Title: Determining Tissue Culture Propagation Procedures for Selected Native Plants

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Abstract

The use of native plant species in managed landscapes throughout the state and region has caught on slowly due to limited availability from wholesale production nurseries. The objective of this study was to determine plant tissue culture techniques that could be used to propagate four improved selections of native species from Dr. Stephen Love's research program. The plants that we attempted to propagate by tissue culture were promising plants selections including: a dwarf selection of western serviceberry (*Amelanchier alnifolia*), three types of skunkbush sumac (*Rhus trilobata*), roundleaf buffaloberry (*Shepherdia rotundifolia*), and firechalice (*Zauschneria garrettii*). Serviceberry and firechalice stems used in tissue culture were obtained from Dr. Love by mid-February. Serviceberry shoots were established on Murashige and Skoog (MS) medium, but firechalice shoots were tested for their ability to grow MS medium and woody plant (WP) medium. Buffaloberry and sumac stems were received in June, July, and September in attempts to establish shoot cultures of these species. A cytokinin (plant growth regulator – phytohormone) was added to the basal salt formulations salts to determine medium requirements for explant establishment. Stem explants from buffaloberry and the sumac selections were heavily contaminated with various fungal species preventing establishments of these two species in culture. Therefore, we concentrated our experiments on the two most promising species (serviceberry and firechalice – based on their acclimation to the tissue culture environment) and completed more in-depth studies with these plant selections. For serviceberry, our goal was to improve shoot growth of stem cultures on MS medium. Experiments with different concentrations (up to four fold) of calcium, zinc, and different forms of nitrogen (ammonium or nitrate) were added to culture medium in different experiments, but the shoots lacked improved growth, as determined by the number of axillary shoots formed, shoot dry weights or shoot heights. Changing three mineral components, $CaCl_2$, $MgSO_4$, KH_2PO_4 in the medium, however, induced serviceberry shoots to grow taller and produce slightly more shoot dry weight than control shoots. Firechalice shoots established much better on MS medium than WP medium. The concentration of 4.4 µM benzyladenine (BA – a cytokinin) promoted establishment of the explants, completing Stage I of micropropagation. Different cytokinins (kinetin, 2iP, thidiazuron, and meta-topolin) were used at concentrations from 0 to 8.8 µM to promote shoot multiplication (Stage II of micropropagation). Preliminary studies on root formation of the multiplied firechalice shoots (Stage III of micropropagation) were completed, and shoots of this species rooted easily without any phytohormones in the culture medium. Although more

research is needed to refine the culture medium for serviceberry shoots, the information from this research will provide plant propagators with the procedures needed to reproduce the selected firechalice plant quickly so that hundreds or thousands of plants can be grown and made available to production nurseries and subsequently retail garden centers.

Materials and Methods

Serviceberry experiments:

Serviceberry shoots had already been established in 2013 for preliminary studies with a dwarf selection of this species from Dr. Love's research program. Since shoots from this selection were moderately difficult to root, we thought that improving shoot growth may improve root formation. In February, experiments were started and different concentrations (up to four fold of the standard salt formulation) of $CaCl_2$, $ZnSO_4$, or NH_4NO_3 (to obtain different forms of nitrogen – ammonium or nitrate) were added to culture medium in different experiments. Since the shoots lacked improved growth, three mineral components – $CaCl_2$, $MgSO_4$, and KH_2PO_4 used together – were changed in the medium. The first study involved increasing the concentration of each component equally 0.75, 1.5, 2.25, or 3 fold (higher than standard MS medium). After looking over the preliminary results from this study, a second study was completed with the concentrations of $CaCl_2$, and $MgSO_4$ held at 0.75 fold, but the concentration of KH_2PO_4 varied from 0 to 3 fold higher than the standard salt formulation. For both of these studies, shoot explants were grown for three subcultures, a little over three months, on fresh medium with each subculture to ensure that the mineral formulations could affect or influence shoot growth.

Firechalice experiments:

Experiments trying to establish firechalice shoots in tissue culture (Stage I of micropropagation) were started in February. After cutting stem sections to a single node (to form explants), these tissues were surface sterilized with 10% sodium hypochlorite for 20 min and then rinsed three times with sterile distilled water. Single-node explants were placed on MS or WP medium supplemented with 1.1 or 2.2 µM BA. Murashige and Skoog medium contained 30 grams per liter (g/l) sucrose and 7 g/l agar and had a pH 5.6, whereas WP medium contained 20 g/l sucrose and 7 g/l agar and had a pH 5.2. The culture media also included Plant Preservative Mixture (PPM) a biocide that is usually effective at controlling or eliminating microbial contamination; the concentration of this ingredient in the culture media was 1 milliliter per liter (ml/l). After about one month, the shoot cultures were subcultured (divided) and placed on fresh MS medium with 4.4 M µBA or fresh WP medium with 3.5 M µBA. After a second subculture (one month later), data showed that MS medium with 4.4 M µBA was the best medium for establishing shoot cultures of firechalice.

The next part of this study (Stage II) involved rapidly multiplying shoots that could be used for later stages of micropropagation. In these experiments, cytokinins were examined for their effects on the number of axillary bud breaks induced on stem explants. In the first study, 0, 1.1, 2.2, 4.4, or 8.8 µM BA was added to the culture medium. In a second study, four other cytokinins including kinetin (abbreviated kin), 6-(γ,γ-dimethlyallylamino)purine (abbreviated 2iP), thidiazuron (abbreviated TDZ), and meta-topolin were used at the following concentrations, 0, 1.1, 2.2, 4.4, or 8.8 µM, to promote axillary bud break and subsequent shoot growth.

Preliminary studies on root formation of the firechalice shoots (Stage III of micropropagation) were completed. In this brief study, firechalice shoots about 1 cm tall were placed on MS medium with 0 or 1 µM naphthaleneacetic acid. After one month, the number of roots were counted, and plantlets were placed in a clam shell container to determine if they could acclimate to conditions outside of the tissue culture vessels (normal environment).

Buffaloberry and sumac experiments:

Shoots of roundleaf buffaloberry and three selections of skunkbush sumac (called Sq2, Sq3, and Sq4) were sent to Moscow from Dr. Love's collections in Aberdeen in June, July and September. The shoots were cut into single-node stem explants ranging from 1 to 3 cm long. Only stems sections that had vegetative buds in the leaf axils were used in this study. In the first and third trials, stem explants were placed in 10% household bleach (v/v) that contained 2 drops of Tween® 20 (a surfactant) and held in this solution for 20 minutes. Since the buffaloberry stems were very hairy and felt gritty, extra steps, which included rinsing stems in a mild detergent solution and then a 30 second dip in 95% ethanol before using the bleach solution, was added to the cleanup protocol. In the second trial, the surface sterilization solution used on buffaloberry stems sections contained 40% bleach and 10% ethanol with 2 drops of Tween® 20. After bleach treatments were completed, stem explants were rinsed three times in sterile distilled water in a laminar flow hood before being placed on either MS medium or WP medium. Both media were supplemented with Plant Preservative Mixture (a biocide and abbreviated PPM) used at a rate of 1 ml/l in the first trial, and then 2 ml/l in the second and third trials. These media also contained 2.2 µM BA. Since a few of the Sq2 stems grew weakly on either medium and most other stem explants were contaminated, the second and third trials involved only stems from buffaloberry along with those from sumac selections Sq3 and Sq4.

Data analyses:

Four or six shoots, depending on the experiment, were used in each of four replicate vessels per treatment. Data taken in each experiment included the number of shoots formed (from axillary buds) per stem explants, the 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 three days. The numbers of shoots per stem was analyzed by using categorical modeling, whereas shoot heights and shoot dry weights were analyzed for treatment significance by one-way analysis of variance (mixed model procedure) for serviceberry experiments and Stage I firechalice experiments. Stage II firechalice experiments were analyzed as two-way analysis of variance (mixed model procedure) with type of cytokinin and cytokinin concentrations used as the independent variables. Significant differences between treatment means were determined by least-square means at the 5% level when comparing plant growth differences in the different media. Significant differences between means were determined only if the $P < 0.05$ for the statistical model for each growth parameter being analyzed.

Results and Discussion

Serviceberry:

Shoots from the dwarf serviceberry plant selected by Dr. Love can be readily multiplied in tissue culture by axillary shoot proliferation, but these shoots can be difficult to root in vitro.

Since the shoots have an off color on standard MS medium, we thought that improving leaf color and improving shoot growth may increase the ability of the shoots to form roots. When different concentrations (up to four fold) of calcium, zinc, and different forms of nitrogen (ammonium or nitrate) were added separately to the culture medium in different experiments, the shoots lacked improved growth, as determined by the numbers of axillary shoots formed, shoot dry weights or shoot heights (data not shown). In addition, the leaves were still an off-green color. When $CaCl_2$, $MgSO_4$, KH_2PO_4, were added together to the media at different concentrations, serviceberry shoots grew taller, produced slightly more shoot dry weight, and had better leaf color than control shoots if the concentrations of these three components were increased either 0.75-fold or 3-fold (data not shown). The second study with the concentrations of $CaCl_2$, and $MgSO_4$ held at 0.75 fold higher but the concentration of KH_2PO_4 varied from 0 to 3 fold higher than standard salt formulation has yet to be statistically analyzed. Visually, shoots receiving 2.25 fold higher KH_2PO_4 had better leaf color and were taller than the control shoots. Statistical analyses of these results is still being completed. Once a better salt formulation is obtained for optimum shoot growth, we will try rooting experiments again to determine if improved shoot growth has a positive influence on rooting shoots from this dwarf serviceberry selection.

Firechalice:

Firechalice shoots were obtained from stock plants from Dr. Love's research program. Single-node stem sections were surface sterilized and placed on either MS or WP medium. After three subcultures, firechalice plants clearly grew better on MS medium. By the third subculture, shoots on MS medium produced 2.6 times more shoots and 2.8 times more shoot dry weight, and grew 2.8 times taller than shoots grown on WP medium (compare the means of 12.6 versus 4.9 shoots formed, 37.6 mg versus 13.3 mg of shoot dry weight , and 2.4 cm versus 0.8 cm shoot height, respectively). Leaf color on shoots grown on MS medium also was greener than the color of leaves on shoots grown on WP medium. This result was somewhat surprising since MS medium is better formulated for herbaceous plants, but WP medium is formulated for woody plants. Since firechalice is a woody plant, we expected it to grow better on WP medium for Stage I of micropropagation.

After firechalice shoots became stabilized in culture (good shoot color and consistent, regular growth of the shoots), Stage II experiments involving rapid shoot multiplication were started. Cytokinins usually induce axillary bud break on shoot cultures, so BA (from 0 to 8.8 µM) was the first cytokinin tested in Stage II and was considered the standard cytokinin to use as a comparison to the others. In the next study, kinetin, 2iP, thidiazuron, and meta-topolin concentrations from 0 to 8.8 µM had different effects on shoot multiplication rates, shoot heights and shoot dry weights. The two-way analyses for all three growth parameters revealed significant interactions ($P < 0.001$) between the type of cytokinin used and the concentrations used. Therefore, data were analyzed for the effects of each separate cytokinin on shoot growth.

The two most effective cytokinins for promoting shoot multiplication were BA and meta-topolin (Tables 1 and 2, respectively). A concentration of 8.8 µM BA induced about 11 shoots to form per explant, whereas 4.4 µM meta-topolin was needed to induce 15 shoots per explant (Tables 1 and 2, respectively). As the concentration of meta-topolin increased from 0 to 8.8 µM, shoot height in culture increased significantly by about 1 cm, but BA concentration failed to affect shoot height (Tables 2 and 1, respectively). The highest BA concentration (8.8 µM)

increased shoot dry weight ~ 2 fold compared to the control stems (Table 1). In contrast, 8.8 µM meta-topolin increased shoot dry weight by over 3 fold compared to control shoots (Table 2).

Table 1: Effects of benzyladenine (BA) on the mean number of shoots, mean shoot heights, and mean shoot dry weights of firechalice shoots grown on MS medium for 4 weeks. Data are means of six shoots in four vessels per treatment.

Concentration (µM)	Number of shoots	Shoot height (cm)	Shoot dry weight (g)
0	1.8 a*	2.5	0.017 a
1.1	6.6 b	2.7	0.022 a
2.2	7.1 b	2.7	0.022 a
4.4	9.0 c	2.7	0.030 b
8.8	11.1 d	2.7	0.037 b

*Different letters within a column indicate significant differences between means as determined by least-squares means tests at $P \leq 0.05$ level (n = 24).

Table 2: Effects of meta-topolin on the mean number of shoots, mean shoot heights, and mean shoot dry weights of firechalice shoots grown on MS medium for 4 weeks. Data are means of six shoots in four vessels per treatment.

Concentration (µM)	Number of shoots	Shoot height (cm)	Shoot dry weight (g)
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

*Different letters within a column indicate significant differences between means as determined by least-squares means tests at $P \leq 0.05$ level (n = 24).

Table 3: Effects of thidiazuron (TDZ) on the mean number of shoots, mean shoot heights, and mean shoot dry weights of firechalice shoots grown on MS medium for 4 weeks. Data are means of six shoots in four vessels per treatment.

Concentration (µM)	Number of shoots	Shoot height (cm)	Shoot dry weight (g)
0	1.9 a*	3.1 b	0.027 a
1.1	6.0 b	2.5 b	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

*Different letters within a column indicate significant differences between means as determined by least-squares means tests at $P \leq 0.05$ level (n = 24).

The other three cytokinins used in this study either had minimal or detrimental effects on the growth of firechalice shoots. For instance, even though 8.8 µM TDZ increased the number of axillary shoots formed by 3.7 fold and shoot dry weight by 4.6 fold over the control treatment (lacked cytokinin in the medium), shoots on medium supplemented with the highest concentration of TDZ were 2.5 times shorter than control shoots (Table 3). The decreasing shoot heights as TDZ concentration increased indicated that this cytokinin had a toxic effect on shoot height. The highest concentration of TDZ also produced the heaviest shoots (0.126 g, Table 3). The highest level of kinetin increased the shoot multiplication rate by over 6 fold compared to the control shoots (Table 4), whereas 8.8 µM 2iP increased shoot multiplication rate by only 2.3 fold over control shoots (Table 5). Neither kin or 2iP concentrations affected shoot heights, yet 8.8 µM kin or 2iP increased shoot dry weights by 3.5 fold or 2 fold compared to controls (Tables 4 and 5, respectively).

Table 4: Effects of kinetin (kin) on the mean number of shoots, mean shoot heights, and mean shoot dry weights of firechalice shoots grown on MS medium for 4 weeks. Data are means of six shoots in four vessels per treatment.

Concentration (µM)	Number of shoots	Shoot height (cm)	Shoot dry weight (g)
0	1.4 a	2.7	0.016 a
1.1	2.9 b	3.0	0.023 a
2.2	3.1 b	3.0	0.028 a
4.4	6.2 c	3.1	0.057 b
8.8	8.9 d	3.1	0.058 b

*Different letters within a column indicate significant differences between means as determined by least-squares means tests at $P \leq 0.05$ level (n = 24).

Table 5: Effects of 6-(γ,γ-dimethlyallylamino)purine (2iP) on the mean number of shoots, mean shoot heights, and mean shoot dry weights of firechalice shoots grown on MS medium for 4 weeks. Data are means of six shoots in four vessels per treatment.

Concentration (µM)	Number of shoots	Shoot height (cm)	Shoot dry weight (g)
0	2.6 a	2.9	0.025 a
1.1	3.0 a	2.8	0.024 a
2.2	5.1 b	2.8	0.036 ab
4.4	5.1 b	3.0	0.041 cb
8.8	6.0 b	3.0	0.051 c

*Different letters within a column indicate significant differences between means as determined by least-squares means tests at $P \leq 0.05$ level (n = 24).

Different cytokinins were tested for their effects on shoot growth since each one can affect different species of plants differently. In our Stage II studies, meta-topolin seemed to promote shoot multiplication the best, even better than BA. This information is good to know for

propagators who have to decide which cytokinins to use in their media. Besides looking for the best plant responses in culture, propagators must also consider the costs of the biochemicals used. The cost of meta-topolin from *Phyto*Technology Laboratories is $257 per gram, whereas the cost of BA from this same company is $5 per gram. The higher cost of meta-topolin fails to justify its use in commercial propagation since BA, which is 51 times cheaper, promoted shoot multiplication almost as well as meta-topolin. This part of the study also showed that TDZ should probably be avoided in firechalice culture medium since shoot heights decreased as its concentration increased, indicating this cytokinin had a toxic effect on shoot height. The cytokinin 2iP had only moderate effects on firechalice shoot multiplication, so this biochemical can be excluded from further Stage II studies.

Preliminary studies on root formation of the multiplied firechalice shoots (Stage III of micropropagation) were completed, and shoots of this species rooted easily without any phytohormones in the culture medium. Auxin is often needed in the medium to promote root initiation on shoots in tissue culture. Firechalice roots easily without auxin added to the medium, indicating that its shoots naturally produce enough auxin to promote root formation. In another preliminary experiment, some of the rooted shoots were placed in a clam shell container to acclimate firechalice shoots to the natural environment. Rooted shoots acclimated well, and survived the transition, but non-rooted shoots (treated as microcuttings) failed to form roots in the containers and died. More experiments need to be completed on Stages III and IV of firechalice micropropagation to determine the best procedures for rooting and acclimatizing this selected specimen.

Buffaloberry and skunkbush sumac

Stem explants from buffaloberry and two of the three sumac selections were heavily contaminated with various fungal species preventing establishments of these two species in culture. Few bacteria appeared to contaminate the stem explants in culture. Some stems from the Sq2 selection (this plant has a compact growth habit and bright red fall color) did start to grow weakly for three subcultures on BA supplemented MS or WP media, but then they died. The Sq3 and Sq4 stem explants were mostly contaminated, but about 25% of the explants formed large amounts of callus at the base of the stem and failed to break bud. Buffaloberry explants had extremely large amounts of pubescence (hairs) on their stems so their high levels of contamination were somewhat expected. Even though extra steps – including rinsing stems in a mild detergent solution and then a 30 second dip in 95% ethanol, or rubbing off stem hairs – were used in addition to a bleach solution, most of the shoots were contaminated by fungi. The PPM biocide used in the culture medium at a concentration of 1 or 2 ml/l per liter failed to stop microbial contamination of buffaloberry stem explants.

The surprising part of the three attempts to establish buffaloberry and sumac stem explants in tissue culture was that most of the stems were contaminated and PPM failed to stop the fungal contamination on the two different species. We are unsure why non-contaminated sumac stem explants formed large amounts of callus without bud break. Both the extent of the contamination and the fact that PPM failed to control or prevent the contamination from appearing indicated that the selected buffaloberry plant and selected Sq3 and Sq4 plants had internal contamination. If fungus and bacteria are growing inside the plant stems (most likely as saprophytes), then these plants may be very difficult to establish in tissue culture. More experiments are needed to

attempt establishment of the stem cultures of these two species by using various combinations of PPM and antibiotics in order to inhibit microbial growth while promoting bud break and subsequent establishment of stem cultures.

Significance to the Nursery Industry

Our studies have shown that adjusting the mineral formulation can improve shoot growth of our western serviceberry selection reproduced in tissue culture. Additional studies are needed to further refine the concentrations of three salts, $CaCl_2$, $MgSO_4$, and KH_2PO_4, and determining the optimum amounts of these salts should improve shoot growth even further. We believe that improved shoot growth of this serviceberry selection will lead to enhanced shoot production and most likely better rooting of the microshoots in culture. The selected firechalice plant from Dr. Love's research program grew well in tissue culture and responded very well to the various stages of culture and cytokinins used in the culture medium. Additional experiments are needed to establish clear rooting and acclimatization protocols for this selected plant. This plant roots easily so additional experiments should be straightforward. The fact that three attempts to establish buffaloberry and skunkbush sumac selections in tissue culture failed indicates that these particular plants will be difficult to establish in vitro.

The goal of this research was to provide information about rapid plant propagation techniques to make Dr. Love's plant selections available to the public in a short amount of time. With a few more experiments, a micropropagation protocol for the firechalice selection can be released to plant propagators. Shoot growth of the dwarf serviceberry plant has been improved, but the medium needs additional refinement. After making the salt modifications, shoot multiplication and growth should be improved even further, and perhaps the shoots will root better than under current protocols. The information from this research will provide plant propagators with procedures needed to reproduce the firechalice and serviceberry plant selections quickly so that hundreds or thousands of these carefully chosen plants can be made available soon to production nurseries and subsequently retail garden centers.