot quality while trying to induce root formation. Firechalice shoots ≈ 1 cm tall were placed on MS medium with various concentrations of BA (0, 1.1, 2.2, or $4.4 \mu\text{M}$) combined with various concentrations of IBA at (0, 1, or $2 \mu\text{M}$). In a second study, firechalice shoots were placed on MS medium with different concentrations of IBA or NAA (0, 3, 6, or $9 \mu\text{M}$). After 4 weeks, shoot growth and rooting parameters were determined (described in the following paragraphs). In a third study, 1-cm-tall shoots taken directly from in vitro cultures had the bottom 3 mm of stem dipped into distilled water. The shoots were treated with 0.2% naphthaleneacetamide (w/w) (Rootone; Smith Industries, Kansas City, MO), 0.3% IBA (w/w) (Hormex #3; Maia Products, Inc., Simi Valley, CA), or distilled water (control) and then placed in Sunshine #1 potting mix (Sun Gro Horticulture, Vancouver, Canada). Treated shoots were placed in three clamshell containers arranged in a randomized complete block design to determine the efficacy of an ex vitro rooting protocol. Ten shoots in one row were placed in each clamshell container ($20 \text{ cm} \times 20 \text{ cm} \times 9 \text{ cm}$) with 30 shoots used per treatment.

Plantlets were placed in clamshell containers to acclimate in vitro-rooted shoots to conditions outside of the tissue culture vessels. In vitro-rooted shoots were used from the IBA and NAA rooting study. The clamshell containers were placed in the growth chamber under the same environmental conditions described previously. The percentage of plantlets that survived was determined 6 weeks after transplanting.

Data analyses. Four experimental units (vessels) with six subsamples (six shoots) were used in each treatment. Vessels were arranged on shelves in the growth chamber in a randomized complete block design with one vessel from each treatment in each block (one block per shelf). Data taken in each experiment included the number of shoots formed (from axillary buds) per stem explant, height of the tallest shoot on the explant, and shoot dry weight. Shoot dry weights were determined by drying the shoots in a 70°C oven for at least 3 days. The number of roots per responding shoot, percentage of explants forming roots, and length of the longest root on each rooted shoot were additional data recorded for the rooting experiments. Growth parameters for the shoots in each vessel were averaged, and the means were analyzed by analysis of variance (ANOVA) (mixed model procedure) (Proc Mixed, SAS, 2012) for shoot establishment data and two-way ANOVA when comparing different plant growth regulators

used at various concentrations for axillary shoot proliferation and rooting studies. The type of cytokinin and their concentrations were used as independent variables for analyses of axillary shoot proliferation, whereas auxin type and concentrations were used as independent variables for analyses of rooting experiments. For all analyses, if the interaction between the growth regulators and their concentrations was significant, then effects of the growth regulator concentrations were tested for each individual growth regulator. Significant differences between treatment means were determined by least-square means at the 5% level when comparing growth differences of explants placed on different media. Plantlet survival data were analyzed by ANOVA assuming a Poisson distribution (Proc Glimmix, SAS, 2012) because count data were evaluated. Single degree-of-freedom contrasts were analyzed to determine the difference in survival between IBA- or NAA-treated plantlets.

Results

Single-node stem sections placed on either MS medium or WPM readily established in culture with $<10\%$ contamination. By the third subculture, firechalice shoots on MS medium produced ≈ 2.6 times more shoots ($P = 0.0001$), 2.9 times more shoot dry weight ($P = 0.0001$), and grew three times taller ($P = 0.0001$) than shoots grown on WPM (Table 1). Leaf color of shoots grown on MS medium was also greener than that of leaves on shoots grown on WPM. If a stem was damaged or crushed rather than cut cleanly or if leaves touched the medium, these tissues produced a black substance, most likely a phenolic compound. Keeping leaves above the medium and cutting the stem without damaging it usually prevented most phenolic production. Firechalice shoots became stabilized after three subcultures (judged by good shoot color and consistent, regular growth of the shoots) and axillary shoot multiplication experiments were started.

Different cytokinins were tested for their ability to induce axillary shoot proliferation. Benzyladenine (from 0 to $8.8 \mu\text{M}$) was considered the standard cytokinin to use as a comparison with other cytokinins. The two-way analyses revealed significant interactions ($P < 0.001$) between the type of cytokinin used and the concentrations used for the number of shoots formed, shoot heights, and shoot dry weights. Therefore, concentration effects on all three shoot growth parameters were analyzed separately for each cytokinin.

The two most effective cytokinins for promoting axillary shoot proliferation were BA and mT (Table 2). A concentration of $8.8 \mu\text{M}$ BA induced ≈ 11 shoots to form per explant, whereas $4.4 \mu\text{M}$ mT was needed to induce 14 shoots per explant (Table 2). As the concentration of mT increased from 0 to $8.8 \mu\text{M}$, shoot height in culture increased significantly by ≈ 1 cm ($P = 0.0059$), but shoot height seemed unaffected by BA

Table 1. Effects of using two different media, Murashige and Skoog (MS) or Woody Plant Medium (WPM), on the mean number of shoots, mean shoot height, and mean shoot dry weight during establishment of *Epilobium canum garretti* shoots in vitro. Plant growth data were from the third subculture of firechalice shoots, and these shoots were grown on MS medium or WPM for 4 weeks for each subculture. Four vessels were used per treatment, and within each vessel, six shoots were averaged before analysis.

Medium	Number of shoots	Shoot ht (cm)	Shoot dry wt (g)
MS	12.6 b ^z	2.4 b	0.038 b
WPM	4.9 a	0.8 a	0.013 a

^zDifferent letters within a column indicate significant differences between means as determined by least-squares means tests at $P \leq 0.05$ level ($n = 4$).

Table 2. Effects of benzyladenine (BA), meta-topolin (mT), thidiazuron (TDZ), kinetin (kin), or 6-(γ,γ -dimethylallylamino)purine (2iP) concentrations on the mean number of shoots, mean shoot heights, and mean shoot dry weights of *Epilobium canum garretti* shoots grown on Murashige and Skoog medium for 4 weeks. Four vessels were used per treatment, and within each vessel, six shoots were averaged before analysis.

Type of cytokinin	Concn (μM)	Number of shoots	Shoot ht (cm)	Shoot dry wt (g)
BA	0	1.8 a ^z	2.5 a	0.017 a
	1.1	6.6 b	2.7 a	0.022 ab
	2.2	7.1 bc	2.7 a	0.022 ab
	4.4	9.0 cd	2.7 a	0.030 bc
	8.8	11.1 d	2.7 a	0.037 c
mT	0	2.0 a	2.3 a	0.025 a
	1.1	10.0 b	2.6 ab	0.048 b
	2.2	10.5 b	2.8 bc	0.060 bc
	4.4	14.4 c	3.1 c	0.071 cd
	8.8	15.0 c	3.2 c	0.088 d
TDZ	0	1.9 a	3.1 c	0.027 a
	1.1	6.0 b	2.5 bc	0.064 b
	2.2	6.2 b	1.8 ab	0.072 b
	4.4	6.3 b	1.5 a	0.073 b
	8.8	7.2 b	1.2 a	0.126 c
kin	0	1.4 a	2.7 a	0.016 a
	1.1	2.9 a	3.0 a	0.023 a
	2.2	3.1 a	3.0 a	0.028 a
	4.4	6.2 b	3.1 a	0.057 b
	8.8	8.9 b	3.1 a	0.058 b
2iP	0	2.6 a	2.9 a	0.025 a
	1.1	3.0 a	2.8 a	0.024 a
	2.2	5.1 b	2.8 a	0.036 ab
	4.4	5.1 b	3.0 a	0.041 ab
	8.8	6.0 b	3.0 a	0.051 b

^zDifferent letters within a column for each separate cytokinin indicate significant differences between means as determined by least-squares means tests at $P \leq 0.05$ level ($n = 4$).

concentration (Table 2). The highest BA concentration (8.8 μM) increased shoot dry weight ≈ 2 -fold compared with the stems grown without added cytokinin (controls) (Table 2). In contrast, 8.8 μM mT increased shoot dry weight by more than 3-fold compared with control shoots (Table 2).

Thidiazuron promoted shoot growth yet had detrimental effects on firechalice shoots. For instance, even though 8.8 μM TDZ increased the number of axillary shoots by 3.7-fold and shoot dry weight by 4.6-fold over the control treatment, shoots on medium supplemented with the highest concentration of TDZ were 2.5 times shorter than control shoots (Table 2, $P = 0.0005$). The highest concentration of TDZ also produced the heaviest shoots (0.126 g, Table 2).

The highest level of kin increased the shoot multiplication rate by more than 6-fold compared with the control shoots (Table 2), whereas 8.8 μM 2iP increased shoot multiplication rate by only 2.3-fold over control shoots (Table 2). Neither kin nor 2iP concentrations affected shoot heights ($P = 0.8382$ or $P = 0.8122$, respectively), yet 8.8 μM kin or

2iP increased shoot dry weights by 3.5-fold or 2-fold compared with controls (Table 2).

Firechalice shoots rooted easily without any phytohormones in the culture medium. Roots failed to form on shoots placed on media with any concentration of BA even if this growth regulator was combined with various concentrations of IBA. Roots formed on 88% of the shoot explants when using IBA without BA, and similar numbers of roots formed on the stem explants regardless of the IBA concentration used ($P = 0.5567$). The mean number of roots formed per responding stem explant was 2.3 for the control, 2.8 for 1 μM IBA, and 2.4 for 2 μM IBA treatments.

When testing IBA or NAA concentrations for their effects on rooting, an interaction between the type of auxin and auxin concentration was absent for the number of shoots formed. Only the main effect for type of auxin was significant ($P = 0.0111$) for the number of shoots formed, and IBA induced $\approx 32\%$ more shoots to form than NAA, regardless of the concentration used for either auxin. The average number of shoots formed per responding explant across

concentrations of IBA was 2.6 vs. 1.8 across NAA concentrations.

Two-way ANOVA for shoot growth and rooting responses showed a significant interaction between the type of auxin used and auxin concentrations on shoot height, shoot dry weight, number of roots formed per responding shoot, mean length of the longest root, and percentage of rooted shoots. For shoot height during rooting, the type of auxin (main effect) was significant ($P = 0.0001$), but auxin concentration lacked significance. Shoot height was unaffected by different concentrations of IBA ($P = 0.2413$), but with NAA in the medium shoot heights decreased with higher concentrations (e.g., control shoots were 2.8-fold taller than shoots treated with 9 μM NAA, Table 3, $P = 0.0001$).

The type and concentration of auxin affected shoot dry weights differently. Naphthaleneacetic acid in the medium failed to affect shoot dry weight ($P = 0.1169$). The highest shoot dry weights were obtained when using the highest concentration of IBA (9 μM), and the mean dry weight of these shoots was ≈ 2.4 times heavier than that of control shoots (Table 3, $P = 0.0017$).

The number of roots formed per responding shoot decreased by at least 36%, and the length of the longest root on rooted shoots decreased at least 3-fold as NAA concentration increased from 0 to 6 μM . Root formation was completely inhibited by 9 μM NAA (Table 3).

The highest IBA concentrations (6 or 9 μM) significantly increased the number of roots per responding shoot ($P = 0.0468$), yet the length of the longest root was unaffected. Indolebutyric acid concentration failed to affect the percentages of shoots that rooted ($P = 0.1828$). In contrast, using NAA strongly inhibited rooting percentage as the concentration increased up to 9 μM (Table 3).

Because in vitro firechalice shoots rooted easily on MS medium without auxin, rooting powders (Rootone and Hormex #3) were used in an attempt to bypass in vitro rooting. Analysis of treated shoots showed that firechalice shoots rooted and survived at similar levels ($P = 0.1714$) regardless of the treatments used. The mean rooting percentages (and subsequent survival) were 47% for Hormex #3-treated shoots, 40% for Rootone-treated shoots, and 20% for control shoots (treated with distilled water).

In a preliminary experiment, rooted shoots acclimated well and survived transplanting, but nonrooted shoots (treated as microcuttings) failed to form roots and died. Half of the shoots from the IBA and NAA rooting experiment were used in the acclimatization study. Although plantlets from the 6 μM IBA treatment survived acclimatization the best (93%), shoots treated with 0, 3, or 9 μM of IBA survived at similar levels (72%, 88%, or 77%, respectively, $P = 0.153$). Only a few (11.1%) rooted shoots treated with 3 μM NAA survived transplanting, and all shoots treated with highest concentrations of NAA (6 or 9 μM) failed to survive transplanting.

Table 3. Effects of different concentrations of indole-3-butyric acid (IBA) and naphthaleneacetic acid (NAA) on the mean number of shoots, mean shoot heights, mean shoot dry weights, mean number of roots per responding shoot, mean length of the longest root per shoot, and mean rooting percentages of *Epilobium canum garretti* shoots grown on Murashige and Skoog medium for 4 weeks. The same control treatment was used for IBA and NAA analyses. Six vessels were used per treatment, and within each vessel, three shoots were averaged before analysis.

Type of auxin	Auxin concn (μM)	Shoot ht (cm)	Shoot dry wt (g)	Number of roots per responding shoot	Length of the longest root (cm)	Percentage rooting
IBA	0	3.4 a ²	0.018 a	3.8 a	1.8 a	99 a
	3	3.4 a	0.020 a	3.8 a	2.2 a	88 a
	6	4.6 a	0.028 ab	5.5 b	2.3 a	75 a
	9	4.6 a	0.044 b	5.9 b	2.7 a	83 a
NAA	0	3.4 c	0.018 a	3.8 b	1.8 c	99 c
	3	2.6 bc	0.019 a	4.6 b	1.1 b	65 b
	6	1.8 ab	0.014 a	2.4 b	0.5 ab	43 b
	9	1.2 a	0.014 a	0 a	0 a	0 a

²Different letters within a column for each separate auxin indicate significant differences between means as determined by least-squares means tests at $P \leq 0.05$ level ($n = 6$).

Discussion

Firechalice stem explants established well on MS medium compared with stem explants on WPM. This result was anticipated because MS medium is formulated for herbaceous plants (Pierik, 1987) compared with WPM, which is formulated for woody plants. Firechalice stem explants exuded a black substance, most likely a phenolic compound, but an antioxidant compound was unnecessary in the medium if leaves were kept above the medium and explant stems were cut without crushing. Leaf, stem, and root cultures of *E. angustifolium* (Dreger et al., 2016) and nodal cultures of *E. parviflorum* and *E. hirsutum* (Deliu et al., 2013) readily established on MS medium. Dreger et al. (2016) used $0.1 \text{ g}\cdot\text{L}^{-1}$ ascorbic acid in the culture medium for stem segments and leaf explants of *E. angustifolium* because of browning and tissue necrosis of the explants. Nodal explants of *E. parviflorum* and *E. hirsutum* also produced phenolic compounds in culture, but the intensity was low enough to avoid the use of antioxidant compounds in the media (Deliu et al., 2013).

In shoot proliferation studies with firechalice, mT promoted shoot multiplication more than all other cytokinin treatments. Besides considering the best plant responses in culture, propagators must also consider the costs of the biochemicals used. The costs of 1 g of mT from two tissue culture supply companies was $\approx \$219$ from one and $\approx \$265$ from the other in June 2017, whereas the cost of BA from both companies was $\approx \$5$ per gram. The higher cost of mT may not be justified for commercial propagation of firechalice given that BA, which is at least 44 times cheaper, promoted shoot multiplication almost as well as mT. Additional plant species observed by other researchers also responded well to mT in Stage II. *Aloe polyphylla* (an endangered medicinal and ornamental aloe) was grown on full-strength basal medium with different concentrations of cytokinins (Bairu et al., 2007). Metatoplin induced the best rate of shoot multiplication, and the optimum concentration was $5 \mu\text{M}$ (Bairu et al., 2007). These results were similar to the firechalice ecotype used in this study. To our knowledge, this study is the first to use mT in an *Epilobium* micropropagation protocol.

The cytokinins 2iP and kin, had only moderate effects on axillary shoot multiplication by firechalice stem explants, which differs with results from Pavingerova et al. (1996) who found that 2iP stimulated adventitious shoot regeneration on leaf and root explants from five cultivars of *Oenothera biennis*, an Onagraceae species. In contrast, Hafez et al. (2015) found that kin-supplemented media induced the highest shoot multiplication rates from apical bud and petiole explants from *O. biennis* compared with BA-amended media. Leaf, petiole, and root explants from *E. angustifolium* on BA-supplemented media produced more adventitious shoots than those explants on kin-supplemented media (Turker et al., 2008).

Thidiazuron is a potent cytokinin-like plant growth regulator that can promote or inhibit the growth of shoot cultures. In this study with firechalice, shoot height reduction associated with increased TDZ concentration indicated that this compound inhibited shoot elongation, similar to results described by Huettelman and Preece (1993) with woody plant tissue culture. Turker et al. (2008) found that 2.3 or $4.5 \mu\text{M}$ TDZ combined with 1.2, 2.5, or $4.9 \mu\text{M}$ IBA failed to induce adventitious shoot regeneration for *E. angustifolium*. In contrast, TDZ can induce high numbers of adventitious shoots to form on cotyledon explants of three commercial cultivars of evening primrose, a related Onagraceae species (DeGyves et al., 2001).

In this study, firechalice rooted easily without auxin added to the medium, indicating that its shoots naturally produced enough auxin to promote root formation. Of the auxins tested, IBA induced more roots to form than NAA for firechalice stem explants (Table 3). Because the higher concentrations of NAA (6 or $9 \mu\text{M}$) decreased shoot height and inhibited root formation, these NAA concentrations appeared to have toxic effects on firechalice, and this auxin should probably be excluded from any firechalice-rooting medium. Turker et al. (2008) found that rooting of *E. angustifolium* shoots decreased from 100% for shoots treated with $2.7 \mu\text{M}$ NAA to 30% for shoots treated with $16.1 \mu\text{M}$ NAA. In our subsequent studies, firechalice shoots rooted well (at least $>80\%$) in vitro

when half-strength MS medium was used without auxin (unpublished data).

Using Rootone or Hormex #3 to bypass in vitro rooting failed to produce acceptable percentages of rooted shoots. Although twice as many firechalice microcuttings treated with Rootone® or Hormex® #3 survived transplanting compared with water-treated controls, less than 50% of the hormone-treated cuttings survived the ex vitro rooting process. These results were unexpected because Anderson and Rupp (2012) showed that almost 100% of *E. canum* stem cuttings formed roots when the cuttings were treated with 0.1% IBA. Rooting the selected firechalice accession in vitro appeared to improve survival of this clone because of at least 72% of the shoots that rooted in vitro without auxin in the rooting medium survived transplanting. Using ex vitro auxin treatments to bypass in vitro rooting was of limited value in this study, but perhaps manipulation of the shoot cultures [e.g., exposing in vitro shoots to a cold treatment (Anderson and Rupp, 2012) before attempting ex vitro rooting] will improve the percentage of ex vitro rooting and survival.

Conclusion

Axillary shoot proliferation is a viable option for rapid propagation of the selected firechalice accession with limited foliage available for propagation. Murashige and Skoog medium was the best medium for establishing firechalice stem explants in culture. Shoot explants multiplied the best when using BA or mT at 4.4 or $8.8 \mu\text{M}$. Firechalice shoots rooted easily without auxin added to the medium, yet adding up to $9 \mu\text{M}$ IBA increased the number of roots per responding shoot although the percentages of rooted shoots were similar to the control treatment. Transplant survival percentages were similar if the shoots were rooted with up to $9 \mu\text{M}$ IBA or without auxin in the medium. Using NAA in the rooting medium should be avoided because it inhibited root formation and decreased transplant survival. The firechalice micropropagation procedures developed in this study can be used by the nursery industry to produce large numbers of tissue culture plantlets quickly.

Literature Cited

- Anderson, R.M. and L.A. Rupp. 2012. Selecting and evaluating accessions of *Epilobium* sect. *Zauschneria* (Onagraceae). Comb. Proc. Intl. Plant Prop. Soc. 62:147–149.
- Bairu, M.W., W.A. Stirk, and K. Dolezal. 2007. Optimizing the micropropagation protocol for the endangered *Aloe polyphylla*: Can metatoplin and its derivatives serve as replacement for benzyladenine and zeatin? Plant Cell Tissue Organ Cult. 90:15–23.
- DeGyves, E.M., C.A. Sparks, A.F. Fieldsend, P.A. Lazzeri, and H.D. Jones. 2001. High frequency of adventitious shoot regeneration from commercial cultivars of evening primrose (*Oenothera* spp.) using thidiazuron. Ann. Appl. Biol. 138:329–332.
- Deliu, C., A. Coste, M. Tămaș, A. Halmagyi, and A. Butiuc-Keul. 2007. *In vitro* propagation of *Epilobium parviflorum* Schreb – An important medicinal plant (in Romanian), p. 212–224. In: Lucrările celui de al XV-lea Simpozion Național de Culturi de Țesuturi și Celule Vegetale “Micropropagatarea speciilor vegetale” Iași, 7 iunie 2006.
- Deliu, C., A. Coste, and M. Tămaș. 2013. *Epilobium* sp. (willow herb): Micropropagation and production of secondary metabolites, p. 149–170. In: S. Chandra, L. Hemant, and A. Varma (eds.). Biotechnology for medicinal plants, micropropagation and improvement. Springer, Heidelberg, Germany.
- Dreger, M., J. Wegenke, J. Makowiecka, T. Michalik, and K. Wielgus. 2016. Application of multi-shoots cultures in micropropagation of willow herb (*Chamaenerion angustifolium* (L.) Scop.). Herba Pol. 62:28–39.
- Hafez, R.F., M. Ghadimzadeh, A.F. Moghaddam, and M. Jafari. 2015. Micropropagation and regeneration potentiality of evening primrose (*Oenothera biennis* L.). Indian J. Fund. Appl. Life Sci. 5:36–42.
- Huetteman, C.A. and J.E. Preece. 1993. Thidiazuron: A potent cytokinin for woody plant tissue culture. Plant Cell Tissue Organ Cult. 33:105–119.
- Kane, M. 2011. Propagation by shoot culture, p. 181–191. In: R.N. Trigiano and D.J. Gray (eds.). Plant tissue culture, development and biotechnology. CRC Press, Boca Raton, FL.
- Lloyd, G. and B. McCown. 1980. Commercially-feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot-tip culture. Comb. Proc. Intl. Plant Prop. Soc. 30:421–427.
- Love, S.L., K. Noble, J.A. Robbins, B. Wilson, and T. McCammon. 2009. Landscaping with native plants. Idaho Agr. Expt. Sta. Bul. 862.
- Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473–497.
- Öztürk, M., K.M. Khawar, H.H. Atar, C. Sancak, and S. Özcan. 2004. *In vitro* micropropagation of the aquarium plant *Ludwigia repens*. Asia Pac. J. Mol. Biol. Biotechnol. 12:21–25.
- Parveen, A. and S. Rasheed. 2013. *In vitro* clonal propagation of *Fuchsia magellanica* Lam. Afr. J. Biotechnol. 12:670–678.
- Pavingerova, D., I. Galis, and M. Ondrej. 1996. Tissue culture and transformation of *Oenothera biennis*. Biol. Plant. 38:27–32.
- Pierik, R.L.M. 1987. *In vitro* culture of higher plants. Martinus Nijhoff, Boston, MA.
- Robson, K.A., A. Richter, and M. Filbert. 2008. Encyclopedia of northwest native plants for gardens and landscapes. Timber Press, Portland, OR.
- SAS. 2012. Users guide statistics Ver. 9.4. SAS Institute Inc., Cary, NC.
- Salinas, R., J. Arellano-Garcia, I. Perea-Arango, L. Alvarez, M.L. Garduno-Ramirez, S. Marquina, A. Zamilpa, and P. Castillo-Espana. 2014. Production of anti-inflammatory compound 6-O-palmitoyl-3-O-b-D-glucopyranosylcampersterol by callus cultures of *Lopezia racemosa* Cav. (Onagraceae). Molecules 19:8679–8690.
- Skrzypczak, L., E. Skrzypczak-Pietraszek, E. Lamer-Zarawska, and B. Hojden. 1994. Micropropagation of *Oenothera biennis* L. and an assay of fatty acids. Acta Soc. Bot. Pol. 63:173–177.
- Tămaș, M., A. Toiu, I. Oniga, C. Deliu, B. Oletean, and G.H. Coldea. 2009. Quantitative determination of total polyphenols and flavonoids from indigenous species of *Epilobium* of wild origin and *in vitro* regenerated plantlets. Contrib. Bot. Cluj-Napoca. 44:119–123.
- Taniguchi, S., T. Hatano, and K. Yazaki. 2006. Production of tannin by tissue culture of woody plants and tannin biosynthesis. Mokuza Gakkaishi 52:67–76.
- Turker, A.U., E.C. Mutlu, and A.B. Yildirim. 2008. Efficient *in vitro* regeneration of fireweed, a medicinal plant. Acta Physiol. Plant. 30: 421–426.
- Unander, D.W., H.H. Bryan, C.J. Lance, and R.T. McMillan. 1995. Factors affecting germination and stand establishment of *Phyllanthus amarus* (Euphorbiaceae). Econ. Bot. 49:49–55.
- Winger, D. 1996. Xeriscape plant guide: 100 water-wise plants for gardens and landscapes. Fulcrum Publishing, Golden, CO.