# Improving Shoot Growth and Multiplication of Native Plants in Tissue Culture

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## ABSTRACT

Propagation techniques are essential for successful production of native plants with commercial value in managed landscapes. Shoots of serviceberry, *Amelanchier alnifolia* Nutt., propagated in tissue culture often fail to form roots readily. Adding mesos components in different concentrations also increased shoot growth and improved overall quality, leaf color, shoot multiplication, shoot dry weight and shoot height. The best treatments were 0.132, 0.054, and 0.204 g $l^{-1}$ or 0.398, 0.054, and 0.102 g $l^{-1}$ for $CaCl_2$, $KH_2PO_4$ and $MgSO_4$, respectively, added to MS medium. These mesos treatments increased the number of shoots per explant by 67% and 43%, shoot height by 91% and 112%, and shoot dry weight by 44% and 77%, respectively, compared to the control shoots.  In vitro cultured shoots from a horticulturally superior dwarf plant were examined for their ability to form roots over a range of basal salt concentrations or inclusion of different auxin concentrations used in Murashige and Skoog (MS) medium. The best rooting was achieved with 10 µM IBA or 10 µM NAA, and the percentage of shoots forming roots was 33% for IBA-treated and 67% for NAA-treated shoots. The percentage of rooting increased to 87% on ½ strength MS medium supplemented with 0.5 µM IBA with 1.6 mg $l^{-1}$ thiamine and 150 mg $l^{-1}$ FeEDDHA. Optimizing shoot multiplication media by adding mesos compounds ($CaCl_2$, $KH_2PO_4$ and $MgSO_4$) to improve serviceberry shoot quality before root formation helped to increase rooting percentage to 100%. *Epilobium canum* (Greene) P.H. Raven subsp. *garrettii* (A. Nelson) P.H. Raven commonly named firechalice was established in tissue culture on MS medium supplemented with 4.4 µM benzyladenine (BA) in Stage I. During Stage II, different

cytokinins, such as BA, kinetin (kin), 6-(γ,γ-dimethlyallylamino)purine (2iP), thidiazuron (TDZ) and meta-topolin (mT), at concentrations of 1.1, 2.2, 4.4 or 8.8 μM were evaluated for axillary shoot proliferation efficacy. The highest numbers of shoots (11.1 or 15) were formed by using the highest concentration (8.8 μM) of BA or meta-topolin, respectively. In Stage III, firechalice shoots rooted easily without auxin added to the medium, yet 0 to 9 μM IBA can be used during Stage III since significantly more roots formed on shoots treated with 9 μM IBA . Overall, 82.5% of the rooted shoots survived in Stage IV when 0 to 9 μM IBA was used in Stage III. The micropropagation protocol developed for firechalice allows large numbers of plantlets to be produced from one stock plant in relatively a short period of time. Micropropagation through tissue culture may positively impact the landscape plant market because a large number of plants can be produced quickly for release to the public. The necessary protocols are now in place so that many of the selected serviceberry and firechalice plants can be produced and used in the nursery and landscape trade.

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## INTRODUCTION TO THE DISSERTATION

Native plants have specific traits, including adaptability and drought tolerance, which make them ideal for creating sustainable urban landscapes. In addition, native plants can match the finest cultivated plants for plant growth and beauty. Once valuable native plants are identified and selected, asexual propagation may be necessary to retain superior characteristics.

The University of Idaho sponsors a native plant domestication project established to select and commercialize drought tolerant, sustainable native plant products. This project is conducted by Dr. Stephen Love at the Aberdeen Research and Extension Center. Among plants selected for their horticultural potential is a serviceberry *Amelanchier alnifolia* Nutt., chosen for its dwarf growth habit (the original plant is 10 years old and only about 1 m tall). In addition, a selection of firechalice *Epilobium canum* (Greene) P.H. Raven subsp. *garrettii* (A. Nelson) P.H. Raven was chosen for its bright red flower color and more upright growth habit. Due a combination of propagation challenges and desire to produce uniform, commercially viable products, these two species are candidates for micropropagation.

Serviceberry can potentially be propagated using seeds, cuttings, or tissue culture. Plants produced from seed display an undesirable range of variability. This species has been consistently difficult to propagate via traditional stem cutting techniques. Also, as with many woody species, serviceberry is difficult to root when propagated by tissue culture.

Firechalice is an herbaceous wildflower with landscape potential, but seeds of some ecotypes are difficult to germinate due to lack of viability or profound dormancy. A selected superior form of firechalice displays these difficult seed characteristics. This species has the potential to be an ideal subject for micropropagation.

To increase the number of these highly desirable plants, commercial micropropagation, usually associated with axillary shoot proliferation, can be used. This technology is based on placing shoots in a sterile growth environment with the correct nutrients and hormonal balance for the formation of shoots and a root system.

The overall goal of this study was to develop tissue culture procedures that would result in producing large numbers of quality plantlets that could be used in commercial plant production. Procedures were developed to produce good shoot quality for the selected serviceberry plant and increase rooting percentages. For firechalice, I developed a protocol to multiply the selected plant quickly through the four stages of micropropagation.

## LITERATURE REVIEW

Plants that reside in a local ecosystem, a state or a region and have not been cultivated are called “native plants” (Love et al 2009). Native woody plants with unique characteristics, such as brilliant fall color, dwarf size or glossy leaves are useful for creating an attractive urban landscape. Choosing non-adapted plants creates a need for additional inputs, such as supplemental watering, and controlling pest problems, to get the plants to thrive in managed landscapes. Native plants help create a more natural landscape and should reduce the resources for maintenance. Understanding the local environment helps landscape designers to choose and grow plants adapted to the local climate and soil.

Water savings and decreased maintenance are the main reasons to consider native plant use in a landscape. In summer, some areas of Idaho are quite dry and require 51 to 76 cm of water to be applied to keep plants green (Love et al 2009). Also, many areas of Idaho receive moderate amount of snow and rain fall in the winter, but only few centimeters of the rain are received for 3 to 4 summer months beginning in June (Love et al 2009). Sustainable landscaping in high desert areas requires drought-tolerant plants. Low humidity, a short frost-free growing season and bright sunny days are three factors that should be considered when choosing plants to grow in Idaho and the intermountain region (Love et al 2009).

In Idaho and parts of the Pacific Northwest, some woody plants, such as serviceberry, grow easily in dry areas because they have low water use requirements. Several species of serviceberry are found in the U.S. including *Amelanchier alnifolia* (USDA Natural Resources Conservation Service 1999).

*Amelanchier alnifolia* has different common names, western serviceberry, Saskatoon serviceberry and Juneberry (Leigh 1999). This species is in the Rosaceae family (Pruski et al 1991). The natural range of *Amelanchier alnifolia* is east of the Cascade and Sierra Nevada mountain ranges, from British Columbia south to central California (USDA Forest Service 1974). It is also found in western Canada and the northwestern region of the United States (Wilson 1993, Leigh 1999, and Pruski et al 1991).

Serviceberry is a shrub or small tree 1 to 4 meters tall with smooth brown bark (Elias 1980). This species can be found in open woods, along canyons or on hillsides (Anonymous 1976). Also, this species grows on forested slopes, cliff edges, and wide prairies or around lakes (Leigh 1999).

Saskatoon serviceberry begins to produce fruit when it is three to five years old and usually reaches full production at three years of age with average crop yields from 3000 to 4000 pounds per acre (Wilson 1993). Floral buds form in the late summer of the first season on one year old branches, and fruits develop the following season (Pierre and Steeves 1990). White flowers appear in the spring before leaves come out and dark purple to black berries are produced in mid- or late summer (USDA Forest Service 1974). Serviceberry is self-pollinated or cross pollinated by insects or wind (Davidson and Mazza 1991).

*Amelanchier alnifolia* has a long history of use by native cultures in the northwestern region of the United States. The berries were eaten raw, cooked or dried and stored. The dried berries were mixed with vegetables or meat or cooked in soups (Gunther 1995). The wood is hard and was used for making digging sticks,

fire drills, tool handles and hoops. Stem, bark, leaves and berries were used as medicine (Turner et al 1990). Saskatoon serviceberry is used for commercial fruit production and is established on many hectares in Alberta, Saskatchewan, and Manitoba (Wilson 1993). Saskatoon serviceberry is attractive to wildlife and useful for landscaping, used as a specimen plant or hedge in shrub or mixed borders, and fits nicely in a naturalistic setting (Winger 1996). This serviceberry species is used as a commercial fruit crop because it can be cultivated on a wide range of soils and can be harvested with the same equipment as blueberry (Pruski et al 1990).

Serviceberry species will tolerate many types of soil (Dirr 1983). This species (*Amelanchier alnifolia*) tolerates soil of various textures and can be found on many sites such as dry or rocky slopes and shaded sites with moist, partial shade or well-drained soil (Dirr 1983, Wasser and Shoemaker 1982, Wilson 1993). In general, this plant is intolerant of saline soils (Wasser and Shoemaker 1982). Soil tests are important to determine fertilization requirements, and yearly fertilization with ammonium nitrate will maximize survival and growth (Harris 1976). Weed control and irrigation during first growing season are important for survival and growth (Wasser and Shoemaker 1982). This species is drought tolerant and very cold hardy to -20ºC. A rich, loamy soil is the favorite soil for this plant, and the best soil pH is between 6.1 and 6.5 (Leigh 1999).

*Amelanchier alnifolia* establishes naturally by seeds and spreads via rhizomes In addition, different methods can be used to propagate serviceberry, such as budding, grafting, root cuttings, layering, division, cold moist seed stratification, and tissue culture (Anonymous 1976). This species is difficult to

propagate by vegetative techniques (Davidson and Mazza 1991). The plant can be produced by seeds, which need to have their dormancy requirements met for germination. Cold stratification for three to six months is needed to break seed dormancy, and the germination rate is less than 50%. The seedling will grow about 30 cm per year, and plants live about 60 years (Leigh 1999). Propagation by seeds is often the least expensive method of production.

Asexual propagation becomes imperative for improving plant qualities (Grainger 1983). Variability of seedlings encourages use of vegetative propagation methods, such as tissue culture, to retain desired plant traits. Successful shoot tip cultures were first used by Harris (1980) for Saskatoon serviceberry. Establishing in vitro cultures of several cultivars of Saskatoon serviceberry can be done by using shoot tips containing dormant buds and active buds (Pruski et al 1985, Pruski 1987). Rapid multiplication was achieved by using different concentrations of $N^6$-benzyladenine (BA), and the average multiplication ratio was seven new shoots produced for every one shoot (Pruski et al 1990). For most cultivars, shoots produced in culture were stunted, had poor leaf development, and were difficult to root when using a high concentration of BA in the multiplication stage. The optimum range of BA for most cultivars was 8.87 to 13.3 μM (Pruski et al 1990).

*Epilobium canum garrettii*, (also known as *Zauschneria garrettii*), common name firechalice or hummingbird flower, is in the Onagraceae family. This species is sometimes called 'Orange Carpet' because the plant spreads as a ground cover, and the flowers are bright orange-red and attractive to hummingbirds (Love et al

2009). This species is relatively small in stature, usually 30 to 45 cm tall and 30 to 60 cm wide.

The plant is dark green with lance shaped foliage that is slightly pubescent. Stems grow upright and are weakly spreading with thin branches that tend to arch. It is an herbaceous perennial plant that grows back each year. After the stems die back in the winter, new ones will emerge from the roots in spring (Winger 1996). This species is native to the Intermountain West, and it grows naturally in all western regions of the United States except Washington, Montana, and Colorado (Love et al 2009).

Firechalice is a good choice for rock gardens and is drought tolerant. Supplemental irrigation and fertilization may help keep the plant attractive throughout the season (Robson et al 2008). It grows in most types of soil except poorly drained soil and tolerates a wide range of soil pH (5.5 to 8.5). The plant grows best in full sun but tolerates partial-shade.

Bloom starts in June and extends to October. Plants bloom the first year, and they increase in width and number of flowering stems as they age. Plants bloom when their stems are about 30 to 45 cm tall, and plant size can be variable with some older plants being much wider than tall. Firechalice is useful for landscaping and tolerates all routine nursery production and handling procedures. It also lacks serious insect or disease problems (Love et al 2009).

Firechalice can be used to add color to almost any site and can be used in either naturalized or formal designs. Firechalice is a good companion to many other

species, such as *Eriogonum umbellatum, Eriogonum compositum, Eriogonum niveum, Baileya multiradiata, Artemisia frigida, Sorghastrum nutans, Chamaebatiaria millefolium, Ribes aureum,* and almost any other species that provides the desired color combinations (Love et al. 2009).

Since unique characteristics of selected individual plants are desired by the landscape industry, selected plants are required to be propagated asexually. Methods of asexual propagation, including grafting, budding, layering and cutting, can be successful with native plants, but sometimes these techniques fail to work or reproduce plants too slowly. Various tissue culture techniques may be successful with native species.

Micropropagation results in true-to-type plants of a selected genotype. Most often micropropagation is associated with mass production at a competitive price (Debergh and Zimmerman 1991). The history of plant tissue culture begins in 1838 - 1839. A successful approach to tissue culture was discovered by Rechinger (1893) who saw the potential to overcome limits of plant divisibility. He used buds, slices of roots, stems and other materials, and then he placed the explants on a sand surface moistened with tap water. He found pieces thicker than 1.5 mm would develop, but his techniques excluded nutrients and aseptic conditions (Gautheret 1983).

In the past, plant tissue culture techniques have been used in academic investigations of totipotency and studies involving the roles of hormones and organogenesis. More recently, tissue culture has been used as a tool for genetic

engineering of plants and provide insight into plant molecular biology and gene regulation (Mineo 1990).

Micropropagation of axillary shoots has five stages (Debergh and Zimmerman 1991). Stage 0 is the preparative stage. Stage I is the initiation of cultures and their stabilization. Stage II is multiplication of axillary shoots. Stage III involves stem elongation and inducing root formation. Stage IV is acclimatization and transfer of rooted shoots (plantlets) to greenhouse conditions.

Stage 0:

This stage involves a treatment to prevent or minimize contamination problems. If the mother plant grew under hygienic conditions, contamination problems can be reduced especially those related to fungi. Stock plants can be grown in a greenhouse to yield more hygienic explants. Stage 0 influences the rate of survival. The most commonly manipulated parameters are light, temperature and growth regulators (Debergh and Zimmerman 1991). Active growth and reduced probability of disease are achieved by maintaining the stock plants in clean controlled conditions. Also, antibiotic sprays help to reduce the contamination potential of candidate explants (Beyl and Trigiano 2008).

Stage I: Establishment and stabilization

The goal of Stage I is initiation and establishment explants in sterile conditions. In this stage, axenic cultures are initiated. Apical or axillary buds are important for their use in micropropagation. An alternate source of explants is leaf pieces (Debergh and Zimmerman 1991). The age of the stock plant and its size can

determine the success of the establishment procedure (Franclet et al 1987). When establishing tissue cultures, potential contamination comes from either carry-over of microorganisms on the explant surface or within explant tissues, or through laboratory procedures (Campbell 1985).

The primary explants are obtained from stock plants, and they can be shoot apical meristems or meristem tips for pathogen elimination or shoot tips from terminal or lateral buds. The surfaces of plants are populated with diverse microflora consisting of bacteria, fungi, yeast and other organisms. Usually, explants are surface disinfected with 70 to 95% alcohol or 0.5% (w/v) sodium hypochlorite. Surface-sterilized explants are transferred into culture vessels with sterilized medium in a laminar airflow transfer hood. Scalpels and forceps are sterilized by flame or dry heat.

Choosing the right culture medium is an important factor, which governs the success of tissue culture. Most plant species can be grown on a properly defined medium. The medium should contain correct amounts and proportions of inorganic nutrients and often has high concentrations of nitrate, potassium, and ammonium relative to other nutrients in the medium. Murashige and Skoog (MS) is the most widely used medium (Gamborg 1976). The medium is completed by adding sucrose, and vitamins supplemented with a cytokinin and solidified with agar or gellan gum. Plant hormones are relatively small molecules that affect and regulate growth at low concentrations. The two types of plant hormones used most frequently in tissue culture are cytokinins and auxins (Ting 1982). The cytokinins used most frequently are $N^6$-benzyladenine (BA), kinetin (kin), or $N^6$-2-Isopentenyl-

adenine (2iP). Of the three, BA is the most widely used cytokinin due to its low cost and high effectiveness (Beyl and Trigiano 2008).

Stage II: Shoot multiplication

Stage II involves repeated enhanced formation of axillary shoots from shoot tips or lateral buds cultured on a medium supplemented with a high concentration of cytokinin. The interval between starting a shoot culture and dividing it after the shoot has grown is generally an interval of four weeks. For some species, shoot explants can produced two to four times the initial number of axillary shoots. With monthly subculturing, some species can be maintained from 8 to 48 months in Stage II (Beyl and Trigiano 2008). In Stage II, selection of cytokinin type and concentration depend on comparative shoot multiplication rate and shoot length. Although shoot proliferation is enhanced at higher cytokinin concentrations, too much cytokinin in the medium can distort or inhibit shoot growth (Beyl and Trigiano 2008).

The propagation ratio can be improved by increasing the amount of cytokinin (Debergh and Zimmerman 1991). During Stage II, different types of cytokinins can be used to multiply shoots. Cytokinins are modified forms of adenine and induce two effects on undifferentiated cells, the stimulation of DNA synthesis and increased cell division (Ting 1982).

Stage III: Root formation

Stage III is often called pretransplant rooting. This step is characterized by preparation of Stage II shoots (microshoots) or shoot clusters for successful transfer to soil (Beyl and Trigiano 2008). Roots are induced to form and develop or elongate

during this stage. The quantity and the quality of shoots from Stage II will determine the approach to be used in this stage.

Auxins are often indole or indole-like compounds that stimulate cell expansion, particularly cell elongation and promote adventitious root formation (Mineo 1990). Herbaceous plants can usually form roots on a medium that lacks auxin. However, with many woody plant species, the addition of auxin (IBA or NAA) in Stage III is required to enhance adventitious rooting (Beyl and Trigiano 2008). The parameters used to judge the efficiency of the rooting treatments in Stage III are the number of shoots formed, shoot length, percentage of rooted shoots, number of roots per shoot, and length of the roots.

Stage IV: Acclimatization

Plantlets, rooted shoots, were developed in culture vessels under low levels of light and aseptic conditions on a medium containing ample sugar and nutrients to allow for heterotrophic growth under a high level of humidity. These factors contribute to a culture-induced phenotype that is unable to survive environmental conditions when directly placed in a greenhouse or field (Hazarika 2003). The greenhouse has lower relative humidity and higher light levels that are stressful to micropropagated plants compared to in vitro conditions. Most species grown *in vitro* require an acclimatization process to ensure plants survive and grow vigorously when transferred to soil. Success in Stage IV involves the ability to transfer and reestablish vigorously growing plants from in vitro conditions to the low relative humidity and high light intensity of the greenhouse. Relative humidity can be

maintained above ambient with humidity tents, single tray propagation domes, intermittent misting, or fog systems (Beyl and Trigiano 2008).

Although Saskatoon serviceberry has been grown in tissue culture, its shoots are difficult to root. During my research, I also noted serviceberry shoots had symptoms of mineral deficiencies, indicating that MS media failed to provide optimum mineral nutrition. Optimizing MS medium for serviceberry and improving shoot quality in the multiplication stage may help shoots to form more roots in the next stage of tissue culture. Our selected ecotype of firechalice (*Epilobium canum garrettii*) is difficult to germinate from seeds. In order to increase the number of selected individuals rapidly, plant tissue culture would be the propagation method of choice.

The goal of this research was to develop a micropropagation procedure for rapid production of selected native plants. I demonstrated that selected plants can multiply quickly in the first two stages of micropropagation and could be rooted and acclimated so that hundreds or thousands of the selected ecotype can be made available to production nurseries.

## REFERENCES


Anonymous (1976) *Amelanchier alnifolia* (Nuttall) Nuttall Saskatoon. Davidsonia 7(1):5-13

Beyl CA, Trigiano RN (2008) Plant Propagation Concepts and Laboratory Exercises. CRC Press, LLC, Boca Raton, Florida, pp 462

Campbell R (1985) Plant Microbiology. Arnold, London, pp 191

Davidson CG, Mazza G (1991) Variability of fruit quality and plant height in populations of Saskatoon berries (*Amelanchier alnifolia* Nutt.) Fruit Varieties Journal. 45:162-165

Debergh PC, Zimmerman RH (1991) Micropropgation Technology and Application. Kluwer Acadamic Publishers, Dordrecht. The Netherlands, pp 1-13

Dirr MA (1983) Manual of Woody Landscape Plants. Stipes Publishing, Champaign, Illinois, pp 826

Elias TS (1980) The Complete Trees of North America. Van Nostrand Reinhold: New York. pp 948

Franclet A, Boulay M, Bekkaoui F, Fouret Y, Verschoore-Martouzet B, Walker N (1987) Rejuvenation. In: Bonga JM, Durzan DJ (Eds) Cell and Tissue Culture in Forestry,Vol 1 Martinus Nijhoff. Dordrecht, Netherlands, pp 232-248

Gamborg OL, Murashiger T, Thorpe TA, Vasil IK (1976) Plant tissue culture media. In Vitro 12:E1-E6

Gautheret RJ (1983) Plant tissue culture: A history. The Botanical Magazine 96:393-410

Grainger G (1983) Propagation of Saskatoons. Alberta Tree Nursery and Horticulture Center, Edmonton, Alberta Agric Publ, pp 15

Gunther E (1995) Ethnobotany of western Washington. University of Washington Press, Seattle, Washington: pp 71

Harris R (1976) Saskatoons (*Amelanchier alnifolia*) Summary report. Proc Western Can Soc Hortic 32:50-59

Harris RE (1980) Propagation of *Amelanchier alnifolia* cv. Smoky in vitro. Proc Can Soc Hortic Sci 19:32-34

Hazarika BN (2003) Acclimatization of tissue culture plants. Curr Sci 85:1704-1712

Leigh M (1999) Grow Your Own Native Landscape: A guide to identifying propagating and landscaping with western Washington native plants. WSU Cooperative Extension, Thurston County

Love SL, Noble K, Robbins JA, Wilson B, McCammon T (2009) Landscaping with Native Plants. University of Idaho Bulletin 862

Mineo L (1990) Plant tissue culture techniques. In Tested Studies for Laboratory teaching. Proceedings of the Eleventh Workshop/Conference of the Association for Biology Laboratory Education (ABLE), Easton, PA, USA 11:151-174

Pierre RG, Steeves TA (1990) Observations on shoot morphology, anthesis, flower number, and seed production in the Saskatoon, *Amelanchier alnifolia* (Rosaceae). Canadian Field Naturalist 104:379-386

Pruski K (1987) Micropropagation of cultivars of the Saskatoon *Amelanchier alnifolia* Nutt. MSc Thesis Dept Plant Sci, Univ Alberta, Edmonton

Pruski K, Grainger G, Nowak J (1985) Micropropagation of Saskatoon. In: Conf Tissue culture as a plant production system for horticultural crops. Beltsville, Maryland, USA, Abstr 26, p 28

Pruski K, Mohyuddin M, Grainger G (1991) Saskatoon (*Amelanchier alnifolia* Nutt.) In: Bajaj Y.P.S (Ed), Biotechnology in Agriculture and Forestry, Vol 16 Trees III Springer-Verlag. Berlin, pp164-179

Pruski K, Nowak J, Grainger G (1990) Micropropagation of four cultivars of Saskatoon *Amelanchier alnifolia* Nutt. Plant Cell Tiss Organ Cult 21:103-109

Rechinger C, (1893) Untersuchungen über die Grenzen der Teilbarkeit im Pflanzenreich. Abh. d. Zool. Bot. Ges. Wien 43: 310-334

Robson KA, Richter A, Filbert M (2008) Encyclopedia of Northwest Native Plants for Gardens and Landscapes. Timber Press, Portland, Oregon. pp 532

Ting IP (1982) Plant Physiology. Addison Wesley. New York. pp 331-363

Turner NJ, Thompson LC, Thompson MT, York AZ (1990) Thompson Ethnobotany. Royal British Columbia Museum: Victoria, British Columbia: Royal British Columbia Museum. pp 335

U.S. Department of Agriculture, Forest Service (1974) Seed of Woody Plants in the United States. Agriculture Handbook 450. Washington, DC. pp 883

U.S. Department of Agriculture, Natural Resources Conservation Service (1999) The PLANTS database http://plants.usda.gov/plants. National. Plant Data Center, Baton Rouge, Louisiana

Wasser CH, Shoemaker J (1982) Ecology and culture of selected species useful in revegetating disturbed lands in the west. FWS/OBS-82/56. pp 177-180

Wilson B (1993) Saskatoon berries. Bemidji, MN: Summer Harvest Berry Farm. pp 5

Winger David (1996) Xeriscape Plant Guide: 100 Water-Wise Plants for Gardens and Landscapes. Fulcrum Publishing, Golden, Colorado. pp 192

# CHAPTER 1

## Altering mineral nutrition to improve shoot growth of serviceberry (*Amelanchier alnifolia*) in tissue culture

## ABSTRACT

A horticulturally superior serviceberry plant (*Amelanchier alnifolia* Nutt.) was propagated by tissue culture, but its shoots appeared to have one or several mineral deficiencies. The objective of this study was to improve shoot growth of in vitro microshoots from this selected serviceberry ecotype. Adjusting mineral concentrations in Murashige and Skoog (MS) medium by adding mesos components ($CaCl_2$, $KH_2PO_4$ and $MgSO_4$) in different concentrations affected overall shoot quality, including leaf color, multiplication rate, height and dry weight. Four different levels of mesos components were used in the first experiment. The best level of these minerals was (in mg $l^{-1}$: 0.331 $CaCl_2$, 0.135 $MgSO_4$ and 0.128 $KH_2PO_4$), and shoot dry weight was 17% heavier compared to the control. In the second experiment, seven levels of mesos were used, and the best treatments were (0.132, 0.054, and 0.204 g $l^{-1}$) or (0.398, 0.054, and 0.102 g $l^{-1}$) for $CaCl_2$, $MgSO_4$ and $KH_2PO_4$, respectively. These treatments increased the number of shoots per explant by 67% and 43%, shoot height by 91% and 112%, and shoot dry weight by 44% and 77%, respectively, compared to the control shoots. This study demonstrated that shoot height, shoot dry weight and the number of axillary shoots produced by the selected serviceberry plant could be improved by adding specific concentrations of mesos components ($CaCl_2$, $MgSO_4$, and $KH_2PO_4$) to MS medium.

# INTRODUCTION

Serviceberry (*Amelanchier alnifolia* Nutt.) is native to North America, extending from Manitoba to the Fraser River Canyon, and from the northwestern United States to the Yukon (Pruski et al. 1991). Serviceberry is occasionally used as a commercial fruit crop and can be cultivated on a wide range of soils. This species can range in height from being a dwarf plant to growing up to 8 m (2 to 26 ft) tall. It is also useful as a landscape plant. White flowers appear in early spring and produce purple fruit in late summer. In fall, leaves turn an attractive dark red color.

Serviceberry can be grown successfully on MS medium in plant tissue culture. In a procedure developed by Pruski et al. (1991), small shoot explants with several nodes were established in the first stage of tissue culture. The second stage involved multiplication and shoot elongation, with a subculture period of four weeks (Pruski et al. 1991). The cytokinin used in Stage II was benzyladenine BA, and increasing the concentration of BA helped to improve and increase the number of shoots per culture up to a certain point, before shoot lengths decreased (Pruski 1987). The optimum range of BA in the proliferation medium was (8.87 to 13.3 μM) for most serviceberry cultivars (Pruski et al.1991). Factors shown to affect in vitro shoot morphology, include type of growth medium, mineral nutrient composition, agar concentration, environment, and type of cytokinin (Bottcher et al.1988; Paques 1991; Debergh et al.1992; Bosela and Michler 2008).

All plants need a certain amount of mineral elements from their environment to ensure successful growth and development of vegetative and reproductive tissues. Abiotic factors, such as pH and temperature can affect nutrient availability.

Mineral deficiencies affect shoot growth by limiting plant biosynthesis and metabolism. For example, N, Fe or Mg deficiencies reduce chlorophyll synthesis and result in chlorosis or yellowing of leaves. Deficiencies of P, K or S impact metabolites or enzymes involved in photosynthesis and respiration. Essential minerals are required for healthy growth (Grusak 2001). Some physiological problems of shoots, such as hyperhydricity, shoot tip necrosis, callus formation, hooked and curled new leaves, fasciation, and hypertrophy can be observed during micropropagation of many species (Reed et al. 2013b). Reed et al. (2013a) found that adjusting mineral nutrition in the culture medium improved pear micropropagation, and they documented a range of shoot responses. Increased mesos minerals ($CaCl_2$ $MgSO_4$, $KH_2PO_4$) in medium improved leaf characteristics, such as darker green color and eliminated most negative leaf symptoms necrosis, spots, and hyperhydricity (Wada et al. 2013).

At the beginning of our research, a serviceberry plant selected for its dwarf growth habit and superior horticultural performance was established in vitro on regular MS medium. Its shoots, however, often had poor leaf color and limited growth. These symptoms indicated the shoots had mineral deficiencies and that regular MS medium was failing to provide optimum mineral nutrition. Optimizing MS medium composition for serviceberry shoot quality during micropropagation was the goal of this study.

## MATERIAL AND METHODS

In this study, the selected serviceberry (*Amelanchier alnifolia*) plant used for micropropagation was obtained from Dr. Stephen Love, University of Idaho,

Aberdeen R & E Center, Aberdeen, ID. Single-node stem explants were taken from stems of the selected plant. The shoots were established on MS medium (Murashige and Skoog 1962). This medium (*Phyto*Technology Laboratories, Shawnee Mission KS, product M524) contained 4.3 g $l^{-1}$ mineral salts and 5.9 μM thiamine-HCl, 8.1 μM nicotinic acid, 4.9 μM pyridoxine-HCl, 53.3 μM glycine, 100 mg $l^{-1}$ myo-inositol, 30 g $l^{-1}$ sucrose, solidified with 7 g $l^{-1}$ agar, (*Phyto*Technology Laboratories, product A111), and had a pH 5.7. The medium included 4.4 μM benzyladenine (BA). The media were dispensed in to GA7 vessels and autoclaved at 120ºC for 20 min. Six shoots were placed in each vessel for all experiments. Cultures were incubated in a SG 30S germinator (Hoffman Manufacturing Inc., Albany, OR) at 25 ± 1ºC under a 16-h photoperiod (cool-white fluorescent lamps), 38 μmol $m^{-2}$ $s^{-1}$ photosynthetic photon flux (PPF). After 30 days, the shoot cultures were subcultured (divided) and placed on fresh MS medium with different treatments. Different minerals were added to the MS medium separately in different concentrations (0, 0.12, 0.23, 0.34 or 0.46 g $l^{-1}$ $Ca(NO_3)_2 \cdot 4H_2O$), (0, 0.08, 0.16, 0.24 or 0.32 g $l^{-1}$ $NH_4NO_3$), (0, 0.000015, 0.00003, 0.000046 or 0.000062 g $l^{-1}$ $NiSO_4$). After these compounds failed to improve shoot growth (number of axillary shoots produced and shoot height), different mesos nutrients were used in two different experiments.

First mesos experiment:

Five different levels of mesos ($CaCl_2$, $KH_2PO_4$ and $MgSO_4$) components were added to the MS media that included 4.4 μM BA (Table 1.1). Cultures were incubated in a SG 30S germinator (described above). Shoots were subcultured

every 3 wk for three subcultures (Reed et al.1998). Shoots were grown for three weeks, and several axillary buds typically broke and grew. After 3 wk, the best shoot (had the best leaf color and appearance) from each shoot explant was cut at its base and transferred to new medium for the second subculture. This same procedure was completed for the third subculture. Six shoot explants of similar size were placed in each of four GA7 boxes for each subculture.

**Table 1.1**: Different concentrations of $CaCl_2$, $MgSO_4$ and $KH_2PO_4$ (mesos minerals) added to MS media used for axillary shoot production of serviceberry shoots.

| Treatment | $CaCl_2$ (g $l^{-1}$) | $MgSO_4$ (g $l^{-1}$) | $KH_2PO_4$ (g $l^{-1}$) |
|---|---|---|---|
| Control | 0 | 0 | 0 |
| 2 | 0.331 | 0.135 | 0.128 |
| 3 | 0.662 | 0.271 | 0.255 |
| 4 | 0.993 | 0.406 | 0.384 |
| 5 | 1.323 | 0.542 | 0.510 |

Second mesos experiment:

One treatment in g $l^{-1}$: (0.331 $CaCl_2$, 0.135 $KH_2$ $PO_4$ and 0.128 $MgSO_4$) from the first study improved shoot quality (leaf color, shoot height and dry weight). Based on these results, seven different levels of mesos components were used in the second experiment (Table 1.2). Since $KH_2PO_4$ can influence shoot quality (Poothong and Reed 2015), different concentrations of $KH_2PO_4$ were used with moderate levels of $CaCl_2$ and $MgSO_4$ (Table 1.2). Shoots explants were placed in each of four GA7 vessels, and the cultures were placed in a SG 30S germinator (described above).

Data analyses:

Six stem explants were placed in each vessel, and four replicate vessels were used for 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 (shelf). Data were taken in each experiment after 9 weeks (three subcultures) on the mesos supplemented media, and they included the number of shoots formed (from axillary buds) per stem explant, 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. Means were analyzed by one-way analysis of variance (mixed model procedure) (Proc Mixed, SAS 2012). 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.

**Table 1.2**: Different concentrations of $CaCl_2$, $MgSO_4$ and $KH_2PO_4$ (mesos minerals) added to MS media used for axillary shoot production of serviceberry shoots.

| Treatment | $CaCl_2$ ($g\ l^{-1}$) | $MgSO_4$ ($g\ l^{-1}$) | $KH_2PO_4$ ($g\ l^{-1}$) |
|---|---|---|---|
| Control | 0 | 0 | 0 |
| z | 0.132 | 0.054 | 0 |
| a | 0.132 | 0.054 | 0.052 |
| b | 0.132 | 0.054 | 0.102 |
| c | 0.132 | 0.054 | 0.154 |
| d | 0.132 | 0.054 | 0.204 |
| e | 0.264 | 0.054 | 0.052 |
| g | 0.398 | 0.054 | 0.102 |

## RESULTS

Using different minerals in different concentrations by themselves (e.g., $Ca(NO_3)_2{\cdot}4H_2O$, $ZnSO_4{\cdot}7H_2O$, $NiSO_4$, etc.) in MS medium failed to improve shoot quality (number of shoots per explant, shoot height or dry weight) compared to control shoots (data not shown). However, adding mesos components ($CaCl_2$, $MgSO_4$ and $KH_2PO_4$) at different concentrations to the MS medium improved shoot heights (P = 0.0009) for some treatments (Table 1.3). In contrast, all treatments in the first experiment failed to affect the number of shoots produced per explant (P = 0.0518). Treatment 5 induced the tallest shoot growth, and this treatment had the highest level of mesos components (in g $l^{-1}$: 1.323 $CaCl_2$, 0.542 $MgSO_4$ and 0.510 $KH_2PO_4$) compared to the other treatments. Shoots receiving the F5 treatment were 72% taller than control shoots. In addition, shoots on treatment 2 (in g $l^{-1}$: 0.331 $CaCl_2$, 0.135 $MgSO_4$ and 0.128 $KH_2PO_4$) grew 44% taller than control shoots. Shoot

**Table 1.3**: Effects of added mesos minerals ($CaCl_2$, $MgSO_4$, $KH_2PO_4$) on the mean number of shoots, mean shoot heights, and mean shoot dry weights of serviceberry shoots grown on MS medium for three subcultures over 9 weeks. Four vessels were used per treatment, and within each vessel six shoots were averaged before analysis.

| Treatment | Number of shoots | Shoot height (cm) | Shoot dry weight (g) |
|---|---|---|---|
| Control | 2.2 a* | 1.8 a | 0.06 bc |
| 2 | 3.3 a | 2.6 c | 0.07 c |
| 3 | 3.1 a | 2.1 ab | 0.05 ab |
| 4 | 2.2 a | 2.2 abc | 0.05 ab |
| 5 | 3.0 a | 3.1 d | 0.07 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 = 4).

dry weight was significantly affected by mesos treatments (P = 0.0013), and treatments 2 and 5 improved shoot dry weight by 40% compared to treatments 3 and 4 (Table 1.3). By the third subculture after 9 weeks, shoot heights and leaf color of shoots grown on treatment 2 medium appeared greener and seemed more healthy than shoots grown on regular MS medium (Figure 1.1).

New concentrations of mesos components were added to MS medium in the second experiment based on the results from the first experiment. In the second experiment, some treatments significantly affected number of axillary shoots formed per explant (P = 0.0014). Shoots grown on different concentrations of all three mesos components ($CaCl_2$, $MgSO_4$ and $KH_2PO_4$) were taller and had a better overall appearance compared to control shoots, those grown on regular MS medium. (Figure 1.2).

The shoots grown on treatments c, d and g produced the most axillary shoots (Table 1.4). Shoot explants on treatment d produced 62% more axillary shoots than

**Figure 1.1**: Effects of added mesos minerals ($CaCl_2$, $MgSO_4$, $KH_2PO_4$) on shoot quality. Shoots were grown on different MS amended media for three subcultures over 9 weeks. Refer to Table 1.1 for the treatments descriptions.

| Trt | Control | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|

the control shoots. In addition, all treatments increased shoot heights (P = 0.0001) compared to control shoots. Heights of shoots on treatments g and d increased about 2 fold compared to control shoots (Table 1.4). In addition, shoot dry weight was significantly affected (P = 0.0026) by the mesos treatments. Shoots on treatment g that had a high level of 0.398 g $l^{-1}$ $CaCl_2$ compared to other treatments produced the most shoot dry weight (0.156 g) which was 75% more than the control shoots (0.089 g).

Optimization of growth media based on mineral nutrition for micropropagation is very challenging due to the unique nutritional requirements of plant species. Replications are an important component of any successful experiment. Treatments d and g were repeated in another experiment to ensure that these two treatments

**Table 1.4**: Effects of added mesos minerals ($CaCl_2$, $MgSO_4$, $KH_2PO_4$) on the mean number of shoots, mean shoot heights, and mean shoot dry weights of serviceberry shoots grown on MS medium for three subcultures over 9 weeks. Four vessels were used per treatment, and within each vessel six shoots were averaged before analysis.

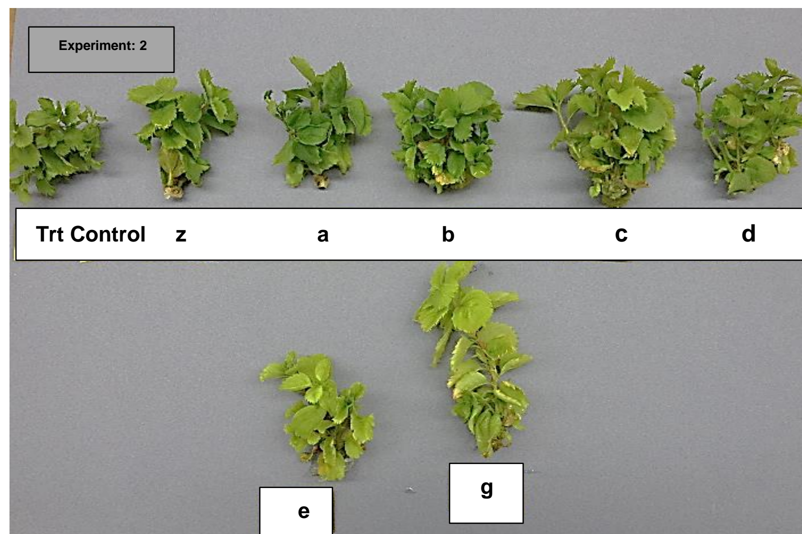
| Treatment | Number of shoots | Shoot height (cm) | Shoot dry weight (g) |
|---|---|---|---|
| Control | 3.7 a | 2.4 a | 0.089 a |
| z | 4.6 bc | 3.2 b | 0.115 ab |
| a | 4.6 bc | 3.4 bc | 0.131 bc |
| b | 4.6 bc | 3.4 bc | 0.133 bc |
| c | 5.0 cd | 3.6 c | 0.128 bc |
| d | 6.0 d | 4.6 d | 0.129 bc |
| e | 4.5 ab | 3.2 bc | 0.100 ab |
| g | 5.3 cd | 5.1 d | 0.156 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 = 4).

yielded similar results.

In the repeated experiment, shoots grown on treatments d and g again grew better than control shoots since the former shoots produced more axillary shoots, grew taller and produced more dry weight compared to the control shoots (Table 1.5

**Figure 1.2**: Effects of added mesos minerals ($CaCl_2$, $MgSO_4$, and $KH_2PO_4$) on appearance of serviceberry that were treated during three subcultures over 9 weeks. Refer to Table 1.2 for the treatments descriptions.



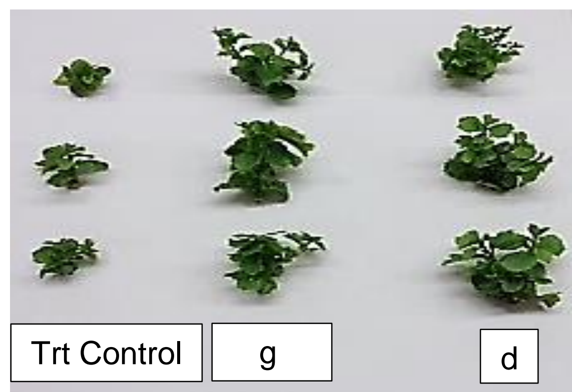

and Figure 1.3). Treatments d and g affected the number of axillary shoots (P = 0.0158), and both treatments increased the number of axillary shoots at least 2 fold compared to control shoots (Table 1.5). Shoot heights increased by about 2.5 fold for both treatments compared to the controls. The increase in shoot dry weights was highly significant ($P < 0.0001$). Mesos d and g treatments increased shoot dry weight at least 4 fold compared to the control shoots (Table 1.5).

**Table 1.5**: Effects of added mesos minerals ($CaCl_2$, $MgSO_4$, $KH_2PO_4$) on the mean number of shoots, mean shoot heights, and mean shoot dry weights of serviceberry shoots grown on MS medium for three subcultures over 9 weeks. Four vessels were used per treatment, and within each vessel six shoots were averaged before analysis.

| Treatment | Number of shoots | Shoot height (cm) | Shoot dry weight (g) |
|---|---|---|---|
| Control | 2.1 a | 1.3 a | 0.025 a |
| d | 5.6 b | 3.3 b | 0.106 b |
| g | 4.2 b | 3.5 b | 0.113 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 = 4).

**Figure 1.3**: Effects of the best level of mesos d and g treatments on serviceberry shoot size and appearance compared to the control. Shoots were grown on mesos supplemented MS medium for three subcultures over 9 weeks. Refer to Table 1.2 for the treatments descriptions.



## DISCUSSION

Mineral nutrients influence shoot growth in tissue culture and can improve shoot quality, yet optimizing mineral nutrition is a difficult challenge in micropropagation. Some shoot growth responses can be improved by adjusting mineral nutrition in vitro.

Adding nutrients to the medium can affect shoot length and influence physiological problems and plant health in tissue culture (Reed et al. 2013a, Wada et al. 2013). The efficacy of adding specific nutrients to the media depends on the type of medium and plant genotype. Among different types of media, concentrations of some minerals are much higher than others. For example, $Ca(NO_3)_2$ concentration in DKW medium is much higher than in WPM or MS medium. Some nutrients are more important for plants than others. For example, N is always an important nutrient, but the type and amount of N needed depends on the species (Hand et al. 2014). Mineral concentrations can be adjusted by using different amounts of individual nutrients or combinations of minerals. In this study, different individual nutrients, such as $Ca\ (NO_3)_2$, $NH_3NO_4$, or $NiSO_4$ in different concentrations were used. Adding any of these minerals separately to MS medium in different concentrations failed to improve serviceberry shoot growth. Other individual elements $ZnSO_4$, $MgSO_4$, were added with 0.5 μM IBA during rooting studies, and these elements also failed to improve shoot response (non-puplished research data). In contrast, some studies (Poothong and Reed 2015, Reed et al. 2013a, Reed et al. 2013b) have demonstrated that combinations of some mineral nutrients added to the culture medium improved shoot growth of some plants. The

mesos minerals ($CaCl_2$, $KH_2PO_4$ and $MgSO_4$) have been shown to be the most important mineral nutrient group that affected leaf color (Poothong and Reed 2015).

In the first mesos experiment, adding a moderate concentration of $CaCl_2$ (0.331 g $l^{-1}$) and low concentrations of $MgSO_4$ (0.135 g $l^{-1}$) and $KH_2PO_4$ (0.128 g $l^{-1}$) to the MS medium improved shoot height and shoot dry weight compared to other treatments. The results from this study indicated that different combinations of mesos minerals were needed to improve shoot growth, and the optimal amount of each individual mesos component needed to be determined. Increasing the concentration of some mesos components should have improved the quality (foliar color and shoot growth) of serviceberry shoots as was found by Poothong and Reed (2015) in their research with 'Trailblazer' raspberry.

In the second mesos experiment, concentrations of mesos components were altered by increasing the $KH_2PO_4$, concentration while using with low concentrations of $MgSO_4$ and $CaCl_2$ or increasing $KH_2PO_4$ and $CaCl_2$ concentrations while using a low concentration of $MgSO_4$ . Shoot number was significantly influenced by $KH_2PO_4$ since treatment d contained a high concentration of $KH_2PO_4$, and explants on this treatment produced the most axillary shoots. These shoots were also the tallest of all other treated shoots except for those on treatment g (Table 1.4). Increased concentrations of $KH_2PO_4$ improved shoot length for most red raspberries cultivars (Poothong and Reed 2015). Also, $KH_2PO_4$ improved *Prunus* shoot multiplication, yet $MgSO_4$ decreased it (Alanagh et al. 2014).

Repeated treatments d and g yielded similar results to the first experiment with treatments d and g, verifying that these two treatment improved growth of the

selected serviceberry plant. Potassium is mainly required for osmotic balance and stomatal function, sulfur is involved in chlorophyll production, and magnesium is the central molecule of chlorophyll (Epstein and Bloom 2005, Bairu et al. 2009). Increased K and decreased Mg enhanced the number of axillary shoots produced by raspberry (Poothong and Reed 2015). In this study, treatment g also improved the shoot growth by increasing the number of shoots, shoot height and shoot dry weight more than other treatments except for treatment d (Table 1.4). Treatment g had a high level of added $CaCl_2$ and low concentrations of added $MgSO_4$ and $KH_2PO_4$. Similar results were found by growing three plant species (*Vriesea friburguensis*, *V. unilateralis*, *V. hieroglyphica*) in media containing high levels of $Ca^{+2}$, which improved plantlet growth for all three species (Aranda-Peres et al. 2009). Calcium may have facilitated absorption of other nutrients by the serviceberry shoots, as is the case for N, K, Z, Mn and B present in high levels in tobacco shoots grown on medium with a high level of $Ca^{+2}$ (Lopez-Lefebre et al. 2001). Calcium is an important macroelement and plays an vital role in cell signaling (Reddy 2001). In addition, $Ca^{+2}$ must always be present in apoplast to protect the plasma membrane and regulate selective ion uptake (Schroeder et al. 2001). High concentrations of $CaCl_2$ or $KH_2PO_4$ used in the medium to multiply serviceberry significantly increased number of shoots, shoot length and shoot dry weight. In addition, leaf color appeared to be a darker green, and some leaves had a glossy appearance (Figures 1.1 and 1.2).

Poothong and Reed (2015) found similar results when they used high concentrations of $CaCl_2$ and $KH_2PO_4$ which resulted in increased shoot length for

most cultivars of red raspberries. They concluded that mesos components ($CaCl_2$, $MgSO_4$, and $KH_2PO_4$) significantly affected raspberry (*Rubus idaeus* L.) shoot quality and growth. Optimizing the growth medium should also provide better commercial production of in vitro shoots (Poothong and Reed 2015). In addition, the concentrations of P, K, Ca and Mg in shoots treated with additional mesos minerals were higher than those in shoots grown on regular MS medium (Poothong and Reed 2015). In this study with a selected serviceberry ecotype, adding d and g mesos concentrations in MS media improved shoot growth, including the number of axillary shoots formed, shoot height and shoot dry weight.

## CONCLUSION

Combinations of mesos nutrients in treatments d and g (in g $l^{-1}$: 0.398 $CaCl_2$, 0.054 $MgSO_4$ and 0.102 $KH_2PO_4$ or 0.132 $CaCl_2$, 0.054 $MgSO_4$ and 0.204 $KH_2PO_4$), respectively, added to MS medium produced high quality shoots by increasing the number of axillary shoots, shoot height, and shoot dry weight. Leaf color and overall appearance of these treated shoots were also improved compared to shoots grown on regular MS medium. Improving shoot quality during the multiplication stage may help shoots to form more roots in the next stage of micropropagation.

**REFERENCES**

Alanagh EN, Garoosi G, Haddad R, Maleki S, Landín M, Gallego PP (2014) Design of tissue culture media for efficient *Prunus* rootstock micropropagation using artificial intelligence models. Plant Cell Tiss Organ Cult 117:349-359

Aranda-Peres AN, Peres LEP, Higashi EN, Martinelli AP (2009) Adjustment of mineral elements in the culture medium for the micropropagation of three *Vriesea* bromeliads from the Brazilian Atlantic Forest: The importance of calcium. HortScience 44:106-112

Bairu MW, Stirk WA, Van Staden J (2009) Factors contributing to in vitro shoot-tip necrosis and their physiological interactions. Plant Cell Tiss Organ Cult 98:239-248

Bosela MJ, Michler CH (2008) Media effects on black walnut (*Juglans nigra* L.) shoot culture growth in vitro: evaluation of multiple nutrient formulations and cytokinin types. In Vitro Cell Dev Biol - Plant 44:316-329

Bottcher I, Zohlauer K, Goring H (1988) Induction and reversion of vitrification of plants cultured in vitro. Physiol Plant 72:560-564

Debergh P, Aitken-Christie J, Cohen D, Grout B, Von Arnold S, Zimmerman R, Ziv M (1992) Reconsideration of the term‘ vitrification’ as used in micropropagation. Plant Cell Tiss Organ Cult 30:135-140

Epstein, E, Bloom, AJ (2005) Mineral nutrition of plants: Principles and perspectives, 2nd Edn. Sinauer Associates, Inc., Sunderland, MA

Grusak MA (2001) Plant Macro- and Micronutrient Minerals. *Encyclopedia of Life Science*. Nature Publishing Group.www.els.net. DOI: 10.1038/npg.els.0001306

Hand C, Maki S, Reed BM (2014) Modeling optimal mineral nutrition for hazelnut micropropagation. Plant Cell Tiss Organ Cult 119:411-425

Lopez-Lefebre LR, Rivero RM, Garcia PC, Sanchez E, Ruiz JM, Romero L (2001) Effect of calcium on mineral nutrient uptake and growth of tobacco. J Sci Food Agr 81:1334-1338

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473-497

Paques M (1991) Vitrification and micropropagation: causes, remedies and prospects. Acta Hortic 289:283-290

Poothong S, Reed B. (2015) Increased $CaCl_2$, $MgSO_4$, and $KH_2PO_4$ improve the growth of micropropagated red raspberries. In Vitro Cell Dev Biol - Plant 51:648-658

Pruski K (1987) Micropropagation of cultivars of the saskatoon, *Amelanchier alnifolia* Nutt. MSc Thesis Dept Plant Sci, Univ Alberta, Edmonton

Pruski K, Mohyuddin M, Grainger G (1991) Saskatoon (*Amelanchier alnifolia* Nutt.) In: Bajaj Y.P.S (Ed), Biotechnology in Agriculture and Forestry, Vol 16 Trees III Springer-Verlag. Berlin, pp.164-179

Reddy AS (2001) Calcium: silver bullet in signaling. Plant Sci 160:381-404

Reed BM, DeNoma J, Luo J, Chang Y, Towill L (1998) Cryopreservation and long-term storage of pear germplasm. In Vitro Cell Dev Biol - Plant 34:256-260

Reed BM, Wada S, DeNoma J, Niedz RP (2013a) Improving in vitro mineral nutrition for diverse pear germplasm. In Vitro Cell Dev Biol - Plant 49:343-355

Reed B M, Wada S, DeNoma J, Niedz RP (2013b) Mineral nutrition influences physiological responses of pear in vitro. In Vitro Cell. Dev. Biol - Plant 49:699-709

SAS (2012) Users Guide statistics VEe.9.4, SAS Institute Inc, Cary, North Carolina

Schroeder JI, Allen G, Hugouvieux V, Kwak JM, Waner D (2001) Guard cell signal transduction. Annu Rev Plant Physiol Plant Mol Biol 52:627-658

Wada S, Niedz RP, DeNoma J, Reed BM (2013) Mesos components ($CaCl_2$ $MgSO_4$, $KH_2PO_4$) are critical for improving pear micropropagation. In Vitro Cell Dev Biol - Plant 49:356-365

# CHAPTER 2

## Improved rooting of a selected ecotype of *Amelanchier alnifolia*

## ABSTRACT

Shoots of serviceberry, *Amelanchier alnifolia* Nutt., propagated in tissue culture often fail to form roots readily. In vitro cultured shoots from a selected dwarf ecotype were examined for their ability to form roots when the basal salt concentration was adjusted or different plant growth regulators were used in the medium. A range of concentrations of Murashige and Skoog (MS) salts were tested (full, ½, ¼ or ⅛ strength). In addition, the plant growth regulators indole-3-butyric acid (IBA) or naphthaleneacetic acid (NAA) at concentrations of 0, 0.5, 1, 5, or 10 µM were tested for their ability to induce root formation. In another study, ½ strength MS medium was supplemented with 0.5 µM IBA, 1.6 mg $l^{-1}$ thiamine and 150 mg $l^{-1}$ FeEDDHA in an effort to improve root formation. The ⅛ strength MS treatment induced 38% of the shoots to form roots, whereas roots failed to form on shoots grown on full strength MS medium. The mean number of roots per responding shoot was 1.6. Indole-3-butyric acid and NAA concentrations induced root formation on full strength MS medium. The best rooting was achieved with 10 µM IBA or 10 µM NAA, and the percentage of shoots forming roots was 33% for IBA-treated and 67% for NAA-treated shoots. The mean number of roots per responding shoot were 6.1 and 2.5 for 10 µM IBA- and 10 µM NAA-treated shoots, respectively. The rooting percentage increased to 87% on ½ MS medium supplemented with 0.5 µM IBA with thiamine and FeEDDHA. Optimizing shoot multiplication media by adding mesos

compounds ($CaCl_2$, $KH_2PO_4$ and $MgSO_4$) to improve shoot quality before root formation helped to increase the rooting percentage to 100%. Although altering salt or auxin concentrations improved rooting response of the serviceberry shoots, the best treatment to induce root formation was ½ strength MS with 0.5 μM IBA with 1.6 mg $l^{-1}$ thiamine and 150 mg $l^{-1}$ FeEDDHA.

## INTRODUCTION

Some woody species are difficult to root when propagated by tissue culture; serviceberry is one of them. Saskatoon serviceberry is native to North America, extending from Manitoba to the Fraser River Canyon, and from the northwestern United States to the Yukon Territory (Pruski et al. 1991). This species can be used in the landscape as a large deciduous shrub, small tree or even a dwarf plant growing from 1 to 8 m (3 to 26 ft). It is also known for its tolerance of dry sites. As part of a native plant domestication project, a dwarf selection of this species has been made. To retain the superior genotype of this selection, asexual propagation is required (Polisetty et al.1996). Axillary shoot proliferation is the best tissue culture technique for true-to-type reproduction (Trigiano and Gray 2011).

Changing MS medium strength can help with root formation on stem explants in tissue culture. Reducing concentrations of basal salts can promote root formation (Polisetty et al. 1996). Activated charcoal can also be added to the medium to help root formation on shoot explants for some species (Thomas 2008). Plant growth regulators, such as cytokinin and auxin can affect organogenesis, and changing the levels of plant growth regulators can induce shoots to form roots. For Dendrobium orchid, the number of roots per plantlet was the highest (2.2 roots per plantlet) with 1

μM IBA followed by 2 μM IBA (1.6 roots per plantlet) after 40 days of culture (Rafique et al. 2012). Since IBA is a rooting hormone, many growers apply IBA rooting solutions to propagate annual, perennial and woody plants (Kroin 2014). Some cultivars are more difficult to micropropagate and may be difficult to establish, multiply or elongate in culture without changing the mineral nutrients (Yeo and Reed 1995, Nas and Read 2001). At one time, micropropagation of pear rootstocks was abandoned due to poor growth in vitro. Development of specialized pear media allowed propagation of all types of pears in vitro. Many rootstock selections now have excellent growth and multiplication as a result of optimizing mineral nutrient concentrations (Reed et al. 2013a, Reed et al. 2013b). Many factors affect root development in vitro. Only healthy explants and proper culture medium produced asparagus plantlets that could be grown successfully in a greenhouse (Ren et al. 2012).

Serviceberry shoots can be readily reproduced by axillary shoot proliferation, but they are difficult to root in vitro. Several possible additions or changes made to culture media may enhance rooting of this recalcitrant species. Murashige and Skoog medium used for axillary shoot proliferation was tested from full strength to ⅛ strength. Indole-3-butyric acid is the most common plant growth regulator used for inducing root formation (Ricardo et al. 2015). Iron is an essential micronutrient for plant tissue culture media. Most iron is complexed to chelating agents, such as EDTA, EDDHA and DTPA, which are absorbed as non-complexed ions by plant roots (George et al. 2008). Two different potential iron sources diethylenetriaminepenta acetic acid (FeDTPA), which is the typical iron source in

tissue culture media, or ethylenediamine-N, N'-bis (2-hydroxyphenylacetic acid) (FeEDDHA) can be used with changes to auxin and salt concentrations to promote root formation. The objective of this study was to induce root formation on serviceberry shoots by adjusting the basal salt concentration, changing plant growth regulator concentrations, or adding chelated iron.

## MATERIALS AND METHODS

In this study, a serviceberry ecotype, *Amelanchier alnifolia* Nutt., selected for its dwarf growth habit (the plant is 10 years old and about 1 meter tall) was cloned via micropropagation. Stems of this specimen plant were obtained from Dr. Stephen Love, University of Idaho, Aberdeen R & E Center, Aberdeen, ID. Single-node stem explants were made from the stems and used to establish cultures. In vitro shoots without roots fro m Stage II of micropropagation were used to determine the effects of in vitro rooting treatments on MS media. These shoots were grown on MS medium (*Phyto*Technology Laboratories, Shawnee Mission KS, product M524) that contained 4.3 g $l^{-1}$ mineral salts and 5.9 μM thiamine-HCl, 8.1 μM nicotinic acid, 4.9 μM pyridoxine-HCl, 53.3 μM glycine, 100 mg $l^{-1}$ myo-inositol, 30 g $l^{-1}$ sucrose, and 4.4 μM BA, solidified with 7 g $l^{-1}$ agar (*Phyto*Technology Laboratories, product A111), and had a pH 5.7.

The media were dispensed into GA7 vessels and autoclaved at 120ºC for 20 min. Six stem explants were placed in each vessel. Four replicate vessels were used for each treatment. Cultures were incubated in a SG 30S germinator (Hoffman Manufacturing Inc., Albany, OR) at 25 ± 1ºC under a 16-h photoperiod (cool-white fluorescent lamps), at 38 μmol $m^{-2}$ $s^{-1}$ photosynthetic photon flux (PPF). Different

strengths of MS medium, full strength salts (4.3 g $l^{-1}$), ½ strength (2.15 g $l^{-1}$), ¼ strength (1.075 g $l^{-1}$) and ⅛ strength (0.5375 g $l^{-1}$) were tested in the first experiment. Although the salt concentrations varied, full strength MS vitamins and myo-inositol were used in all media in this experiment. In the second experiment, different concentrations of growth regulators IBA or NAA or NAA combined with 2 μM benzyladenine (BA) were tested for their effects on root initiation. The concentrations of IBA or NAA were (0, 0.5, 1, 5 or 10 μM).

Shoots grown on MS multiplication media with two different concentrations of mesos minerals ($CaCl_2$, $KH_2PO_4$ and $MgSO_4$) were tested for their ability to form roots on MS medium that included different concentrations (0, 0.5, 1, 5, 10 μM) of NAA. One shoot multiplication medium included full strength MS medium supplemented with 0.398 g $l^{-1}$ $CaCl_2$, 0.054 g $l^{-1}$ $MgSO_4$ and 0.102 g $l^{-1}$ $KH_2PO_4$ (referred to hereafter as mesos treatment 1). A second shoot multiplication medium included full strength MS medium supplemented with 0.132 g $l^{-1}$ $CaCl_2$, 0.054 g $l^{-1}$ $MgSO_4$ and 0.204 g $l^{-1}$ $KH_2PO_4$ (referred to hereafter as mesos treatment 2). Both modified shoot multiplication media contained 4.4 μM BA.

In another experiment, two different iron sources (150 mg $l^{-1}$ of FeDTPA or FeEDDHA) were combined with 1.6 mg $l^{-1}$ thiamine and 0.5 μM IBA added to the full or ½ strength MS medium and tested for their ability to induce root formation. The ½ strength MS medium included half the MS salts and half of the MS vitamins and myo-inositol. In another part of this study, shoots grown on MS multiplication media with two different concentrations of mesos minerals (mesos treatment 1 or mesos treatment 2) were tested for their ability to form roots on FeEDDHA supplemented

medium. The shoots were grown for three weeks during each of three subcultures. After the third subculture, shoots were transferred to full or ½ strength MS that included 0.5 μM IBA, 150 mg $l^{-1}$ FeEDDHA and 1.6 mg $l^{-1}$ thiamine. Also, some shoots were transferred to ½ strength MS that included 0.5 μM IBA, 150 mg $l^{-1}$ FeEDDHA and 1.6 mg $l^{-1}$ thiamine and mesos minerals (mesos treatment 2) were added to the rooting medium.

Data analyses:

Six shoots were used in each of four replicate vessels per treatment. The GA7 vessels were arranged in a randomized complete block design with one replicate vessel per treatment placed on one shelf in the Hoffman germinator. Data taken in the first and second experiments included the percentage shoots forming roots and mean number of roots per responding shoot. Mean length of the longest root formed on each shoot was also measured in the second experiment. In the third experiment, shoot height, number of roots per responding shoot, length of longest root, root percentage and shoot dry weights were determined. Shoot dry weight was determined by drying the shoots in a 70°C oven for at least three days. Growth parameters for the shoots in each jar were averaged, and the means were analyzed by analysis of variance mixed model procedure (Proc Mixed, SAS 2012). One-way ANOVA was used to analyze data from the MS concentration experiment and iron treatment experiment, whereas growth data from the auxin concentration experiment were analyzed by using two-way ANOVA. For analyses involving the auxin treatments, if the interaction between the growth regulators and their concentrations was significant, then effects of the growth regulator concentrations

were tested for each auxin separately. Significant differences between treatment means were determined by least-square means at the 5% level when comparing plant growth differences of explants placed on different media. Single degree-of-freedom contrasts were used to determine the effects of MS medium concentrations on the growth parameters in the iron treatments experiment.

## RESULTS

In the first experiment that involved different levels of MS salts, roots failed to form on shoots placed on full strength MS medium even though the shoots appeared to be relatively healthy. Roots also failed to form on shoots in half strength medium. Roots formed on shoots placed in ¼ and ⅛ strength media, and both treatments induced on the same mean number of roots per responding shoot (Table 2.1). In contrast to those on full strength medium, shoots placed on ⅛ strength medium appeared discolored and stressed with some leaves turning brown.

In the second set of experiments, different concentrations of plant growth regulators (NAA + BA, IBA or NAA) were tested. All shoots failed to form roots when a range of different concentrations of NAA (0, 0.5, 1, 5 or 10 μM) were combined with 2 μM BA. Almost all the shoots (22 of 24 shoots) formed callus at their base except for 0 NAA treatments. In another study, MS medium was supplemented with 0, 0.5, 1, 5 or 10 μM IBA or NAA. Since the interaction between auxin type and concentration was quantitative for rooting percentage (P = 0.0408) and mean number of roots per responding shoot (P = 0.0001), the effects of IBA or NAA concentrations on rooting were analyzed separately. For shoots treated with IBA,

**Table 2.1**: Rooting response of serviceberry shoots on different concentrations of MS medium from full to ⅛ strength. Four vessels were used per treatment, and within each vessel six shoots were averaged before analysis.

| MS strength | Percentage rooting | Mean number of roots per responding shoot |
|---|---|---|
| full | 0 a* | 0 a |
| 1/2 | 0 a | 0 a |
| 1/4 | 21 b | 1.6 b |
| 1/8 | 38 b | 1.6 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 = 4).

the highest percentage of shoots that formed roots was in the medium that contained 0.5 µM IBA (Table 2.2). Mean length of the longest root decreased 48% as IBA concentration increased to 10 µM (Figure 2.1), but the mean number of roots per responding shoot was highest when 10 µM IBA was included in the culture medium (Table 2.2).

Different concentrations of NAA also affected in vitro root formation by serviceberry shoots. Roughly one-third of the shoots formed callus at their bases for all NAA concentrations in this experiment. Ten µM NAA induced 67% of the serviceberry shoots to form roots, yet the most roots formed per shoot when the medium contained 1µM NAA (Table 2.2). The mean length of the longest root increased 32% with increasing concentration of NAA (Figure 2.1).

**Table 2.2**: Effects of IBA or NAA in different concentrations (0, 0.5, 1, 5 or 10 µM) on rooting response by serviceberry shoots. Four vessels were used per treatment, and within each vessel six shoots were averaged before analysis.

| Growth regulator concentration (µM) | Percentage rooting | | Mean number of roots per responding shoot | |
|---|---|---|---|---|
| | IBA | NAA | IBA | NAA |
| 0 | 0 a* | 0 a | 0 a | 0 a |
| 0.5 | 42 b | 25 c | 2.9 c | 2.9 c |
| 1 | 25 b | 13 b | 1.7 b | 3.5 d |
| 5 | 29 b | 37 c | 3.1 c | 2.8 bc |
| 10 | 33 b | 67 d | 6.1 d | 2.5 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 = 4).

Shoots grown on different concentrations of $CaCl_2$, $KH_2PO_4$ and $MgSO_4$ (mesos treatments 1 or 2) during Stage II were placed on MS medium with different concentrations (0, 0.5, 1, 5 or 10 µM NAA). Shoots receiving either mesos treatment rooted at similar percentages to regular MS treated shoots receiving up to 10 µM NAA (data not shown), but neither the mesos shoot treatments nor NAA treatments affected the mean number of roots per responding shoot (P = 0.0726).

In the third experiment, full or ½ strength MS medium with different combinations of FeEDDHA and 0.5 µM IBA affected root formation on serviceberry shoots (P < 0.0001). The most effective root-inducing treatment was half strength MS medium with 0.5 µM IBA combined with FeEDDHA, which produced 3.4 roots formed per responding shoot (Table 2.3). Adding the additional FeEDDHA to the half strength MS medium with 0.5 µM IBA increased the root percentage to 87%,

which was significantly higher compared to treatments of full or half strength MS medium containing 0.5 µM IBA without iron (Table 2.3). In addition, shoot dry weight was significantly affected (P = 0.0070) by the strength of the MS salts. Full strength MS medium with 0.5 µM IBA and additional FeEDDHA had the highest shoot dry weight (0.057 mg) compared to other treatments (Table 2.3). Full strength and half strength MS medium lacked an effect on shoot height and mean length of the longest root (P = 0.9220 and P = 0.5703, respectively). In contrast, more roots formed on each responding shoot when ½ strength MS salts were used in the medium compared to full strength salts (P = 0.0075).

**Figure 2.1:** Effects of different IBA or NAA concentrations on the mean length of the longest root formed on serviceberry shoots after 4 weeks on MS medium supplemented with an auxin. Data are means of three roots per treatment. Due to a qualitative interaction between auxin type and concentration, the effects of IBA or NAA concentrations were analyzed separately. Four vessels were used per treatment, and within each vessel six shoots were averaged before analysis. The error bars represent ± standard error.

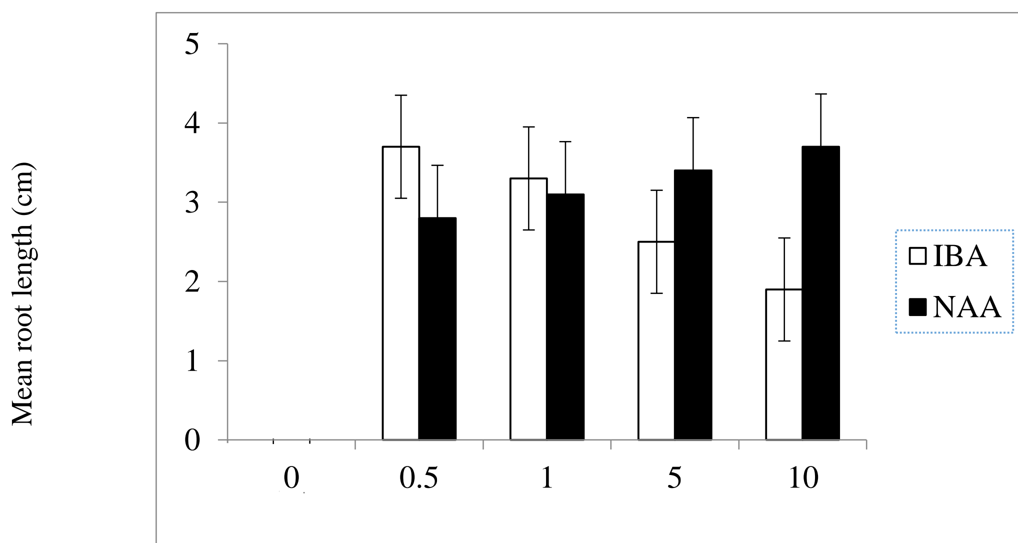


**Table 2.3:** Effect of using two levels of MS medium (1/2 or full strength), on the mean number of roots, mean shoot height and mean shoot dry weight, mean length of the longest root and mean rooting percentage during root formation by serviceberry shoots in vitro. These shoots were grown on MS medium for 4 weeks. Iron FeEDDHA at 150 mg $l^{-1}$ was used as an additional source of Fe in the media. Four vessels were used per treatment, and within each vessel six shoots were averaged before analysis.

| Treatments | | | Shoot height (cm) | Shoot dry weight (g) | Number of roots per responding shoot | Length of longest root (cm) | Percentage rooting |
|---|---|---|---|---|---|---|---|
| MS Conc | Fe | IBA (0.5 µM) | | | | | |
| 1/2 | - | - | 1.0 a | 0.017 a | 0 a | 0 a | 0 a |
| 1/2 | - | + | 1.0 ab | 0.018 a | 2.8 b | 1.5 b | 33 b |
| 1/2 | + | - | 1.1 b | 0.015 a | 0 a | 0 a | 0 a |
| 1/2 | + | + | 1.7 cd | 0.038 bc | 3.4 b | 3.7de | 87 c |
| Full | - | - | 1.0 ab | 0.016 a | 0 a | 0 a | 0 a |
| Full | - | + | 1.1 ab | 0.027 ab | 2.6 b | 0.4 ab | 25 ab |
| Full | + | - | 1.1 ab | 0.015 a | 0 a | 0 a | 0 a |
| Full | + | + | 1.5 c | 0.057 d | 2.1 b | 2.8 cd | 54 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 = 4).

Two different sources of iron, 150 mg $l^{-1}$ of FeDPTA or FeEDDHA, were added to ½ strength MS medium plus 1.6 mg thiamine with 0.5 µM IBA to compare their effects on shoot growth and root formation. Shoot height, shoot dry weight, number of roots per responding shoot, length of the longest root and rooting percentage were significantly enhanced by the addition of FeEDDHA compared the FeDTPA (Table 2.4). Adding FeEDDHA to the ½ strength MS medium including 0.5

μM IBA increased the number of roots by almost 2 fold, and the root percentage was 87% compared to the FeDTPA-treated shoots that formed 1.9 roots per responding shoot with only 29% of the shoots forming roots. In addition, the longest root on each responding shoot was significantly longer (3.7 cm) when using FeEDDHA compared to FeDTPA-treated shoots (mean of the longest root was 1.0 cm). Shoot height and shoot dry weight also improved with FeEDDHA in the medium, and the shoots were 54% taller and produced twice as much dry weight compared to the mean height and dry weight of FeDTPA-treated shoots.

Serviceberry shoots that received mesos treatment 1 or 2 during Stage II were placed on full or half strength MS medium containing FeEDDHA and IBA to determine if they had an improved rooting response. Enhancements of Stage II shoot multiplication medium increased the rooting percentage to 100% during Stage III for shoots that received mesos treatment 1 or mesos treatment 2 for nine weeks (three subcultures). The rooting percentages increased to 100% for shoots receiving either mesos treatments. In addition, the number of roots per responding was 4.7 or 3.6 for mesos treatments 1 or 2, respectively. When mesos treatment 2 shoots were transferred to full strength MS medium with FeEDDHA and 0.05 μM IBA, shoot dry weight increased to 0.053 g and the rooting percentage was 75% (Table 2.4).

**Table 2.4:** Effect of using different iron treatments or shoots with improved mineral nutrition placed on MS medium with 0.5 µM IBA on the mean shoot height, mean shoot dry weight, mean number of roots per responding shoot, mean length of the longest root and mean rooting percentage during root formation by serviceberry shoots in vitro. The shoots were grown on MS rooting medium for 4 weeks. Either 150 mg $l^{-1}$ FeEDDHA or FeDTPA were added to MS medium. Four vessels were used per treatment, and within each vessel six shoots were averaged before analysis.

| Treatments | | Shoot height (cm) | Shoot dry weight (g) | Number of roots per responding shoot | Length of the longest root (cm) | Percentage rooting |
|---|---|---|---|---|---|---|
| MS conc | Fe | | | | | |
| ½ MS | FeEDDHA | 1.7 a[w] | 0.038 bc | 3.4 b | 3.7 b | 87 b |
| ½ MS | FeDTPA | 1.1 a | 0.019 a | 1.9 a | 1.0 a | 29 a |
| ½ MS[x] | FeEDDHA | 2.0 a | 0.042 bcd | 4.7 c | 3.1 b | 100 b |
| ½ MS[y] | FeEDDHA | 1.8 a | 0.039 bc | 3.6 b | 3.4 b | 100 b |
| Full MS[z] | FeEDDHA | 1.9 a | 0.053 cd | 3.4 b | 4.8 c | 75 b |

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

[X] shoots grown on MS medium included additional mesos treatment 1 and 4.4 µM BA for three weeks for three subcultures, then the shoots were transferred to ½ strength MS medium that included 0.5 µM IBA + FeEDDHA and extra 1.6 mg $l^{-1}$ thiamin.

[Y] shoots grown on MS medium included mesos treatment 2 and 4.4 µM BA for three weeks for three subcultures, then the shoots were transferred to ½ strength MS medium that included 0.5 µM IBA + FeEDDHA and extra 1.6 mg $l^{-1}$ thiamin with mesos treatment 2 minerals added to the rooting medium.

[Z] shoots grown on MS medium included mesos treatment 2 and 4.4 µM BA three weeks for three subcultures, then the shoots transferred to full strength MS medium that included 0.5 µM IBA + FeEDDHA and extra 1.6 mg $l^{-1}$ thiamin.

# DISCUSSION

In this study, using either ¼ or ⅛ strength MS medium induced shoots to form roots, whereas shoots treated with full strength MS medium failed to form roots. Even though reducing MS salt concentration to ⅛ or ¼ strength helped shoots to form roots, the shoots appeared unhealthy and appeared to have mineral deficiencies. Different strengths of MS medium can be used for obtaining root formation by shoots from different species. Half or quarter strength MS medium are better for in vitro rooting of chickpea shoots than full strength MS medium (Polisetty et al. 1996). Fade et al. (2010) found that inorganic salt concentration affects in vitro root organogenesis of spearmint (*Mentha spicata* L.). Roots formed after two weeks on ½ strength MS medium, and the highest number of shoots and roots induced per explant (3.5 and 10, respectively) was on ½ strength medium supplemented with 0.2 mg $l^{-1}$ NAA and 1 mg $l^{-1}$ kinetin. During the rooting stage, ½ strength MS medium produced double the number of roots compared to the full strength MS medium (Fade et al. 2010).

In this study with a selected dwarf serviceberry ecotype, the application of high concentrations (up to 10 μM) IBA or NAA to micropropagated shoots helped induce root formation, with 10 μM NAA inducing 67% of the shoots to form roots, which was double the percentage of 10 μM IBA treated shoots (Table 2.2). In addition, NAA increased the mean length of the longest root per shoot as the concentration increased to 10 μM (Figure 2.1). In vitro shoots of *Aegle marmelos* (L.) Corr were transferred to root induction medium with half-strength MS supplemented with IAA, IBA, or NAA, and the best treatment was 14.7 μM IBA.

Rooted plantlets were acclimatized and transferred to the field with 80% survival rate (Nayak et al. 2007).

In general, NAA seemed to be the better auxin to promote root formation on the selected serviceberry ecotype since fewer IBA treated shoots formed roots (Table 2.1), and the longest roots were shorter than those on NAA treated shoots (Figure 2.1). Although, different concentrations of NAA and IBA promoted root formation on the serviceberry shoots, and the mean number of roots formed per responding shoot for shoots receiving 10 μM IBA was double that of shoots treated with 10 μM NAA. Since the mean length of the longest root on IBA treated shoots decreased as the concentration increased, the high IBA concentrations seemed to show an overdose effect (Figure 2.1). In general, serviceberry shoots treated with NAA induced a higher percentage of shoots to form roots than IBA. A higher concentration of IBA was reported to reduce root number and root length of woody plants (George et al. 2008). For Dendrobium orchid, the highest number of roots per explant was found on MS medium with 1 μM IBA followed by 0.5 μM or 1.5 μM IBA. Roots formed in 11 days on the lower concentration of IBA, which proved that low concentrations promoted root formation (Rafique et al. 2012). Although, 10 μM NAA improved root formation by serviceberry shoots, the percentage of rooting was still only 67%, so additional experiments were completed to improve the rooting percentage.

Adding iron (FeEDDHA) to ½ strength MS medium marginally increased the number of roots per responding shoot, but the percentage of rooted shoots increased over 2.5 fold (Table 2.3). In addition, roots formed in 10 days after shoots

were placed on FeEDDHA rooting medium compared to roots forming in 20 days when placed on medium with auxin alone. This added Fe source also helped to improve shoot quality (shoot height and shoot dry weight) compared to MS medium with only 0.5 μM IBA (Table 2.3) or FeDTPA (Table 2.4). These results indicated that the form in which iron was supplied was clearly important. Similar results with FeEDDHA have been obtained for *Coryllus* shoots (Nas and Read 2001) and *Rosa* shoots (VanDer Salm et al.1994). The beneficial effects of FeEDDHA also included improving the rooting response by increasing the percentage of rooted microshoots, the number of roots per explant and the length of the longest root on the serviceberry shoots (Table 2.4).

The form of the iron complex is important for in vitro culture of many species, and FeEDDHA is more beneficial for some species than FeEDTA. When FeEDTA was replaced by FeEDDHA for walnut, micropropagation shoot quality improved, as seen by taller and healthier microshoots (better foliage color) (Ricardo et al. 2015). The average shoot height of an iron-sensitive walnut genotype treated with FeEDDHA was over double the height of shoots on medium supplemented with FeEDTA (compare 3.9 cm versus 1.5 cm). In the present study with serviceberry, the average length of shoots on the FeEDDHA medium was 1.7 cm, but shoots on FeDTPA were 1.1 cm, 35% shorter. Mean length of the longest root was 3-fold longer for FeEDDHA treated shoots compared to the FeDTPA treated shoots (Table 2.4). Skoog and co-workers began to use FeEDTA in media for tobacco callus cultures in 1956 and discussed their findings in the same paper (Murashige and Skoog 1962).

Although most iron complexed to chelating agents, such as FeEDTA and FeEDDHA, is absorbed as non-complexed ions by plant roots, chelating agents can be taken up into plant tissue (Weinstein et al. 1951; Tiffin et al. 1960). Growth and morphogenesis in tissue cultures have been noted to be influenced by chelating agents. The physiological effect of FeEDTA and FeEDDHA as chelating agents was clearly different as found by Chopra and Rashid (1969) who observed that the moss *Anoectangium thomsonii* failed to form buds as other mosses do, when grown on a simple medium containing ferric citrate or FeEDTA, but did so when 5 to 20 mg $l^{-1}$ FeEDDHA was added to the medium instead. For tissue culture of rose (VanDer Salm et al 1994), *Prunus* (Mallosiotis et al. 2003), citrus (Dimassi et al. 2003) and red raspberry (Zawadzka and Orlikowska 2006) shoots, use of FeEDDHA rather than FeEDTA was advantageous for root formation. Additional thiamine 1.6 mg $l^{-1}$ added to the basal medium may affect root formation on the selected serviceberry ecotype. Many media formulations include trace amounts of certain organic compounds, such as vitamins and plant growth regulators, to improve growth.

Using EDTA in the medium can also cause toxicity problems for in vitro cultures. High levels of EDTA added to MS medium clearly inhibited and prevented endive shoot formation (Legrand 1975). Likewise, Dalton et al. (1983) found that 0.3 mM EDTA compared to the 0.1 mM in MS medium reduced the growth rate of *Ocimum*. In vitro root growth of wheat in the dark was inhibited by EDTA (Burstrom 1961). Chelating agents act as auxin synergists by isolating Ca from the cell wall (Thimann and Takahashi 1958). Chelating agents, such as EDTA, may interact with metabolism and transport of endogenous auxin. Also, EDTA acts like polyphenols in

depressing the decarboxylation of IAA (Tomaszewski and Thimann 1966). The FeEDDHA may have had a similar role in preventing auxin degradation, resulting in improved serviceberry root formation.

Some organic compounds are able to form complexes with metal cations in which the metal is held with fairly tight chemical bonds. This complex formation is called a chelate. Some complexes can be more chemically reactive than the metals themselves. For example, $Cu^{2+}$ complexed with amino acids is more active than the free ion (Cruickshank et al.1987). Complexes have a higher stability than the free ions of $Cu^{2+}$, $Ni^{2+}$, $Al^{3+}$, $Zn^{2+}$, $Fe^{2+}$ or $Ca^{2+}$. Metals in very stable complexes can be unavailable to plants; copper in EDTA chelate is an example (Coombes et al 1977). In plants, iron is used in the chloroplasts, mitochondria and peroxisomes, affecting oxidation reduction (redox) reactions. Iron is also a component of ferredoxin proteins, which function as electron carriers in photosynthesis, and it is an essential micronutrient for plant tissue culture media. When $Fe^{+2}$ and $Fe^{+3}$ ions escape from the chelating agent, they can precipitate as iron phosphate, and they may be unavailable to plant cells. Iron present in the form of ferric sulfate is much less effective than iron in chelated form (George et al. 2008). Street et al (1952) found that iron in chelated form was less toxic and could be utilized by in vitro culture.

The rooting results comparing the two iron sources in this study were surprising in that the FeDTPA treated shoots produced fewer roots per shoot and fewer shoots rooted compared to FeEDDHA treated shoots (Table 2.4). These results were unexpected since FeEDDHA is preferentially used to enhance plant growth in alkaline and calcareous soils with a pH greater than 7, whereas FeDTPA

should be used in slightly acid soils (BASF Corporation 2013). Since the pH of the rooting medium was 5.7, FeDTPA was expected to provide a better rooting response than the FeEDDHA supplemented medium. Perhaps the FeEDDHA was a better auxin synergist than the FeDTPA or the FeEDDHA was more stable and provided more iron to the shoots than the FeDTPA. Additional studies would be needed to determine a clear role for the root promoting effects of the FeEDDHA treatment.

In vitro shoots of the selected serviceberry ecotype on MS medium appeared to have slight to moderate mineral deficiencies based on their leaf color. By adjusting the shoot multiplication medium before the rooting stage, perhaps improved shoot quality would lead to increased root formation. For healthy and vigorous growth, plants need balanced amounts of some inorganic elements (N, K, Ca, P, Mg and S) and small quantities of other elements (Fe, Ni, Cl, Mn and Zn) (George et al 2008). According to Epstein (1971), a plant fails to complete its life cycle without esential elements. The most commonly used tissue culture medium is MS medium (Murashige and Skoog 1962). This medium was developed for optimal growth of tobacco callus, and medium development involved a large number of dose response curves for the various essential minerals. Murashige and Skoog medium has low levels of Ca, P and Mg. Each species has the ability to adapt and acclimate to a medium formulation. Using shoots grown on MS medium that included additional mesos components and 4.4 μM BA in multiplication stage before use in the root formation stage helped to increase the rooting percentage (Table 2.4), and the shoots appeared healthier (dark green leaves).

The FeEDDHA treatment appeared to provide some essential component needed for improving the rooting percentages of serviceberry stem explants. For example, optimizing the shoot multiplication medium to obtain better quality shoots by itself was insufficient to improve rooting percentages on NAA supplemented media (data not shown). However, optimizing MS medium and improving shoot quality before use in the root formation stage seemed to promote root formation and increased the root percentage to 100% when FeEDDHA was included in the medium (Table 2.4). Both types of mesos treated shoots formed roots, but the percentages were statistically similar to non-mesos treated shoots. Improving shoot nutrition via mesos treatment was worthwhile because 100% of the shoots rooted and resulted in highly efficient plantlet production. In a study that used high concentrations of $CaCl_2$ and $KH_2PO_4$, Poothong and Reed (2015) found that these minerals increased shoot length for most cultivars of red raspberries, their results demonstrated that mesos components ($CaCl_2$, $MgSO_4$, and $KH_2PO_4$) significantly improved raspberry (*Rubus idaeus* L.) shoot quality and growth. In addition, optimizing the growth medium for shoot production would provide better commercial production. The addition of mesos to tissue culture media during Stage II can help to make macro- and micronutrients more accessible to plant cells.

## CONCLUSION

Plant propagation success in tissue culture is influenced by the nature of the culture medium used. Shoots from some cultivars or clones of woody plants, such as *Amelanchier alnifolia*, can be difficult to root. One-eighth strength MS medium induced some root formation but resulted in poor shoot growth. Shoots formed roots

with 10 µM IBA or 10 µM NAA, yet the percentage of shoots that formed roots was below 70% when either auxin was used. The best rooting of serviceberry shoot cultures was achieved by reducing MS medium to ½ strength and adding 150 mg $l^{-1}$ FeEDDHA and 1.6 mg $l^{-1}$ thiamine with 0.5 µM IBA. Improving shoot quality by optimizing the multiplication medium (by adding mesos minerals) increased the root percentage to 100%, and the shoots looked healthier and roots formed quickly, within 10 days.

## REFERENCES

BASF Corporation (2013) Sequestrene® 138 Fe Sequestrene® 330 Fe High performance iron chelates for foliar and soil applications. http://m.agproducts.basf.us/products/research-library/sequestrene-tech-sheet.pdf. Accessed 28 March 2016

Burstrom H (1961) Growth action of EDTA in light and darkness. Physiol Plant 14:354-377

Chopra RN, Rashid A (1969) Auxin-cytokinin interaction in shoot-bud formation of a moss, *Anoectangium thomsonii* Mitt. Z Pflanzenphysiol 61:192-198

Coombes AJ, Phipps NW, Lepp NW (1977) Uptake patterns of free and complexed copper in excised roots of barley (*Hordeum vulgare* L. var. Zephyr). Z Pflanzenphysiol 82:435-439

Cruickshank IAM, Dudman WF, Peoples MB, Smith MM (1987) Elicitation of pisatin in pea (*Pisum sativum* L.) by copper-asparagine complexes. Aust J Plant Physiol 14:549-559

Dalton CC, Iqbal K, Turner DA (1983) Iron phosphate precipitation in Murashige and Skoog media. Physiol Plant 57:472-476

Dimassi K, Chouliaras V, Diamantidis G, Therios I ( 2003) Effect of iron and auxins on peroxidase activity and rooting performance of three citrus rootstocks *in vitro*. J Plant Nutr 26:1023-1034

Epstein E (1971) Mineral Nutrition of Plants. Principles and Perspectives. John Wiley and Sons Inc., New York.

Fade D, Kintzios S, Economou AS, Moschopoulou G, Constantinidous HA (2010) Effect of different strength of medium on organogenesis, phenolic accumulation and antioxidant activity of spearmint (*Mentha spicata* L.). Open Horticulture Journal 3:31-35

George EF, Hall MA, Deklerk GJ (2008) Plant propagation by tissue culture. 3rd Edn, Springer, Dordrecht, The Netherlands

Kroin J (2014) Development and application of foliar applied rooting solutions. Hortus USA Corp, New York

Legrand B (1975) Action of iron and EDTA on the neoformation of buds, by the leaf fragments of endive cultivated *in vitro*. Compt Rend Acad Sci Paris 280D: 2215-2218

Mallosiotis AN, Dimassi K, Therios I, Diamantidis G (2003) Fe-EDDHA promotes rooting of rootstoock GF-677 (*Prunus amygdalus* x *P. persica*) explants *in vitro*. Biol Plant 47:141-144

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473-497

Nas MN, Read PE (2001) Micropropagation of hybrid hazelnut: medium composition, physical state and iron source affect shoot morphogenesis, multiplication and explant vitality. Acta Hortic 556: 251-258

Nayak P, Behera PR, Manikkannan T (2007) High frequency plantlet regeneration from cotyledonary node cultures of *Aegle marmelos* (L.) Corr. In Vitro Cell Dev Biol – Plant 3:231-236

Polisetty RP, Patil JJ, Deveshwar S, Khetarpal R, Chandra (1996) Rooting and establishment of in vitro grown shoot tip explants of chickpea. Indian J Exp Biol 34:806-809

Poothong S, Reed B (2015) Increased $CaCl_2$, $MgSO_4$, and $KH_2PO_4$ improve the growth of micropropagated red raspberries. In Vitro Cell Dev Biol - Plant 51*:* 648-658

Pruski K, Mohyuddin M, Grainger G (1991) Saskatoon (*Amelanchier alnifolia* Nutt.) In: Bajaj Y.P.S (Ed), Biotechnology in Agriculture and Forestry, Vol 16 Trees III Springer-Verlag. Berlin, pp.164-179

Rafique R, Fatima B, Mushtaq S, Iqbal MS, Rasheed M, Ali M and Hasan SZU (2012) Effect of Indole-3-butyric acid (IBA) on in vitro root induction in Dendrobium orchid (Dendrobium Sabin H.) African Biotech 20:4673-4675

Reed BM, Wada S, DeNoma J, Niedz RP (2013a) Improving in vitro mineral nutrition for diverse pear germplasm. In Vitro Cell Dev Biol - Plant 49:343-355

Reed BM, Wada S, DeNoma J, Niedz RP (2013b) Mineral nutrition influences physiological responses of pear in vitro. In Vitro Cell Dev Biol - Plant 49:699-709

Ren J, Chen W, Knaflewski M (2012) Factors affecting asparagus (*Asparagus officinalis* L.) root development *in vitro*. Acta Sci Pol Hortorum Cultus 11:107-118

Ricardo J, Moreno L, Contreras A, Morales A, Urban I, Daquinta M, and Gomez L (2015) Improved walnut mass micropropagation through the combined use of phloroglucinol and FeEDDHA Plant Cell Tiss Organ Cult 123:143-154. DoI:10.1007/s11240-015-0822-3

SAS (2012) Users Guide statistics VEe.9.4, SAS Institute Inc. Cary, North Carolina

Street HE, McGonagle MP, McGregor SM (1952) Observations on the ‘staling’ of White’s medium by excised tomato roots. II. Iron availability. Physiol Plant 5:248-276

Thimann KV, Takahashi N (1958) The action of chelating agents on growth of *Avena*. Plant Physiol 33, Suppl., 33

Thomas D (2008) The role of activated charcoal in plant tissue culture. Biotech Adv 26:618-631

Tiffin LO, Brown JC, Krauss RW (1960) Differential absorption of metal chelate components by plant roots. Plant Physiol 35:362-367

Tomaszewski M, Thimann KV (1966) Interactions of phenolic acids, metallic ions and chelating agents on auxin-induced growth. Plant Physiol. 41(9):1443-1454. DOI: 10.1104/pp.41.9.1443.

Trigiano RN, and Gray DJ (2011) Plant tissue culture, development and biotechnology. CRC Press, Boca Raton, Florida

VanDer Salm TPM, VanDer Toorn CJG, Hänish Ten Cate CH, Dubois LAM, DeVries DP, Dons HJM (1994) Importance of the iron chelate formula for micropropagation of *Rosa hybrida* L. 'Moneyway'. Plant Cell Tiss Organ Cult 37:73-77

Weinstein LH, Robbins WR, Perkins HF (1951) Chelating agents and plant nutrition. Science 120:41-43

Yeo DY, and Reed BM (1995) Micropropagation of three *Pyrus* rootstocks. HortScience 30:620-623

Zawadzka M, Orlikowska TK (2006) The influence of FeEDDHA on red raspberry cultures during shoot multiplication and adventitious regeneration from explants. Plant Cell Tiss Organ Cult. 85:145–149. DOI: 10.1007/s11240-005-9063-1

# CHAPTER 3

## Establishment and production of firechalice in plant tissue culture

## ABSTRACT

*Epilobium canum* (Greene) P.H. Raven subsp. *garrettii* (A. Nelson) P.H. Raven, (also known as *Zauschneria garrettii* or firechalice) is an herbaceous wildflower with landscape potential. Lack of seed viability combined with seed dormancy within some ecotypes of this species hinders seed propagation. Plant tissue culture may be the propagation method of choice for rapid commercial-scale reproduction. Single-node stem explants from a selected plant were examined for their ability to establish on Murashige and Skoog (MS) medium or Woody Plant Medium (WPM) during Stage I of micropropagation. Murashige and Skoog medium was found to be the better salt formulation particularly when supplemented with 4.4 μM benzyladenine (BA). During Stage II, different cytokinins, such as BA, kinetin (kin), 6-(γ,γ-dimethlyallylamino)purine (2iP), thidiazuron (TDZ) and meta-topolin (mT), at concentrations of 1.1, 2.2, 4.4 or 8.8 μM were evaluated for shoot proliferation efficacy. Each of the cytokinins tested at 4.4 or 8.8 μM in the medium induced the explants to form numerous shoots and increased shoot dry weight. The highest number of shoots were formed at the highest concentration (8.8 μM) of BA (11.1 shoots) and mT (15 shoots). Benzyladenine, 2iP and kin failed to affect shoot height even at the highest concentrations used, but 4.4 or 8.8 μM TDZ inhibited shoot height compared to control shoots. In Stage III, firechalice rooted easily without auxin added to the medium, but roots failed to form when using

combinations of BA and indole-3- butyric acid (IBA). However, 4 to 6 roots per shoot formed when using up to 9 µM of IBA alone. In contrast, naphthaleneacetic acid (NAA) inhibited root formation as the concentration increased from 3 to 9 µM. When using 0 to 9 µM IBA during Stage III, 82.5% of the rooted shoots survived in Stage IV. Based on these results, optimum in vitro propagation of firechalice may be achieved with shoots established on MS medium plus 4.4 µM BA for Stage I, a concentration of 4.4 or 8.8 µM mT in the medium during Stage II, and use of up to 9 µM IBA during Stage III root induction resulted in successful acclimatization after transplanting.

## INTRODUCTION

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 find limited use in the landscape trade due to seed-based propagation difficulties. Vegetative propagation could provide an alternate tool for commercial production of these plants. Two reasons asexual plant production may be a viable option for reproducing plants are low seed germination rates and slow seedling growth (Unander et al. 1995).

*Epilobium canum garrettii*, (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 (Love et al. 2009). Firechalice spreads as a ground cover, and the flowers are bright orange-red and attractive to hummingbirds (Love et al. 2009). Plants are relatively small, usually 30 to 45 cm tall

and 30 to 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 about 30 to 45 cm tall (Love et al. 2009). Plants 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 an outstanding xeric landscape plant that deserves more attention in the nursery trade.

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 to 8.5).

Various techniques can be used to propagate firechalice. Seeds of this species are difficult to harvest and seeds from some ecotypes germinate poorly, meaning asexual propagation would be an appropriate alternative. This plant can be propagated from stem cuttings with the highest success when using the basal parts of young tender stems without flowers or seed capsules. 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. For plants in general, axillary shoot proliferation is the

best tissue culture technique for true-to-type reproduction (Trigiano and Gray 2011). Plants used in axillary shoot proliferation will undergo the four stages of micropropagation. Stage I is establishment and stabilization of shoot cultures. Stem tissue must be free from microbial contamination, and stem cultures used for further propagation can be obtained by explant establishment under aseptic culture conditions (Einset 1986). The optimum basal medium to use during this stage must first be determined. For example, MS medium, WPM or Driver-Kuniyuki walnut (DKW) medium can be used to establish shoots in vitro.

Stage II involves inducing shoot proliferation by increasing the level of cytokinin in the medium. Cytokinin is an important class of phytohormone and is used in species-appropriate concentrations to multiply and improve plant growth in culture. Explants usually respond to high concentrations of cytokinin and produce many shoots. Einset (1986) reported that one million shoots can be produced from a single growing tip, meaning this stage is critical in establishing a successful micropropagation protocol. Benzyladenine is the most widely used cytokinin in the micropropagation industry, although meta-topolin can be used as an alternative to BA or zeatin (Bairu et al. 2007).

Stage III involves inducing in vitro root formation on shoots (Trigiano and Gray 2011). According to Pierik (1987), most plants require auxins for root regeneration. Shoots from herbaceous plants generally form roots easier than those from trees and shrubs (woody plants). Also, stems from herbaceous plants require low auxin concentrations for inducing root formation compared to stems from woody plants (Pierik 1987). Indoleacetic acid can be used during rooting of stems from

herbaceous plants, but IBA and NAA usually promote the most root formation by stem cuttings. In general, adventitious root formation is inhibited by cytokinins, gibberellins and abscisic acid (Pierik 1987). Stage IV involves acclimatization of the shoot cultures to the outside environment. Clamshell containers can be used as small, interim growth chambers to increase humidity and decrease light intensity before transferring rooted shoots to the greenhouse.

The goal of this research was to develop a micropropagation procedure 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 throughput propagation of the selected clone.

## MATERIAL AND METHODS

A selected firechalice accession, (*Epilobium canum* var. *garrettii*) used for micropropagation was obtained from Dr. Stephen Love, University of Idaho, Aberdeen R & E Center. Aberdeen, ID. The selection was derived from a wild collection made in 2008 near Tony Grove Lake, Cache County, Utah, USA. This plant was selected for upright growth habit, long bloom period, intense red flower color and limited rhizomatous spreading compared to the species norm. Single-node stem explants were taken from rooted cuttings of the selected plant.

Stage I: Explant establishment and stabilization

a. Surface Sterilization

Leaves were removed from stems and approximately 2 cm stem explants that contained a single node were surface sterilized for 20 min in 0.6% (v/v) sodium hypochlorite containing 2 drops of Tween® 20 (a surfactant).

b. Shoot Establishment

Two media formulations 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 (*Phyto*Technology Laboratories, Shawnee Mission KS, product M524) contained 4.3 g $l^{-1}$ mineral salts and 5.9 µM thiamine-HCl, 8.1 µM nicotinic acid, 4.9 µM pyridoxine-HCl, 53.3 µM glycine, 100 mg $l^{-1}$ myo-inositol, and 30 g $l^{-1}$ sucrose, solidified with 7 g $l^{-1}$ agar, (*Phyto*Technology Laboratories, product A111), and had a pH 5.7. The medium included 3.5 µM BA in the first subculture which was increased to 4.4 µM in the second subculture. Woody plant medium (*Phyto*Technology Laboratories, product L449) contained 2.3 g $l^{-1}$ salts, and the same concentrations of thiamine, nicotinic acid, pyridoxine, glycine, and myo-inositol as MS medium and contained 20 g $l^{-1}$ sucrose, was solidified with 7 g $l^{-1}$ agar, was adjusted to pH 5.2 and included 3.5 µM BA. The media were dispensed into GA7 vessels and autoclaved at 120ºC for 20 min. After inoculation, stem cultures were incubated in a SG 30S germinator (Hoffman Manufacturing Inc., Albany, OR) at 25 ± 1ºC under a 16-h photoperiod (cool-white fluorescent lamps),

with 38 μmol $m^{-2}$ $s^{-1}$ photosynthetic photon flux (PPF). After 30 days, the shoot cultures were subcultured (divided) and placed on fresh MS medium or WPM two times before Stage II was initiated. Given that stem explants grew best on the MS salt formulation, this medium supplemented with 4.4 μM BA was used in subsequent experiments directed at optimizing shoot proliferation and root formation in Stages II and III.

Stage II: Shoot multiplication

Studies were conducted to optimize the Stage II medium for rapidly multiplying shoots in preparation for later stages of micropropagation. Selected cytokinins were examined mainly for their effects on the number of axillary shoots produced on stem explants. In the first experiment, 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 kin, 2iP, TDZ, and mT 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. After one month, shoot growth parameters were determined (described below).

Stage III: Root Initiation

In Stage III, combinations of BA and auxin were evaluated for their ability to maintain or improve shoot quality while trying to induce root formation. Firechalice shoots about 1 cm tall were placed on MS medium with various concentrations of BA (0, 1.1, 2.2 or 4.4 μM) combined with various concentrations of IBA at (0, 1 or 2 μM). In a second study, firechalice shoots were placed on MS medium with different concentrations of IBA or NAA (0, 3, 6 or 9 μM). After one month, shoot growth and

rooting parameters were determined (described below). In a third study, 1 cm tall shoots were treated with Rootone® (Smith Industries, Kansas City, MO), Hormex® #3 (Maia Products, Inc. Simi Valley, CA), or distilled water (control) then placed in Sunshine #1 potting mix (Sun Gro® Horticulture, Vancouver, Canada) 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 for a total of 30 shoots per treatment.

Stage IV: Acclimatization

Plantlets, meaning rooted shoots, were placed in clamshell (20 cm x 20 cm x 9 cm) containers to acclimate rooted shoots to conditions outside of the tissue culture vessels. Rooted shoots were used from the Stage III IBA and NAA rooting study. The percentage of plantlets that survived was determined six weeks after transplanting.

Data analyses:

Six shoots were used in each of four replicate vessels per 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 (shelf). Data taken in each experiment for Stage I, II and III included the number of shoots formed (from axillary buds) per stem explant, 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 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 during Stage III. For all Stage I, II and III experiments, growth parameters for the shoots in each vessel were averaged, and the means were analyzed by analysis of variance (mixed model procedure) (Proc Mixed, SAS 2012) for Stage I data and two-way ANOVA when comparing different plant growth regulators used at various concentrations for Stages II and III. For Stage II analyses, cytokinin type and cytokinin concentrations were used as independent variables. For Stage III analyses, auxin type and auxin concentrations were used as independent variables. 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 plant growth differences of explants placed on different media. Survival data from Stage IV were analyzed by ANOVA assuming a Poisson distribution (Proc Glimmix, SAS 2012) since count data were evaluated. Single degree-of-freedom contrasts were analyzed to determine the difference in survival between IBA- or NAA-treated plantlets.

## RESULTS

Stage I Experiments:

For Stage I, single-node stem sections were surface sterilized and placed on either MS medium or WPM. Benzyladenine at 3.5 μM was added to MS and WP media in the first culture. The concentration of BA was increased to 4.4 μM in the second subculture for MS medium. After three subcultures, firechalice plants clearly grew better on MS medium (Table 3.1). By the third subculture, shoots on MS medium

produced 2.6 times more shoots, 2.8 times more shoot dry weight, and grew 2.8 times taller than shoots grown on WPM. Leaf color of shoots grown on MS medium also was greener than the color of leaves on shoots grown on WPM.

**Table 3.1**: Effect of using two different media, MS or WPM, on the mean number of shoots, mean shoot height and mean shoot dry weight during establishment of firechalice shoots in vitro. Plant growth data were from the third subculture of firechalice shoots, and these shoots were grown on MS 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 height (cm) | Shoot dry weight (g) |
|---|---|---|---|
| MS | 12.6 b* | 2.4 b | 0.04 b |
| WPM | 4.9 a | 0.8 a | 0.01 a |

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

By the third subculture, firechalice shoots became stabilized in culture (judged by good shoot color and consistent, regular growth of the shoots), and Stage II experiments involving rapid shoot multiplication were started.

Stage II Experiments:

In this stage, different forms of cytokinin were tested for their ability to induce axillary shoot proliferation. Benzyladenine (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 others. In the next study, the effects of kin, 2iP, TDZ, and mT concentrations from 0 to 8.8 μM on shoot multiplication rates, shoot heights and

shoot dry weights were determined. The two-way analyses revealed significant interactions ($P < 0.001$) between the type of cytokinin used and the concentrations used for all three growth parameters.

Interaction between cytokinin type and concentration were quantitative for the number of shoots and shoot dry weight per explant but were qualitative for shoot height (see results below). Therefore, concentration effects on shoot growth were analyzed separately for each cytokinin.

The two most effective cytokinins for promoting shoot multiplication were BA and mT (Tables 3.2 and 3.3, respectively). A concentration of 8.8 μM BA induced about 11 shoots to form per explant, whereas only 4.4 μM mT was needed to induce 14 shoots per explant (Tables 3.2 and 3.3, respectively). As the concentration of mT increased from 0 to 8.8 μM, shoot height in culture increased significantly by about 1 cm, but BA concentrations failed to affect shoot height (Tables 3.3 and 3.2, respectively). The highest BA concentration (8.8 μM) increased shoot dry weight ~ 2 fold compared to the stems grown without added cytokinin (controls) (Table 3.2). In contrast, 8.8 μM mT increased shoot dry weight by over 3 fold compared to control shoots (Table 3.3). For either BA or mT shoot growth was similar whether 4.4 or 8.8 μM concentrations were used in the media.

The other three cytokinins used in this study had either mixed or detrimental effects on the growth of 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 (lacked cytokinin in the medium), shoots on medium supplemented with the highest concentration of TDZ were 2.5 times shorter than

control shoots (Table 3.4). The highest concentration of TDZ also produced the heaviest shoots (0.126 g, Table 3.4).

**Table 3.2:** Effects of BA concentrations on the mean number of shoots, mean shoot heights, and mean shoot dry weights of firechalice shoots grown on MS medium for 4 weeks. Four vessels were used per treatment, and within each vessel six shoots were averaged before analysis.

| Concentration (µM) | Number of shoots | Shoot height (cm) | Shoot dry weight (g) |
|---|---|---|---|
| 0 | 1.8 a* | 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 |

*Different 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 3.3:** Effects of mT concentrations on the mean number of shoots, mean shoot heights, and mean shoot dry weights of firechalice shoots grown on MS medium for 4 weeks. Four vessels were used per treatment, and within each vessel six shoots were averaged before analysis.

| 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 = 4).

The highest level of kin increased the shoot multiplication rate by over 6-fold compared to the control shoots (Table 3.5), whereas 8.8 μM 2iP increased shoot multiplication rate by only 2.3-fold over control shoots (Table 3.6). Neither kin nor 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 3.5 and 3.6, respectively).

**Table 3.4:** Effects of TDZ concentrations on the mean number of shoots, mean shoot heights, and mean shoot dry weights of firechalice shoots grown on MS medium for 4 weeks. Four vessels were used per treatment, and within each vessel six shoots were averaged before analysis.

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

*Different 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 3.5:** Effects of kin concentrations on the mean number of shoots, mean shoot heights, and mean shoot dry weights of firechalice shoots grown on MS medium for 4 weeks. Four vessels were used per treatment, and within each vessel six shoots were averaged before analysis.

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

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

Stage III Experiments:

The initial discovery in Stage III was that 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

**Table 3.6:** Effects of 2iP concentrations on the mean number of shoots, mean shoot heights, and mean shoot dry weights of firechalice shoots grown on MS medium for 4 weeks. Four vessels were used per treatment, and within each vessel six shoots were averaged before analysis.

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

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

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 stem explant was 2.3 for the control, 2.8, for 1 μM IBA, and 2.4 for 2 μM IBA treatments.

In the second Stage III study, two types of auxins (NAA or IBA) were added to MS medium in an attempt to further stimulate root formation and improve explant condition. An interaction between 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 about 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 versus 1.8 across NAA concentrations.

Two-way analysis of variance for shoot growth and rooting responses showed a significant interaction between the type of auxin used and auxin concentrations; these interactions were qualitative for their effects 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, but auxin concentration lacked significance. Different concentrations of IBA failed to affect shoot height (P = 0.2413), but with NAA shoot heights decreased with higher concentrations (e.g., control shoots were 2.8-fold taller than shoots treated with 9 μM NAA, Table 3.7).

A significant interaction between the type of auxin and concentration affected shoot dry weight. Naphthaleneacetic acid included 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 about 2.4 times heavier than that of control shoots (Table 3.7).

The number of roots formed per responding shoot decreased by at least 36%, and the length of the longest root for the rooted shoots decreased at least 4-fold and 3-fold, respectively, as NAA concentration increased from 0 to 6 μM. Root formation was completely inhibited by 9 μM NAA (Table 3.7).

**Table 3.7:** Effects of different concentrations of IBA and 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 firechalice shoots grown on MS 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 concentrations (µM) | Shoot height (cm) | Shoot dry weight (g) | Number of roots per responding shoot | Length of the longest root (cm) | Percentage rooting |
|---|---|---|---|---|---|---|
| **IBA** | 0 | 3.4 a | 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 |

*Different 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).

The highest IBA concentrations (6 or 9 µM) increased the number of roots per responding shoot, 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 3.7).

Because cultured firechalice shoots rooted easily on MS medium without auxin, rooting powders (Rootone ® and Hormex® #3) were used at the end of Stage II in an attempt to bypass Stage III. 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).

Stage IV Experiments:

In a preliminary Stage IV experiment, rooted shoots from Stage III acclimated well and survived the transition, but non-rooted shoots (treated as microcuttings) failed to root or to form roots and died. Half of the shoots used in the IBA and NAA rooting experiment from Stage III were used in Stage IV to determine the ability of these shoots to acclimate to the natural environment.

Although plantlets from the 6 μM IBA treatment (Stage III) survived the best (93%) in Stage IV, 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%) of rooted shoots treated with 3 μM NAA in Stage III survived transplanting, and all shoots treated with highest concentrations of NAA (6 or 9 μM) failed to survive transplanting.

## DISCUSSION

Murashige and Skoog medium is used extensively and is useful for culturing in vitro plants. This medium contains high concentrations of nitrate, potassium and ammonium relative to other nutrient media (Gamborg et al. 1976). A medium should

contain the correct amounts and proportions of inorganic nutrients, and in this study, MS medium promoted shoot growth better than WPM for firechalice stem explants. These results make sense because MS medium is formulated for herbaceous plants (Pierik 1987) compared to WPM, which is formulated for woody plants.

Different cytokinins were tested for their effects on shoot proliferation since each can uniquely affect different species of plants. In Stage II of micropropagation, high concentrations of cytokinin promoted axillary bud break and overcame apical dominance, as demonstrated by Murashige who was involved in the early development of tissue culture techniques used for propagation (Einset 1986). The optimal BA concentration for *Vitis vinifera* cv. Pinot noir culture establishment was 8.9 µM added to MS basal medium (Heloir et al. 1997).

In Stage II studies with firechalice, mT promoted shoot multiplication the best, even better than BA. This information may prove valuable to propagators attempting to optimize propagation efficiency for firechalice. Besides looking for the best plant responses in culture, propagators must also consider the costs of the biochemicals used. The cost of mT from *Phyto*Technology Laboratories in January 2016 was $257 per gram, whereas the cost of BA from this same company was $5 per gram. Since similar concentrations of mT or BA were used for shoot multiplication, the higher cost of mT may not be justified for commercial propagation of firechalice given that BA, which is 51 times cheaper, promoted shoot multiplication almost as well as mT. Additional plant species observed by other researches 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). The best rate of multiplication in Stage II was found with mT, and the optimum concentration was 5 µM (Bairu et al. 2007). These results were similar to the firechalice ecotype used in this study. In contrast, Niedz and Evens (2011) found that different concentrations of BA and mT improved shoot quality and the number of explants that produce buds and shoots for (Hamlin) sweet orange (*Citrus sinensis* L.), but BA was more effective than mT for shoot multiplication.

The cytokinin 2iP had only moderate effects on firechalice shoot multiplication, so this biochemical could be excluded from further Stage II studies. Four different cytokinins: BA (4.4, 11.1, or 22.2 µM), kin (4.7, 11.6, or 23.3 µM), 2iP (4.9, 12.3 or 24.6 µM), TDZ (4.5, 11.4 or 22.7 µM) were tested for their effects on shoot multiplication of *Rheum rhaponticum* 'Karpow Lipskiego' on MS medium (Kozak and Sałata 2011). These cytokinins induced new shoots to form, and the highest number of axillary shoots formed on media supplemented with 11.1 or 22.2 µM BA. Treatment with 4.7 to 11.6 µM kin or 12.3 µM 2iP improved shoot length and leaf blade size (Kozak and Sałata 2011). *Pyrus pyrifolia* N. 'Hosui' and 'Kosui' shoots subcultured on a WPM supplemented with kin, BA or TDZ in different concentrations formed the most shoots if using 11 µM BA (Kadota and Niimi 2003).

Thidiazuron is a potent cytokinin-like plant growth regulator that can promote or inhibit the growth of shoot cultures. In this study with firechalice, shoots on medium that contained 8.8 µM TDZ were less than half the height of control shoots (Table 3.4). Shoot height reduction associated with increased TDZ concentration indicated that this cytokinin had a harmful effect on shoot growth. Likewise, TDZ

concentrations inhibited shoot elongation of *Rheum rhaponticum* L. (Kozak and Sałata 2011).

The most prominent types of plant growth regulators used in tissue culture are auxins and cytokinins. The effects of auxin and cytokinin were tested by Skoog and Miller (1957) on tobacco tissue culture, and their results are still the basis for plant tissue culture manipulation today (Trigiano and Gray 2011). Auxins are used in various concentrations to promote root formation, and cytokinin may be decreased or eliminated entirely from the medium during root induction depending on the species. Auxins can affect many developmental processes in plant, including cell elongation and swelling of tissue, apical dominance, adventitious root formation and somatic embryogenesis. The main auxin used to root in vitro shoots is NAA (Trigiano and Gray 2011).

Auxin is often needed in the medium to promote root initiation on shoots during Stage III. In this study, firechalice rooted easily without auxin added to the medium, indicating that its shoots naturally produce enough auxin to promote root formation. When IBA was combined with BA, firechalice roots formation was completely inhibited. These results are in contrast with those of Abrie and Staden (2001) who found that low BA concentrations combined with IBA improved shoot appearance and induced root formation. Of the auxins tested, IBA induced more roots to form than NAA for firechalice stem explants (Table 3.7). Shoot explants treated with 6 or 9 μM IBA formed at least 44% more roots than control shoots, whereas up to 9 μM NAA decreased shoot height, number of roots per responding shoot, rooting percentage and length of the longest root per rooted shoot, indicating

this high NAA concentration had toxic effects on firechalice shoot growth and root formation. For *Vitis vinifera* cv. Pinot noir, NAA or IBA at 2.5 to 5 μM induced 100% of the shoots to form roots. The highest number of roots were induced by using NAA, and the initiated roots were shorter compared to those on IBA-treated shoots at the same concentration (Heloir et al. 1997).

Using Rootone® or Hormex® #3 to bypass Stage III failed to produce rooted shoots at acceptable levels. Although twice as many firechalice microcuttings treated with Rootone® or Hormex® #3 survived Stage IV transplanting compared to water-treated controls, less than 50% of the hormone-treated cuttings survived the ex vitro rooting process. In contrast, at least 72% of the firechalice shoots rooted in vitro without auxin in Stage III medium survived Stage IV transplanting. Since firechalice shoots root readily in vitro without auxin in the medium (Table 3.7) and more than 70% of these shoots survived Stage IV, using ex vitro auxin treatments to bypass Stage III seems to be of limited value.

During Stage IV, rooted shoots survived transplanting, but non-rooted shoots usually failed to survive, regardless of the type of rooting treatment used. If using auxin during Stage III, IBA treated shoots survived at similar levels to those of control shoots in Stage IV. Since control- and IBA- treated shoots acclimatized well to ex vitro conditions compared to NAA treated shoots, using NAA for root formation should be avoided.

# CONCLUSION

In vitro culture is a viable option for rapid propagation of firechalice. Murashige and Skoog medium was the best medium for establishing firechalice stem explants in Stage I. During Stage II, shoot explants multiplied the best when using BA or mT at 4.4 or 8.8 μM. Firechalice shoots, rooted easily without auxin added to the medium, yet adding up to 9 μM IBA increased the number of roots per responding shoot although the percentages of rooted shoots were similar to the control treatment. Using a range of IBA concentrations in Stage III yielded similar transplant survival results as the control treatment (lacked auxin). Using NAA in Stage III should be avoided because it inhibited root formation and decreased transplant survival. The firechalice micropropagation procedures developed in this study can be used to produce large numbers of tissue culture plantlets for use in the nursery industry.

## REFERENCES

Abrie AL, Staden JV (2001) Micropropagation of the endangered *Aloe polyphylla*. Plant Growth Regul 33:19-23

Bairu MW, Stirk, WA, Dolezal K (2007) Optimizing the micropropagation protocol for the endangered *Aloe polyphylla*: can meta-topolin and its derivatives serve as replacement for benzyladenine and zeatin? Plant Cell Tissue Organ Cult 90:15-23

Einset JW (1986) A practical guide to woody plant micropropagation. Arnoldia. 46:36-44

Gamborg OL, Murashige T, Thorpe TA, Vasil LK (1976) Plant tissue culture media. In Vitro 7:473-478

Heloir MC, Fournioux JC, Oziol L, Bessis R (1997) An improved procedure for the propagation in vitro of grapevine (*Vitis vinifera* cv. Pinot noir) using axillary bud microcuttings. Plant Cell Tiss Organ Cult 49:223-225

Kadota M, Niimi Y (2003) Effects of cytokinin types and their concentrations on shoot proliferation and hyperhydricity in *in vitro* pear cultivar shoots. Plant Cell Tiss Org 3:261-265

Kozak D, Sałata A (2011) Effect of cytokinins on *in vitro* multiplication of rhubarb (*Rheum rhaponticum* L.) 'Karpow Lipskiego' shoots and *ex vitro* acclimatization and growth. Acta Scientiarum Polonorum - Hortorum Cultus 10:75-87

Lloyd G, McCown B (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 SL, Noble K, Robbins JA, Wilson B, McCammon T (2009) Landscaping with Native Plants. University of Idaho Bulletin 862

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473-497

Niedz RP, Evens TJ (2011) The effects of benzyladenine and meta-topolin on in vitro shoot regeneration of sweet orange. ARPN J Agric Biol Sci 6:64-73

Pierik RLM (1987) In vitro culture of higher plants. Martinus Nijhoff, Boston

Robson KA, Richter A, Filbert M (2008) Encyclopedia of Northwest native plants for gardens and landscapes. Timber Press, Portland, Oregon

SAS (2012) Users Guide statistics VEe.9.4, SAS Institute Inc. Cary, North Carolina

Skoog F, Miller CO (1957). Chemical regulation of growth and organ formation in plant tissue cultures *in vitro*. Symp Soc Exp Biol 11:118-131

Trigiano RN, and Gray DJ (2011) Plant tissue culture, development and biotechnology. CRC Press, Boca Raton, Florida

Unander DW, Bryan HH, Lance CJ, McMillan RT (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, Colorado

## CONCLUSION TO THE DISSERTATION

A selected serviceberry ecotype (*Amelanchier alnifolia*) grew successfully in vitro on MS medium supplemented with mesos nutrients, and these shoots produced more axillary shoots of good quality compared to shoots grown on regular MS medium. Combinations of mesos nutrients in two tested treatments (in g $l^{-1}$: treatment d – 0.398 $CaCl_2$, 0.054 $MgSO_4$ and 0.102 $KH_2PO_4$ or treatment g – 0.132 $CaCl_2$, 0.054 $MgSO_4$ and 0.204 $KH_2PO_4$) added to MS medium increased the number of shoots, shoot height, and shoot dry weight. The best rooting of serviceberry shoot explants was achieved by reducing MS medium to ½ strength and supplementing the medium with 150 mg $l^{-1}$ FeEDDHA and 1.6 mg $l^{-1}$ thiamine with 0.5 µM IBA. This rooting medium was by far the best, inducing up to 87% of the shoots to root. Improving shoot quality by optimizing the multiplication medium (by adding mesos minerals) increased the serviceberry rooting percentage to 100%, and the shoots looked healthier (dark green color) and formed roots quickly, within 10 days.

In vitro culture is a viable option for rapid propagation of firechalice, and I demonstrated that firechalice can multiply quickly in the first two stages of micropropagation so that high throughput propagation of the selected ecotype can make many plantlets available to production nurseries. MS medium was best for establishing firechalice stem explants in Stage I. During Stage II, the most axillary shoots were produced by using BA or mT at 4.4 or 8.8 µM. Firechalice shoots, rooted easily without auxin added to the medium. In Stage III, IBA improved rooting, and plantlets from the 6 µM IBA treatment (Stage III) survived the highest levels

(93%) during Stage IV. The procedures described here can be used to produce high numbers of firechalice plants that can be used in commercial production in the nursery trade.