Micropropagation of Douglas Maple (*Acer glabrum* Torr. var. *douglasii* (Hook.) Dipple [Aceraceae])

A Thesis

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Authorization to Submit Thesis

The thesis of Noel A. Hathaway, submitted for the degree of Master of Science with a major in Plant Science and titled, "Micropropagation of Douglas Maple (*Acer glabrum* Torr. var. *douglasii* (Hook.) Dipple [Aceraceae])," has been reviewed in final form. Permission, as indicated by the signatures and dates given below, is now granted to submit final copies to the College of Graduate Studies for approval.

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Abstract

Douglas maple (*Acer glabrum* Torr. var. *douglasii* (Hook.) Dipple [Aceraceae]) is a potentially valuable landscape species with significant propagation difficulties. Micropropagation has potential to resolve these issues and allow production of marketable plants in commercial quantities. A research project was initiated to refine procedures for successful tissue culture multiplication of Douglas maple. A series of experiments were designed to optimize factors that impact propagation efficiency and efficacy, including: explant establishment, cytokinin type and concentration for enhancing shoot multiplication, length of subculture period, explant density in culture, light intensity, nutrient formulation of culture medium, rooting methods and their effect on acclimatization, and auxin type and concentration for rooting stem explants. Establishment (decontamination and initiating shoot growth) was accomplished by surface sterilizing explants with a 20% bleach solution (v/v) and then culturing on DKW medium salts and zeatin. This surface sterilization method resulted in 46% contamination-free explants and many expanded shoots. Assessment of cytokinin compound and concentration optimization was conducted to optimize shoot growth during multiplication. Three experiments were conducted, in which, benzyl-adenine (BA), kinetin, meta-topolin (MT), thidiazuron (TDZ) and zeatin were all tested at various concentrations. The cytokinin concentration and compound producing the longest average shoots (39 mm (1.54 in) in a 31 day subculture was 2 µM MT. In an experiment testing shoot growth rates to optimize subculture length, two different genotypes were tested over 6 weeks in culture. The shoot growth of the fastest growing genotype was modeled linearly from week 3 to week 6 by $Y = (1.456) X – 7.937$, where X = days in culture, and Y = shoot length (mm), suggesting that a subculture of 6+ weeks is best for resource and time efficiency. This model had an adjusted r-squared value of 0.81. An explant density experiment was conducted to maximize resource efficiency. Explant densities at 4, 6 and 8 shoot explants per tissue culture vessel (GA-7) failed to significantly affect shoot length, number of nodes or dry weights, meaning 8 explants per vessel is likely more efficient. Shoot health was evaluated at 3 light intensities (7, 17 and 37 µmols•m^{-2}•s^{-1} (0.65, 1.58 and 3.44 µmols•ft^{-2}•s^{-1}) photosynthetic photon flux). Dry weights of shoots receiving the highest and lowest light intensities were significantly different from each (the lowest light treatment had lowest

weight), and plants exposed to the highest light intensity became chlorotic. Deficiency symptoms, such as chlorosis and shoot tip necrosis of Douglas maple plantlets grown on Driver Kuniyuki Walnut (DKW) medium salts, prompted nutrient experimentation. Informal experimentation with 2x iron (relative to Fe content in DKW medium salts) remediated the chlorosis issue. Standard commercial salts (DKW, Murashige and Skoog (MS) and Woody Plant Medium (WPM) medium salts) with supplemental iron were tested for their ability to induce rapid shoot growth, increase node number, and increase dry weights. The DKW medium was statistically superior at promoting shoot growth and the most number of nodes. Another experiment tested various fortifications to DKW medium with supplemental iron and was primarily focused on remediating the shoot tip necrosis. Additional boric acid (12.0 mg/l (0.0016 oz/l)) significantly improved shoot length (51 mm (2.01 in)) in comparison to control DKW medium with supplemental iron (29 mm (1.14 in)), and also reduced necrosis issues over a period of subcultures. A final nutrient experiment tested whether nutrient ratios within *Acer glabrum* sap were appropriate for formulating medium. Shoots grown on the sap nutrient ratio medium grew slightly less than the other control treatments included in this experiment, but were statistically similar. Sap-based nutrient ratio formulation showed potential, though more experimentation is still needed to improve the methods and strategies used for this nutrient optimization approach. In vitro and ex vitro rooting procedures were tested for their ability to root high percentages of shoot explants, and to acclimatize plantlets efficiently. The in vitro rooting factors tested were: auxin type (indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) or both in combination), light vs dark, apical vs nodal explants, half vs full strength DKW medium salts, IBA concentration (0, 1, 2, 4 or 8 μM), and the length of the explant (2.0 to 3.0 cm (0.79 to 1.18 in), or 3.5 to 4.5 cm (1.38 to 1.77 in)). The best in vitro rooting treatment, 1 μM IBA in half-strength DKW regardless of explant length, yielded 75% survival after rooting and acclimatization. Ex vitro rooting treatments included explant length as a covariate and a treatment (concentrations of IBA (0.1 and 0.3%) in talcum based rooting powders, and a control (water) group). The most successful treatments were 0.1 and 0.3% IBA talcum powder, both yielding a 92% survival rate after rooting and acclimatization. Ex vitro rooting methods were deemed better because of better time and resource efficiency (1 month of vitro culture subtracted), and higher survival rates after rooting and acclimatization. A second ex vitro experiment involving another Douglas maple

genotype further validated the effectiveness of ex vitro rooting using 0.1% IBA talcum powder. Overall, these experiments have optimized micropropagation methods such that an estimated 12 fold multiplication of explants can be achieved every 6 week subculture, and only 8% of plantlets fail to survive after the rooting and acclimatization stages.

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Dedication

I am thankful to God for this opportunity to get a Master of Science degree. I am thankful that I got to study His creation in such depth. He made His creation wonderful and it proclaims His glory. I thank my wonderful wife, Maritza, for her encouragement and support through the difficulties of research and writing, and for her hard-work supporting us financially during the summers. I am thankful to my loving parents, Jerry and Maxine, for consistently pushing, encouraging, and showing me that, with hard work and determination, I could accomplish difficult things such as a master's degree. I thank my grandfather, Maxwell Vogel, for inspiring my curiosity in science and having genuine interest in my studies. I thank my 6th to 9th grade science teacher, Mrs. Karen Hull, for giving me a solid scientific foundation and showing me the awesomeness of God's creation. To all mentioned, and many others unmentioned, I sincerely thank you.

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INTRODUCTION TO THESIS

Native plants placed within the urban landscape can be beneficial to homeowners for many reasons. Native plants in landscapes can potentially reduce the impact of exotic invasive species, decrease time and costs associated with landscape maintenance, reduce water delivery costs, improve health of landscape specimens through improved adaptability, and create attractive scenes that incorporate a sense of place (Love and others 2009). Moreover, in dry climates, drought tolerance is an important plant trait which can be invaluable during periods of short water supply (Love and others 2009). Conserving water in urban landscapes could potentially provide broader environmental and economic benefits such as preservation of groundwater resources, deceleration in rates of soil salinization, and conservation of valuable water resources for environmental, domestic, agricultural and industrial purposes.

Developing native plants with magnified environmental benefits is the overriding goal of a University of Idaho native plant development project led by Dr. Stephen Love. One specific objective of the program is to produce aesthetically superior native trees, shrubs, and forbs by employing a domestication process that involves multiple cycles of mass selection of wild-collected germplasm. The ultimate output of the program is superior native plant products - with consistent landscape performance quality – for use by the landscape and nursery industries. In addition to producing attractive, valuable plants, concurrent development of efficacious propagation protocols (via seed or vegetative methods) is critical to successful marketing of native plants. A focal species for the native plant development project is Douglas maple, also known as Rocky Mountain maple (*Acer glabrum* Torr. *Douglasii* (Hook.) Dipple [Aceraceae]).

Douglas maple is a species native to western North America, having a large native range which encompasses much of the western United States of America and Canada, including California, Idaho, Montana, Nevada, Oregon, Washington, Wyoming, Alberta and British Columbia (USDA NRCS Plants Database 2019). Douglas maple is a multi-stemmed, large shrub to small tree; it typically grows to a height of 6.10 to 9.14 m (20 to 30 ft) at full maturity (Anderson 2001). Its medium-green, simple, opposite, palmately-lobed leaves have a width of 6 to12 cm (2.4 to 4.7 in) (Breen 2019, Hitchcock and Cronquist 1973). Some of this species' most attractive attributes for urban landscaping are bright red color of new-

growth stems, red to yellow fall foliage, low to moderate water usage, ability to thrive in varied sun exposure, and a moderate growth rate (Breen 2019, Hitchcock and Cronquist 1973, USDA NRCS Plants Database 2019). Of the maple species native to North America, *Acer glabrum* is the most adapted to growing in low water conditions according to data provided by the USDA NRCS Plants Database (2019), and can be observed growing in areas receiving just 23 cm (9 in) of annual precipitation.

The subspecies *douglasii* (Douglas maple) naturally grows in areas receiving 41 to 127 cm (16 to 50 in) of annual precipitation (USDA NRCS Plants Database 2019). Douglas maple is hardy with respect to temperature extremes to USDA hardiness zone 5 and is resilient to fire via resprouting, wildlife browsing, and other damage (Anderson 2001, USDA NRCS Plants Database 2019). Douglas maples are able to grow well in most soils, except those that have chronically poor drainage, or have pH values below 5.5 or above 7.5 (USDA NRCS Plants Database 2019). *Acer glabrum* is also a highly valuable plant for habitat establishment, because it thrives in disturbed sites and is an important winter food source for many animal species. For example, some populations of deer, elk and moose rely heavily on this species' young shoots for browse during the winter months (Anderson 2001). In addition, mature trees can be tapped for syrup (Naughton and others 2006). Douglas maple wood has appropriate characteristics for wood working. Native Americans used Douglas maple for medicinal and functional purposes such as drum frames, snowshoe frames and toys (Nesom 2006). Overall, this maple is aesthetically attractive, adaptable, hardy and useful to wildlife.

Propagation of Douglas maple is most commonly accomplished by seed, but due to poor natural seed quality and recalcitrant dormancy characteristics, this process is inefficient. Also, seed propagation fails to conserve superior traits that make selected Douglas maple genotypes desirable as urban landscape plants. Seed germination rates are low and variable, ranging from 1 to 52% (Flessner and Trindle 2008, Hutton personal communication 2018). Propagation using stem cuttings is partially successful but is difficult, and overwintering death is an issue (Hartmann and others 2011, Rupp and Wheaton 2014). A high throughput asexual propagation protocol for Douglas maple is needed such that superior genotypes can be conserved and rapidly propagated. In vitro propagation techniques would satisfy these needs, but protocols appropriate for this species are currently lacking. In vitro nodal culture,

or axillary shoot proliferation, is a good technique for maintaining true-to-type plantlets, and also potentially achieving high propagation ratios. In developing such a protocol for Douglas maple, resource efficiency and cost effectiveness were goals such that commercial usefulness could be maximized.

In accordance with Hartmann and Kester's book Plant Propagation Principles and Practices (2011), micropropagation is a good choice for commercial production when the cultured species: 1) has a low propagation rate by conventional methods, 2) has high market demand, 3) has high market value, 4) is difficult to propagate clonally, and/or 5) is an endangered species. Douglas maple, in my opinion, satisfies the first and fourth reasons for choosing micropropagation, but also has strong potential to be valuable and in high demand, thus satisfying Hartmann and Kester's second and third qualifications.

Micropropagation typically involves 4 distinct stages: 1) establishment (including surface sterilization, shoot growth initiation, and stabilization), 2) multiplication, 3) rooting and 4) acclimatization to the natural environment. Most experiments within this thesis were designed to improve propagation efficiency, particularly efficacy during stages 2 (multiplication) and 3 (rooting). In published literature, stages 1 (establishment) and 4 (acclimatization) were easily accomplished for maple species. The overall objective of this research was to develop a simple, cost effective, propagation protocol for Douglas maple – a protocol that can be shared with, and used by native plant growers.

In summary, native plants in managed landscapes can provide many valuable benefits and attributes. Douglas maple has been identified as a species of high value because of its positive landscape attributes, including its ability to grow in dry climates. Propagation by seed is the current standard for this species, but problems associated with this method of multiplying plants could be overcome by using micropropagation. Propagation recalcitrance and potential commercial value make Douglas maple a good candidate for this approach. Objectives of the experiments documented in this thesis were directed towards development of a time-wise and economically efficient micropropagation protocol that can be used by nursery industry personnel.

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LITERATURE REVIEW

Asexual and Sexual Propagation Considerations for *Acer glabrum*

Plant propagation methods can be divided into asexual and sexual procedures. Sexual methods are carried out via seed. Common asexual methods of propagation for woody species include: layering, rooted stem cuttings, grafting, and micropropagation using tissue culture. Asexual (also called vegetative or clonal) methods of propagation have the unique advantage of producing genetically identical plants (clones).

Asexual propagation has additional advantages over sexual propagation. With species recalcitrant from seeds, asexual propagation can be more efficient (Hartmann and others 2011). This procedure also allows easy conservation of superior phenotypic traits that make certain plant accessions valuable (Hartmann and others 2011). However, clones produced via asexual propagation have disadvantages too. Clonal populations are susceptible to widespread damage caused by environmental conditions and pests (Hartmann and others 2011). They are also typically more expensive to produce than plants reproduced by seeds (Hartmann and others 2011).

Acer glabrum can be reproduced by using seeds, but reproduction by seeds will result in phenotypic variation (Brown and others 2014). In addition, seed germination can be erratic and unpredictable. The reputation and value of a landscape plant is contingent upon its consistency in possessing key phenotypic traits. Furthermore, if a cultivar is to be successfully marketed, phenotypic variation, which may create customer dissatisfaction, would be undesirable or even entirely unacceptable. For cultivar acceptance, the following 3 criteria must be satisfied: distinctiveness, uniformity, and stability (Brown and others 2014). Acceptable non-clonal cultivars could probably be developed through breeding, but the long, 10+ year generation spans of *Acer glabrum* would make this a time-consuming and impractical undertaking. However, if asexual propagation methods are used, then uniformity and stability would be accomplished with ease. And because registered cultivars are nonexistent for *Acer glabrum*, distinctiveness would be automatically accomplished.

Layering is suggested as an asexual propagation method on some internet webpages, but success rates are unknown. Nesom (2006) purports that *Acer glabrum* is difficult to

propagate by cuttings. Moreover, Rupp and Wheaton (2014) state in their publication on native plant propagation that, “there are no published techniques for its [*Acer glabrum*] vegetative propagation.” Negative reports in combination with a lack of established protocols for asexual propagation provide ample justification for micropropagation research on *Acer glabrum.* In essence, micropropagation seems to be a reasonable asexual propagation procedure given Douglas maple’s propagative resistance to standard methods, its potentially high market value, and potentially significant market demand.

Micropropagation

The following summary of basic micropropagation concepts is derived largely from chapters 17 and 18 of Hartman and Kester (2011) 8th edition book, Plant Propagation Principles and Techniques, except where otherwise cited.

Micropropagation is defined as the propagation of plants in tissue culture; and tissue culture is defined as the establishment and maintenance of plant cells, organs or tissues in aseptic, in vitro culture. Micropropagation inherently has major advantages and disadvantages. The first advantage is partial reversion of cultured plant tissue from a more mature ontological state to a more juvenile state. Developmental state is important because juvenile tissue is easier to establish in culture and root. Second, micropropagation precludes propagation restrictions caused by seasons or weather. The last notable advantage is that micropropagation gives propagators very precise morphological and physiological control over the growth and development of plantlets. The main disadvantages of micropropagation are expenses related to start-up costs, lack of trained technical help, and the vigilance necessary to identify and eliminate problems unique to this process, including culture contamination and somaclonal variation.

Several cultural techniques are used with micropropagation. A common technique is nodal culture – a form of axillary shoot culture, in which, “long shoots are cut into single nodes and planted vertically in the medium.” This type of culture relies on the pre-formed meristems of axillary buds to form new shoots. The process of forming new shoots from preformed meristems of nodal cuttings is primarily mediated by the alteration of plant growth regulators. Nodal culture is the best procedure for limiting somaclonal variation, and

therefore, maintaining genetically true-to-type plantlets. An alternative to axillary shoot culture is adventitious shoot culture. Adventitious shoot culture is the culture of shoots from cells that must be dedifferentiated and then re-differentiated to form new apical meristems and eventual shoots. This process is also mediated by the alteration of plant growth regulators. This form of culture can have a higher output of shoots, but mutations (somaclonal variation) are considered to be more common using this method. Maintaining true-to-type plants is paramount to the reputation and integrity of a plant producer. Given the objectives associated with propagation of Douglas maple, the logical choice is axillary shoot culture rather than adventitious shoot culture. From this point forward, all mentions of micropropagation will be in reference to a nodal culture system.

The process of micropropagation is divided into 4 stages: 1) establishment, 2) shoot multiplication, 3) root formation, and 4) acclimatization to the natural environment. Establishment involves 3 sub-stages: sterilization, and initialization of shoot growth in culture, and stabilization of in vitro shoot growth. Stabilization of tissue is often considered an aspect of establishment, however, stabilization takes place on a continuum and is hard to define until the growth characteristics of the species in tissue culture have been determined. For this research, stabilization was not measured or considered for determining establishment success. Transition from stage to stage is made possible by the totipotency of plant cells, and primarily mediated by manipulating plant growth regulators in the growth medium.

Totipotency is defined as, "the concept that a single cell has the genetic program to grow into an entire plant." In plant tissue culture, plant growth regulators (PGR) (also known as plant hormones) are used to manipulate genetic expression and the subsequent development of tissue. Certain PGRs are used to enhance the development of particular plant organs. Two very important PGRs for micropropagation are cytokinins and auxins. Practically speaking, auxins are the PGR primarily responsible for the formation of lateral and adventitious roots, whereas cytokinins are the PGR primarily responsible for shoot formation and bud break. These PGRs are naturally produced within plant tissues, but can also be exogenously supplied by adding them to growth medium. For many species, supplemental exogenous PGR is needed for the induction of a desired response.

Auxin is naturally produced in shoot meristem regions and young leaves. Naturally occurring auxins include indole-3-butyric acid (IBA) and indole-3-acetic acid (IAA). Both are relatively labile to heat and light. Synthetic auxins α-naphthaleneacetic acid (NAA) and 2,4-dichloro-phenoxy-acetic acid (2,4-D), are often more potent and stable than naturally occurring auxins. In addition to root formation, auxins play a major role in phototropism, gravitropism, thigmatropism, apical dominance, floral bud development, prevention of leaf abscission, fruit development, and vascular differentiation (Taiz and Zeiger 2010).

Cytokinin is produced in young leaves, developing embryos, fruits and roots. The most common naturally occurring plant cytokinin is zeatin (Taiz and Zeiger 2010). Other common non-plant and synthetic cytokinins include thidiazuron (TDZ), meta-topolin (MT), 6-benzylaminopurine or benzyl adenine (BA), kinetin and 6-(γ,γ-dimethylallylamino)purine (2iP). Cytokinins play a role in shoot growth, promotion of lateral bud growth, inhibition of root growth, aspects of mitosis, delayed leaf senescence, and nutrient mobilization (Taiz and Zeiger 2010).

Ratios of auxin and cytokinin concentrations are very important in controlling growth and development of plants. In an in vitro environment, generally speaking, high auxin:cytokinin ratios stimulate root formation and growth, low auxin:cytokinin ratios stimulate shoot formation and growth, and simultaneous intermediate levels of auxin and cytokinin stimulate callus production (Taiz and Zeiger 2010).

Micropropagation of *Acer* Species Stage by Stage

Many published research articles address micropropagation for species within the genus *Acer*. In preparation for this in vitro research project, many of these publications were reviewed on the assumption that they may have relevance to propagation of Douglas maple. Using a sequential stage by stage presentation, this section will highlight the most important elements of in vitro research-based propagation techniques of *Acer* species.

Establishment: Several *Acer* species have been successfully established in tissue culture from nodal stem explants. Though many variations exist between procedures, a number of steps and details are common. Tissue selected for culture is typically derived from young, juvenile material, of trees 5 years old or less (Ďurkovič 2003, Bowen-O’Connor and others

2007, Lattier and others 2013). Ontologically mature tissues can also be used but may establish at a lower rate of success than juvenile tissues (Wann and Gates 1993). The timing of tissue collection may also play a role in the success of establishment; spring is indicated as being the best time (Wann and Gates 1993).

Surface sterilization of stem segments was most commonly accomplished by rinsing the tissue under tap water, soaking in a bleach solution, followed by repeatedly soaking in sterile water. Bleach solution concentrations were usually 20 to 35% (v/v) in water. The duration of bleach solution exposure ranged from 5 to 20 minutes (Wann and Gates 1993, Bowen-O'Connor and others 2007). Sometimes, as a second sterilizing strategy, tissue was soaked in Plant Preservative Mixture (PPM™) or other antimicrobial compounds (Brassard and others 2003, Ďurkovič 2003).

Contamination rates were seldom mentioned in published research reports, except in Ďurkovič (2003) where 39.6% of 2 year old plant explants and 20% of 4 to 5 year old plant explants were contaminated. Based on published data, decontamination of stem explants from *Acer* species is not a major obstacle to the establishment of plant tissue in culture.

Exudation of dark colored compounds from explants into the media after surface sterilization was common with most cultured *Acer* species (Wann and Gates 1993, Ďurkovič 2003, Bowen-O'Connor and others 2007). Such exudate accumulation in tissue culture medium can cause necrotic effects on explant tissues. As a remedy, explants were transferred to fresh medium frequently, or to less discolored sections of the same medium (Wann and Gates 1993, Bowen-O'Connor and others 2007). Some researchers added ascorbic or citric acid to establishment media, presumably, to help minimize the effects of these exudates (Brassard and others 2003).

Shoot growth initiation (the second step in tissue establishment) rates were only addressed within a few of the reviewed research papers. Thirty-three percent of surface sterilized Bigtooth maple (*Acer grandidentatum* Nutt. [Aceraceae]) stem explants placed on Driver Kuniyuki Walnut (DKW) medium with 10 µM zeatin were successfully established after 120 days (Bowen-O'Connor and others 2007). Other media types tested in this experiment were Murashige and Skoog (MS), Linsmaier and Skoog (LS), and Woody Plant Medium (WPM),

but these formulations had significantly lower establishment rates (Bowen-O'Connor et al., 2007). Kawakami maple (*Acer cauditifolium* Hayata [Aceraceae]) was successfully established on WPM salts with BA alone, or in combination with NAA (Ďurkovič 2003). The highest documented establishment rate was with Kawakami maple at 72.7% on WPM with 0.7 mg/l (0.000093 oz/gal) BA and 0.05 mg/l (0.0000067 oz/gal) NAA. Red maple (*Acer rubrum* L. [Aceraceae]) sapling nodal cuttings were established at a rate of 80% on MS media salts with 0.01 mg/l (0.0000013 oz/gal) TDZ (Wann and Gates 1993).

Even though most research reports lack details on explant establishment, relevant information about media used for successful culture can be gleaned from careful inspection of employed methods. Silver maple (*Acer saccharinum* L. [Aceraceae]) and sycamore maple (*Acer pseudoplatanus* L. [Aceraceae]) were cultured on DKW medium salts. Norway maple (*Acer platanoides* L. [Aceraceae]) was successfully cultured on MS media salts (Lattier et al., 2013), and sugar maple (*Acer saccharum* Marshall [Aceraceae]) was established on MS medium salts.

In summary, *Acer* species are likely best established when starting with juvenile tissue with actively growing shoots. Surface sterilization using bleach is a proven and common method, which seems to entail minimal complication. Initialization of shoot growth can be accomplished on sundry types of basal salt formulations and plant growth regulators, however, DKW and MS medium salts were most commonly used.

Shoot Multiplication: Experiments conducted on silver maple, sugar maple, red maple, sycamore maple and Montpellier maple (*Acer monspessulanum* L. [Aceraceae]) showed that thidiazuron (TDZ) alone or in combination with other plant growth regulators was most effective at promoting shoot growth (Preece and others 1991, Wann and Gates 1993, Wilhelm 1999, Brassard and others 2003, Heidari and Safarnejad 2015). Benzyladenine, when used alone, was inferior or even inhibitory to shoot growth, except with Norway maple, which optimally grew with 2 to 4 µM BA (Lattier and others 2013). Sycamore maple shoots grew best with a combination of TDZ and BA (Wilhelm 1999). Kawakami maple grew best with a combination of BA and NAA (Ďurkovič 2003). Bigtooth maple grew best with zeatin (Bowen-O'Connor and others 2007). Benzyladenine and TDZ were tried on bigtooth maple, but failed to induce shoot growth or elongation (Bowen-O'Connor and others 2007).

Preece and others (1991) completed some of the most comprehensive cytokinin experiments on maple to date. Kinetin, 2iP, BA, TDZ and zeatin were tested for their ability to promote shoot formation and elongation on several genotypes of silver maple. An experiment tested, zeatin, 2iP, BA and kinetin at 1 µM and 10 µM. Zeatin at 10 µM induced the most shoots, and 2iP at 1 µM induced the longest shoots. Thidiazuron was also tested at nM concentrations and was found to produce the most shoots over 5 mm (0.20 in) at 10 nM. Thidiazuron at 100 nM produced similar results. Preece and others (1991) concluded that TDZ at 10 nM was the optimum type and rate of cytokinin for multiplication of silver maple.

Cytokinin is perhaps the most crucial element for enhancing multiplication of plant material during micropropagation of maple. As such, most research involved investigations of cytokinins and their concentrations. Research involving inorganic nutrients, vitamins, and sugar sources was more infrequent. However, some in vitro nutrient related research on *Acer* species has been reported. Heidari and Safarnejad (2015) reported that *Acer monspessulanum* benefitted from supplemental iron above what is provided in MS medium salts. In general, however, multiplication media were usually based on DKW or MS medium salts and included agar at 6 to 8 g/l (0.80 to 1.07 oz/gal), vitamins, 20 to 30 g/l (2.67 to 4.00 oz/gal) of sucrose, and some cytokinin.

Root Formation: The best rooting treatments for *Acer* species often included an auxin in the medium. *Acer*-specific rooting experiments yielded rooting percentages between 65 and 100% (Ďurkovič and Andrea 2008). In vitro adventitious rooting via use of 1.0 mg/l (0.00013 oz/gal) IBA in medium is among the most common treatments (Ďurkovič and Andrea 2008). Some maple species rooted successfully without any exogenous auxin, but this response appeared to be highly dependent on genotype (Bowen-O'Connor and others 2007). Various medium salt mixtures were used during the rooting stage, and sometimes applied at half-strength (Ďurkovič and Andrea 2008).

Preece and others (1991) demonstrated that silver maple microshoots could be rooted ex vitro by applying a liquid IBA treatment to the base of shoots – resulting in a rooting rate of 70%. Overall, successful rooting is readily accomplished for most maple species.

Acclimatization: Acclimatization is easily accomplished by transferring rooted plantlets to soilless potting medium, and keeping environmental humidity high (Wilhelm 1999, Bowen-O'Connor and others 2007). Survival rates after acclimatization across several *Acer*-specific experiments ranged from 77 to 100% (Ďurkovič and Andrea 2008).

Influence of Environmental Conditions within In Vitro Culture

Another important aspect of micropropagation is the environmental conditions in which explants are cultured. Temperature, photoperiod, light source, and light intensity are carefully controlled in reported studies. Cultures of *Acer* species were grown at constant temperatures or with a day to night temperature differential. Cultures managed at constant temperatures were held between 20° and 28° C (68° and 82° F) (Preece and others 1991, Bowen-O'Connor and others 2007). Cultures managed with a day and night temperature change had a maximum 10° C (18° F) differential, with temperatures fluctuating from 26° C (79° F) during the day to 16° C (61° F) during the night (Brassard and others 2003; Ďurkovič 2003).

Most *Acer*-specific cultures were maintained on a 16 hour photoperiod. Nearly all cultures were illuminated with cool white fluorescent lamps. Supplied light intensity was highly variable between cultures. In some studies, explant quality was improved by decreasing light intensity (Wann and Gates 1993, Singh and others 2017). Singh and others (2017) explores this factor in much detail and found that under full-spectrum white light, sugar maple was best grown at 16 $\mu mols \cdot m^{-2} \cdot s^{-1}$ (1.48 $\mu mols \cdot ft^{-2} \cdot s^{-1}$). Even 40 $\mu mols \cdot m^{-2} \cdot s^{-1}$ (3.72 $\mu mols \cdot ft^{-2} \cdot s^{-1}$) photosynthetic photon flux was too high for sugar maple, and this intensity is on the lower end of what is typically used in micropropagation systems. Light intensity failed to significantly affect shoot length or node number, but it did significantly affect dry and fresh weights (Singh and others 2017). Explants cultured at 40 $\mu mols \cdot m^{-2} \cdot s^{-1}$ (3.72 $\mu mols \cdot ft^{-2} \cdot s^{-1}$) actually had the higher weights, but showed visual signs of stress, and low leaf chlorophyll content. Another sugar maple research article reports using a light intensity of 80 $\mu mols \cdot m^{-2} \cdot s^{-1}$ (7.43 $\mu mols \cdot ft^{-2} \cdot s^{-1}$) (Brassard and others 2003), but whether this intensity caused any detrimental effects is unknown. Excluding sugar maple, cultures of other *Acer spp.* were grown with a light intensity between 33 and 75 $\mu mols \cdot m^{-2} \cdot s^{-1}$ (3.06 and 6.97 $\mu mols \cdot ft^{-2} \cdot s^{-1}$) (Preece and others 1991, Lattier and others 2013).

Nutritional Considerations

In reviewing a wide range of micropropagation papers, inorganic nutrition appears to be a crucial component to protocol success. A well-documented fact is that individual species often have specific nutritional requirements, a factor that can even vary from genotype to genotype within species (Nas and Read 2004).

Frequently, nutrient optimization in tissue culture is accomplished by adding specific nutrients to common media formulations such as DKW, WPM and MS medium salts. Formulas for the commercially available salt mixtures were developed by testing levels of a nutrient and finding the optimum concentration. Specific strategies, models and methods varied, but they often represented very time-consuming and laborious projects. For example, the MS medium salt formula took 5 years to finalize (Nas and Read 2004). Given that the optimal nutrition for Douglas maple may be unique – and being limited by time – I searched for an accelerated/abbreviated method of inorganic nutrient optimization, or even a diagnostic-like test that might give insight into specific nutritional needs of this species.

Moderate success in media development has been achieved by using media formulations based on nutrient content of seeds (Nas and Read 2004); however, some nutrient concentrations in the seed were not usefully appropriate to media formulation. Copper and nitrogen levels, for example, were at toxic concentrations in proportion to other nutrients. Tissue nutrient analysis has also been used to guide and optimize concentrations of inorganic media components in tissue culture, again with moderate success (Monteiro and others 2000).

Logically, sap analysis may be a good candidate for generating nutrient values and ratios for use in optimizing micropropagation media because it is the actual liquid that supplies organic and inorganic compounds essential for in vivo growth. Some nutrients are also stored in a physical matrix within the plant, but again, these must be mobilized and translocated to a sink via the sap. Sap samples may be a better indicator of nutrient requirements than tissue samples because a sap sample would show the nutrients in solution that are supplied from an external source to the cells, whereas, a tissue sample is indicative of what the cells have already accumulated. This accumulation in tissues of certain nutrients might lead to false

predictions of ideal relative nutrient concentrations for optimizing in vitro growth. These inaccurate proportions could be detrimental to optimization, because some nutrients are optimally assimilated in specific proportions (Halloran and Adelberg 2011).

Sap nutrient analysis has been done before, but for purposes other than developing tissue culture nutrient formulations. Some researchers have collected sap for constituent analysis by using excised aphid stylets in order to minimize the alteration of in vivo sap constituents (Gaupels and others 2008). Companies such as Advancing Eco Agriculture have adopted sap analysis as a commercial agronomic service. They claim it to be a very effective way of identifying near-deficiencies before they fully develop. Nevertheless, the application of sap analysis to in vitro nutrient optimization is a concept never before discussed in the literature.

Summary to the Literature Review

Developing asexual methods of propagation for Douglas maple are crucial to the conservation of key characteristics. Micropropagation appears to be a reasonable choice given the potential challenges entailed in using other propagation methods with this species. All stages of micropropagation have been completed on other *Acer* species and have provided an outline of acceptable procedures and treatments for the culture of Douglas maple. Establishment is accomplished by the collection of young tissue, surface sterilization by a bleach solution, and then placement of tissue on a medium with DKW, WPM or MS medium salts, and a cytokinin such as BA, TDZ or zeatin. Multiplication of *Acer* species has been successful on media made with DKW, WPM or MS medium salts supplemented with BA, TDZ or zeatin. In vitro rooting was often accomplished with IBA in media. An ex vitro method has also been shown to be effective. One maple species, *Acer grandidentatum,* rooted well on a PGR-free medium. Acclimatization was simple and easily accomplished by keeping humidity high. Environmental conditions of in vitro culture ranged considerably between *Acer*-specific protocols, but light intensity seemed to be an important factor as evidenced by findings on sugar maple. For this species, high light intensities were detrimental to overall explant health. Inorganic nutrient optimization, specifically for Douglas maple, may also be beneficial to maximizing the potential of a micropropagation protocol. Fast-track methods for nutrient optimization, such as using ratios of nutrients found in tissue or seed samples, could be used, but sap may also provide an effective estimation of

accurate nutrient ratios ideal for growth. Using sap analysis for tissue culture nutrient optimization appears to be a novel approach.

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CHAPTER 1 – Establishment and Multiplication Stages of Douglas Maple: Cytokinin, Resource and Time Efficiency, and Light Intensity Optimization

ABSTRACT

Douglas maple (*Acer glabrum* Torr. var. *douglasii* (Hook.) Dipple [Aceraceae]) is a potentially valuable landscape plant that currently lacks proven asexual propagation protocols. Micropropagation has potential as an efficacious method for propagating Douglas maple. Establishment methods, cytokinin optimization, efficiency optimization, and light intensity optimization were researched. Cytokinin optimization with respect to shoot length was done in 3 experiments, in which, BA, kinetin, MT, TDZ and zeatin were all tested at various concentrations. In addition, 2 sterilization methods of zeatin were tested, which were autoclaving zeatin in medium, and filter sterilizing zeatin solution prior to adding to the autoclaved medium. The sterilization methods were tested to assess altered potency of zeatin. Autoclaved zeatin at 4 µM and 2 µM MT were the best 2 cytokinin compounds and concentrations for promoting shoot growth. Efficiency experiments tested growth rates of different genotypes and the effect of explant density on shoot length, number of nodes and average dry weights. The shoot growth of 1 genotype was modeled linearly by $Y = (1.456) X - 7.937$, where X = days in culture and Y = shoot length (mm). This model had an adjusted R^2 of 0.81. Explant densities at 4, 6 or 8 explants per tissue culture vessel failed to significantly affect shoot length, number of nodes, or average dry weights. Three light intensities – 7, 17 and 37 $\mu mols \bullet m^{-2} \bullet s^{-1}$ (0.65, 1.58 and 3.44 $\mu mols \bullet ft^{-2} \bullet s^{-1}$, respectively) PPF – were tested for their effect on chlorophyll content, shoot length, number of nodes and average dry weight. The highest and lowest PPFs caused significant differences only in average dry weight. Shoots receiving the higher light intensity produced the higher average dry weights. Overall, the experiments have optimized methods such that an estimated 12 fold multiplication of explants can be achieved every 6 week subculture.

KEY WORDS: micropropagation, in vitro, tissue culture, *Acer glabrum*, multiplicative efficiency.

NOMENCLATURE:

USDA NRCS (2019)

Abbreviations: 2iP – 6-(γ,γ-dimethylallylamino)purine, MT – 6-(3-hydroxybenzylamino)purine or meta-topolin, BA – 6-benzylaminopurine or benzyladenine, kinetin – N-(2-furanylmethyl)-1H-purin-6-amine, zeatin – (E)-2-methyl-4-(1H-purin-6ylamino)-2-buten-1-ol or trans-zeatin, TDZ – N-phenyl-N'-1,2,3-thidiazol-5-ylurea or thidiazuron, DKW – Driver and Kuniyuki Walnut, WPM – Woody Plant Medium, MS – Murashige and Skoog, LS – Linsmaier and Skoog, PPF – photosynthetic photon flux, SPAD – soil plant analysis development, PPM – Plant Preservative Mixture.

INTRODUCTION

Douglas maple, also known as Rocky Mountain maple, (*Acer glabrum* Torr. var. *douglasii* (Hook.) Dipple [Aceraceae]) possesses excellent landscape plant characteristics, including high adaptability to a variety of climates and soil types, bright red or yellow fall foliage, attractive red young stems, and a moderate degree of drought tolerance. In spite of excellent landscape potential, use of this species is limited, in part, due to a lack of published protocols for asexual (vegetative) propagation. Rupp and Wheaton (2014) state in their publication on native plant propagation that, “there are no published techniques for its [*Acer glabrum*] vegetative propagation.”

Propagation of Douglas maple is most commonly accomplished by seed, but due to poor natural seed quality and recalcitrant dormancy characteristics, this process is inefficient. In addition, seed propagation does not allow conservation of superior traits that make selected Douglas maple genotypes desirable as urban landscape plants. Propagation by cuttings is usually difficult and inefficacious, and a tendency for overwintering death of cuttings may also be a detriment (Nesom 2006, Hartmann and others 2011). A high throughput asexual propagation protocol for Douglas maple is needed such that superior genotypes can be genetically conserved and rapidly propagated. High landscape potential combined with a lack of established protocols for asexual propagation provides ample justification for micropropagation research on Douglas maple. A positive attribute of micropropagation is that it typically causes a partial reversion of cultured plant tissue to a more juvenile state

(Hartmann and others 2011). This is important because juvenile tissue is easier to root (Hartmann and others 2011). Micropropagation via nodal culture is the technique researched herein. Adventitious shoot proliferation methods could likely achieve higher propagative increase ratios than nodal culture, but somaclonal variation is considered to be more common (Hartmann and others 2011). Somaclonal variation is important to avoid for maintaining true-to-type plantlets, thus nodal culture was chosen over an adventitious method.

Within this chapter the first 2 stages of micropropagation – establishment and multiplication – of Douglas maple are addressed. Establishment is herein defined as the surface sterilization of tissue such that no signs of contamination are visible in aseptic culture, and that buds break and shoots start to grow. Stabilization of tissue is often considered an aspect of establishment; however, stabilization takes place on a continuum and is difficult to define until the growth characteristics of the species within in vitro culture have been determined. For this research, stabilization was not measured or considered for determining establishment success. Additionally, light intensity was tested as a factor of explant quality.

Establishment:

Several maple species have been successfully established in vitro from nodal stem explants. Tissue selected for culture is typically derived from young, juvenile material, of trees 5 years old or less (Ďurkovič 2003, Bowen-O'Connor and others 2007, Lattier and others 2013). Ontologically mature tissues can also be used but may establish at a lower rate of success than juvenile tissues (Wann and Gates 1993). The timing of tissue collection may also play a role in the success of establishment; spring is indicated as being the best time (Wann and Gates 1993).

A review of past research reveals that surface sterilization of maple stem explants was most commonly accomplished by rinsing stems under tap water and then soaking in a bleach solution, which was then followed by repeatedly soaking in sterile water. Effective bleach concentrations usually ranged from 20 to 35% (v/v) in water. The duration of bleach solution exposure has typically ranged from 5 to 20 minutes (Wann & Gates, 1993; Bowen-O'Connor et al., 2007). Based on published data, it appears that decontamination of *Acer* species plant material is of minimal hindrance to the establishment of tissue in culture.

Exudation of dark colored compounds from explants into the media after surface sterilization was common with most cultured *Acer* species (Wann and Gates 1993, Ďurkovič 2003, Bowen-O'Connor and others 2007). It is a well-known problem that exudate accumulation in tissue culture medium can cause necrotic effects on explant tissues. As a remedy in earlier research, explants were transferred to fresh medium frequently, or to less discolored sections of the same medium (Wann and Gates 1993, Bowen-O'Connor and others 2007).

Though most research reports on *Acer* species lack details on explant establishment, relevant information about media used for successful establishment can be gleaned from careful inspection of employed methods. Silver maple (*Acer saccharinum* L.) and sycamore maple (*Acer pseudoplatanus* L.) were cultured on DKW medium salts (Ďurkovič and Andrea 2008). Norway maple (*Acer platanoides* L.) was successfully cultured on MS medium salts (Lattier and others 2013). And sugar maple (*Acer saccharum* Marshall) was established on MS medium salts (Ďurkovič and Andrea 2008). Cytokinin type and amount also varied, but BA, TDZ and zeatin were all indicated as efficacious cytokinin molecules (Ďurkovič and Andrea 2008).

Shoot growth initiation (the second part in tissue establishment) rates were only addressed within a few of the reviewed papers. Thirty-three percent of surface sterilized bigtooth maple (*Acer grandidentatum* Nutt.) stem explants placed on DKW medium with 10 μM zeatin successfully established after 120 days (Bowen-O'Connor and others 2007). Other medium types tested in this experiment were MS, LS, and WPM, but these formulations had significantly lower establishment rates.

In summary, *Acer* species are often established using juvenile tissue with actively growing shoots. Surface sterilization using bleach is a proven and common method, which seems to entail minimal complication. Initialization of shoot growth can be accomplished on sundry types of basal salt formulations and plant growth regulators.

Shoot Multiplication: Multiplication medium constituents typically included a commercial nutrient salts mixture, 6 to 8 g/l (0.79 to 1.06 oz/gal) agar, vitamins, 20 to 30 g/l (2.69 to 4.01 oz/gal) of sucrose, cytokinin, and sometimes an auxin. Cytokinin is perhaps the most crucial element for enhancing multiplication of plant material during micropropagation. As such,

most experimentation of *Acer* species involved investigations of cytokinin type and the concentrations thereof. Experiments conducted on silver maple, sugar maple, sycamore maple, red maple (*Acer rubrum* L.) and Montpellier maple (*Acer monspessulanum* L.) showed that TDZ alone or in combination with other plant growth regulators was most efficacious at promoting shoot growth (Preece and others 1991, Wann and Gates 1993, Wilhelm 1999, Brassard and others 2003, Heidari and Safarnejad 2015). Benzyl adenine, when used alone, was inferior or even inhibitory to shoot growth, except with Norway maple, which grew optimally with 2 to 4 µM BA (Lattier et al., 2013). Sycamore maple grew best with a combination of TDZ and BA (Wilhelm, 1999). Kawakami maple grew best with a combination of BA and NAA (Ďurkovič 2003). Bigtooth maple grew best with zeatin. Benzyladenine and TDZ were tried on bigtooth maple, but failed to induce shoot elongation (Bowen-O'Connor and others 2007).

Light Intensity: Most *Acer*-specific cultures are maintained with a 16 hour photoperiod. Most culture systems use cool white fluorescent lamps as their light source. Supplied light intensity was highly variable between maple cultures of different species by different researchers. Excluding sugar maple, cultures of other *Acer* species were grown with a light intensity between 33 and 75 $\mu mols{\bullet}m^{-2}{\bullet}s^{-1}$ (3.07 and 6.97 $\mu mols{\bullet}ft^{-2}{\bullet}s^{-1}$) PPF (Preece and others 1991, Lattier and others 2013). In some studies, explant quality was improved by decreasing light intensity (Wann and Gates 1993, Singh and others 2017). Singh and others (2017) explored this factor in great detail and found that under full-spectrum white light, sugar maple was best grown at 16 $\mu mols{\bullet}m^{-2}{\bullet}s^{-1}$ (1.49 $\mu mols{\bullet}ft^{-2}{\bullet}s^{-1}$) PPF. Even 40 $\mu mols{\bullet}m^{-2}{\bullet}s^{-1}$ (3.72 $\mu mols{\bullet}ft^{-2}{\bullet}s^{-1}$) PPF was too high for sugar maple, and this intensity is on the lower end of what is typically used in micropropagation systems. Light intensity failed to significantly affect shoot length or node number, but it did significantly affect dry and fresh weights (Singh and others 2017). Explants cultured at 40 $\mu mols{\bullet}m^{-2}{\bullet}s^{-1}$ (3.72 $\mu mols{\bullet}ft^{-2}{\bullet}s^{-1}$) PPF actually had the higher weights, but showed visual signs of stress, and low leaf chlorophyll content. Another sugar maple research article reports using a light intensity of 80 $\mu mols{\bullet}m^{-2}{\bullet}s^{-1}$ (7.43 $\mu mols{\bullet}ft^{-2}{\bullet}s^{-1}$) PPF (Brassard and others 2003), but whether this intensity caused detrimental effects is unknown.

The overall goal of the research described in this chapter is to determine methods and materials that effectively and efficiently support explant establishment and shoot multiplication of Douglas maple. Herein, experiments are focused on cytokinin optimization, resource and time efficiency, and light intensity for rapid plant reproduction.

MATERIALS AND METHODS

Plant Material: Two accessions of Douglas maple were used in the experiments. Tissue of the first accession, herein called genotype 1 (G1), was collected from the University of Idaho Arboretum in Moscow, Idaho. The plant was obtained by the arboretum staff from a nursery called Plants of the Wild in Tekoa, Washington. Plants of the Wild staff were not able to provide precise provenance information, but said the seed used was likely from Bonner County in northern Idaho. The tree was ontologically immature, having not yet produced seed or flowers. The plant material used in this experiment was collected and inducted into tissue culture on August 9, 2017.

The second accession, herein called genotype 2 (G2), was obtained from Dr. Stephen Love, extension/research professor at the University of Idaho Aberdeen Research and Extension Center. The source plant was located on the research station in Aberdeen. The accession was originally collected in the Albion Mountains, near the community of Oakley in southern Idaho. Ontologically immature stem cuttings were used as a source of propagation material and inducted into tissue culture July 30, 2018.

All experimentation made use of G1. Genotype 2 was used only in 1 experiment which compared in vitro growth rates of the 2 genotypes on a standardized medium optimized for G1.

The following italicized, bold headings and subsequent sections expound key details which are referenced throughout the chapter. These details are common to several experiments and sections.

Standard Data Collection Procedures:

- Total number of shoots was recorded by observation. The main apical shoot was included in the count. A minimum of 5 mm (0.20 in) stem length was required to be considered a shoot.
- Total shoot length was recorded by measuring from the base of the explant (excluding basal callus tissue) to the apical meristem of the shoot. To qualify for a recorded length, the shoot needed to be at least 5 mm (0.20 in) in length.
- Number of nodes was counted only on explants that were 5+ mm (0.20 in) in length. Visible buds, or any 2 oppositely arranged leaves originating from the same position on the stem counted as a node. The uppermost node was not included if leaves associated with it were still crinkled due to their lack of development. The goal was to include only nodes that could be cut and used as an explant.
- Dry weights were recorded by removing basal callus tissue and placing all stem and leaf material into an envelope. The envelope was put in a drying oven for a minimum of 3 days at ~77° C (171° F). The dried plant material was then weighed.
- SPAD chlorophyll meter readings were conducted by selecting the largest 3 leaves from each explant and recording 2 samples from each leaf. The 6 SPAD samples were then averaged for the explant, and the explant averages were averaged per tissue culture vessel.

Standard Medium:

The base medium recipe used for all experiments will be referred to as the *standard medium.* Some experiments had minor changes or additional, unique specifications which will be stated within the respective experiment materials and methods sections. The *standard medium* recipe per liter (33.81 oz) was: 5.2 g (0.18 oz) DKW medium salts, 45.4 mg (0.0016 oz) NaFeEDTA, 30.0 g (1.06 oz) D-sucrose, 5.0 ml (0.17 oz) stock G vitamin solution (20 mg (0.00071 oz) thiamine-HCl, 10 mg (0.00035 oz) nicotinic acid, 10 mg (0.00035 oz) pyridoxine HCl and 40 mg (0.0014 oz) glycine in 100 ml (3.38 oz) of reverse osmosis (RO) water), 5.0 ml (0.17 oz) stock H vitamin solution (2.0 g (0.071 oz) myo-inositol in 100 ml (3.38 oz) of RO water), the appropriate amount of cytokinin as per treatment (except for when filter sterilization is needed), 1 M or 0.1 M HCl and/or NaOH to adjust the pH of the medium to 5.80 ± 0.02, and 8.0 g (0.28 oz) agar. Medium was then poured into individual 7.7

x 7.7 x 9.7 cm (3.0 x 3.0 x 3.8 in) tissue culture vessels (Magenta™ GA-7) before autoclaving. For treatments needing filter sterilized constituents, media were autoclaved in flasks and then cooled to a touchable temperature. In a laminar flow hood, constituents were added via filter sterilization (Corning Incorporated 25mm syringe filter – 0.20 microns) and mixed by swirling. Then the media were poured into autoclaved tissue culture vessels, also in a laminar flow hood. The autoclave cycles ran for 25 minutes at 121° C (250° F) and 103.4 kPa (15 psi). Roughly 50 ml (1.69 oz) of media were aliquoted per tissue culture vessel. The *standard medium* recipe changed for the efficiency experiments, which included the addition of 12 mg/L (0.0016 oz/gal) of boric acid (H_3BO_3).

Stock Explants: the term *stock explant* used herein refers to shoot explants that have been cultured on *standard medium*. *Stock explants* were usually grown at a density of 5 explants per Magenta™ GA-7 vessel. *Stock explants* were subcultured about every 5 weeks.

Establishment

Establishment procedures used for Douglas maple were primarily influenced by findings from research on bigtooth maple in Bowen-O'Connor and others (2007). Bigtooth maple is native to some of the same regions as Douglas maple. Given this similarity, both species may have similarities in nutrient and cytokinin requirements for growth in vitro. Essentially, bigtooth maple initiated shoot growth best on DKW medium salts with zeatin. Therefore, DKW medium salts with zeatin was evaluated as a treatment for establishment of Douglas maple. In addition, DKW medium salts with addition of BA was also tried. A factorial treatment design was used in this experiment. Both cytokinins were tested at 4 concentrations (0, 7, 14, or 21 μM) and the number of nodes on the explant at 2 levels (1 or 2 nodes).

Establishment began with the collection of G1stem tissue of the current year's growth. Collection was done early in the morning to minimize water loss in the tissue. Explant material was put in a cooler with moist paper towels and quickly transferred to the lab for sterilization. Sterilization was accomplished by cutting the stems into 1 or 2 node sections, and then placing in a 20% (v/v) bleach solution with a few drops of Tween 20 for 15 to 20 minutes. The stem segments were then transferred to 3 sterile water baths for 2 minutes each.

Cut surfaces exposed to bleach were cut a second time (approximately 1 to 4 mm (0.04 to 0.16 in) removed) to expose fresh tissue, and then stem segments were placed into media.

Standard medium was used but with the following differences and specifications. The pH of the media was adjusted to 5.7 ± 0.12. Roughly 10 ml (0.34 oz) of medium were aliquoted per vessel (glass culture tube). Media was autoclaved for 20 minutes at 121° C (250° F) and 103.4 kPa (15.0 psi). Zeatin was added to the media after autoclaving by filter sterilization, whereas BA was added before autoclaving. Each treatment combination had 3 replicates.

Dependent variable data collection took place 28 days after the start of the first culture, and again 29 days after the first subculture. The dependent variables included contamination presence or absence, and how many buds initiated growth (having opened bud scales and produced visible foliage).

Two months after induction of tissue into culture, filter sterilized zeatin at 4 levels (0, 7, 14 and 21 µM) was combined with 0.75 µM IBA (compared to a zero-level control) to see if it enhanced shoot growth. This was not a formal experiment, and no data was recorded, but visual observations indicated that IBA didn't enhance shoot growth.

Multiplication

Three cytokinin optimization experiments were completed. The first (*Cytokinin Experiment 1*) compared several cytokinin types and concentrations in an attempt to identify an alternative cytokinin to zeatin. The results from *Cytokinin Experiment 1* directed the design of the second and third cytokinin experiments called *Cytokinin Experiment 2* and *Cytokinin Experiment 3,* respectively. *Cytokinin Experiment 2* tested zeatin levels at higher resolution, and under autoclaved and filter sterilized treatment. *Cytokinin Experiment 3* tested other cytokinin alternatives absent from *Cytokinin Experiment 1*.

The goal for *Cytokinin Experiment 1* was to: 1) to identify the most efficacious cytokinin compounds and concentrations thereof by quantitatively measuring their performance, and 2) determine if autoclaving changes zeatin efficacy/potency by contrasting with filter sterilized zeatin effects on shoots. *Cytokinin Experiment 1* design was a randomized complete block with a factorial treatment structure. All plant material was G1. Limited explant material

required that explant number was maximized by using plant material derived from 4 different medium types; in other words, uniform *stock explants* were not used. To account for variation caused by explant derivation, the experiment was blocked on previous medium type so that there were 4 blocks. In addition, each block was placed on a different shelf within the growth chamber; so this confounded medium type and shelf position in the blocking structure. The independent variables tested were: cytokinin compound (kinetin, 2iP, BA and zeatin (autoclaved and filter sterilized)) and cytokinin concentration (0, 1, 2, 4, 8, 16, or 32 μM). Kinetin, 2iP, and BA were chosen because they were the cheapest cytokinin compounds, while filter sterilized zeatin served as a positive control (having shown its efficacy during establishment). In addition, sterilization method of zeatin during medium preparation was explored as a factor in this experiment; thus, filter sterilized zeatin and autoclaved zeatin were essentially tested as 2 separate cytokinin types. The dependent variables were the number of shoots ≥ 5 mm (0.20 in) per explant, the number of nodes per explant, and the total shoot length per original explant. Thirty-five vessels were used per block (35 treatments with 1 treatment replicate each). Each tissue culture vessel had 2 explants within it. *Standard medium* recipe (aforementioned) and procedures were used.

The experiment involved 2 subcultures: the first of 29 days and the second of 29 days for block 1, 30 days for blocks 2 and 3, and 31 days for block 4. Explant specifications for the first subculture were 6 to 14 mm (0.24 to 0.55 in) in shoot length with 2 to 3 leaves, and in the second subculture they were 4 to 13 mm (0.16 to 0.51 in) in length and also 2 to 3 leaves. Explant cuttings were prepared using a scalpel to cut plant material to specified lengths. Data were collected at the end of the second subculture.

Cytokinin Experiment 2 was conducted with the purpose of quantifying the efficacy of zeatin and to determine if sterilization method was of importance to potency. The design was a randomized complete block with a factorial treatment structure and 3 block replications. The blocking variable started as the shelf within the growth chamber. The second subculture started as being blocked by shelf, but the blocking variable was changed to the row on a single shelf. This change of blocking variable came from observations about how light intensity was affecting explant quality and morphology. The third subculture was blocked according to row on the shelf (see Figure 1.1). Blocking according to row helped to isolate

variation due to light intensity. *Cytokinin Experiment 2* had independent variables of: sterilization method of zeatin (autoclaved or filter sterilized) and concentration of zeatin at 5 levels (4.0, 6.5, 9.0, 11.5 and 14.0 µM). The independent variables collected were total shoot length, number of nodes per explant, and dry weights (see *standard data collection procedures* aforementioned). Genotype 1 *stock explants* were used for this experiment. This experiment lasted 3 subcultures: the first and second both being 28 days, and the last being 29 to 30 days depending on block. A total of 20 vessels were used per block (10 treatments with 2 treatment replicates each). Each vessel contained 4 explants. Explants used in the experiment were 8 to 18 mm (0.31 to 0.71 in) in length. Lengths of explants at start of final subculture were 5 to 15 mm (0.24 to 0.59 in) and had 2 to 3 leaves. Data were collected at the end of the third subculture.

The *standard medium* (aforementioned) recipe was used for making the treatments.

Cytokinin Experiment 3 was conducted for the purpose of identifying an efficacious cytokinin alternative to zeatin. The continued importance of finding an alternative has to do with the cost of zeatin. For perspective, on January 14, 2019, from PhytoTechnology Laboratories®, the price of zeatin was $1065.07/g, whereas BA was just $5.20/g.

This experiment had a randomized complete block design with 4 block replications and factorial treatment structure. The blocking variable was light intensity, which was blocked by rows with varying exposure to the light source (see figure 1.1). Independent variables for this experiment were: no cytokinin compound (negative control), zeatin (positive control), MT and TDZ), and cytokinin concentration (zeatin: 6.5 µM; MT: 2, 4, 8 or 16 µM; TDZ: 50, 100, 200 or 400 nM). The dependent variables collected were: total shoot length per explant, the number of nodes per explant, and the dry weight (see *standard data collection procedures,* aforementioned). Genotype 1 *stock explants* were used in this experiment. Ten vessels were used per block (10 treatments with 1 treatment replicate each). Each vessel had 4 explants within it. This experiment lasted 2 subcultures: the first being 32 days in duration and the second being 31 days in duration. Data were collected at the end of the second subculture.

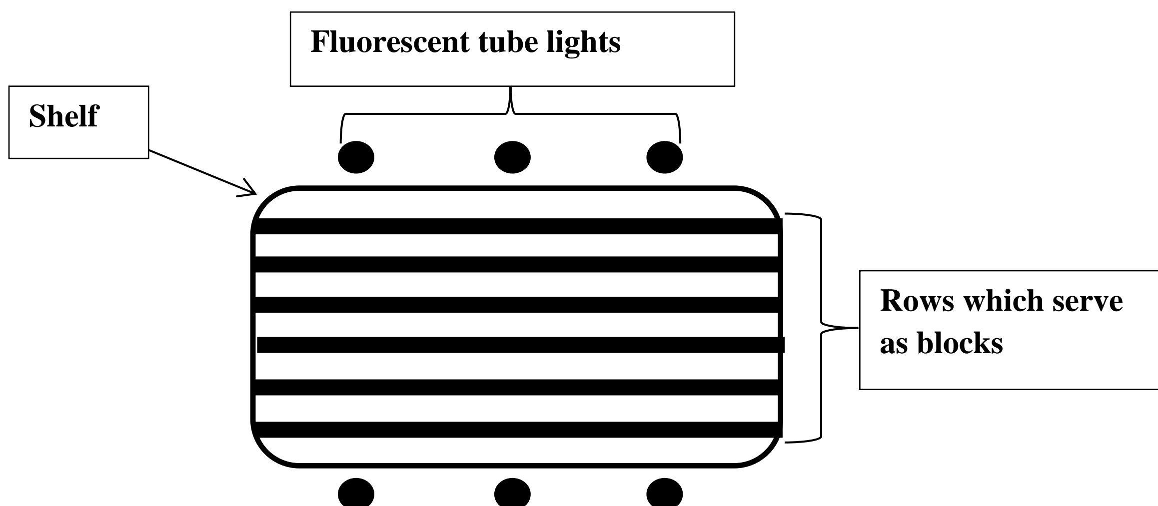

Figure 1.1. Top-view diagram of the blocking structure used in *Cytokinin Experiment 2* and *Cytokinin Experiment 3.* Rows were of GA-7 tissue culture vessels.

Efficiency

Two multiplication efficiency experiments were conducted. The first, called *Efficiency Experiment 1*, was a growth rate experiment aimed at comparing the difference in growth between G1 and G2 over the course of 6 weeks in vitro. The second, called *Efficiency Experiment 2*, tested the effect of explant density at 3 levels (4, 6 and 8 explants per GA-7 box).

Efficiency Experiment 1 was conducted with the purpose of determining the efficacy of *standard medium*, which was optimized for G1, on a second genotype (G2). Growth rates were also of interest as a factor effecting resource cost and efficiency and the propagative increase rate. *Efficiency Experiment 1* was designed using a completely randomized arrangement. There were a total of 24 tissue culture vessels – 12 of each genotype (Figure 1.2). The independent variables were genotype (G1 or G2) and collection date (4 dates at roughly 3, 4, 5 and 6 weeks after experiment commencement). The dependent variables were the total shoot length, number of nodes per explant and dry weight of all plant tissue per vessel (refer to *standard data collection procedures* aforementioned). Four explants were in each vessel. Both G1 and G2 were previously cultured as per *stock explant* specifications aforementioned. There were 2 subcultures – the first being 29 days for G2 and 30 days for G1 (the 1-day difference due to logistical complications), and the second subculture being of

roughly 3, 4, 5, or 6 weeks duration depending on data collection date. The number of days after commencing the second subculture until data collection were precisely 21, 27, 35, 42 days for G1 and 22, 28, 36, and 43 days for G2. The first subculture explant specifications were highly variable because G2 had limited plant material and was just starting to stabilize in culture. Eighteen tissue culture vessels (4 explants in each) were used per genotype in the first subculture. After the first subculture it was determined by visual evaluation that only 12 vessels of G2 could be cultured because some of the explants appeared to have irregular growth and development (not yet stabilized) in tissue culture; thus, 12 vessels of G1 were also cultured in the second subculture. The specifications of explants for both genotypes of the second subculture were 6 to 12 mm (0.24 to 0.47 in) in length, 2 to 3 leaves, and all apical explants. One layer of ~55% shade cloth was placed around the vessels to reduce light intensity.

All explants were grown on the *standard medium* recipe. Autoclaved zeatin at 4 µM concentration was the choice of growth regulator.

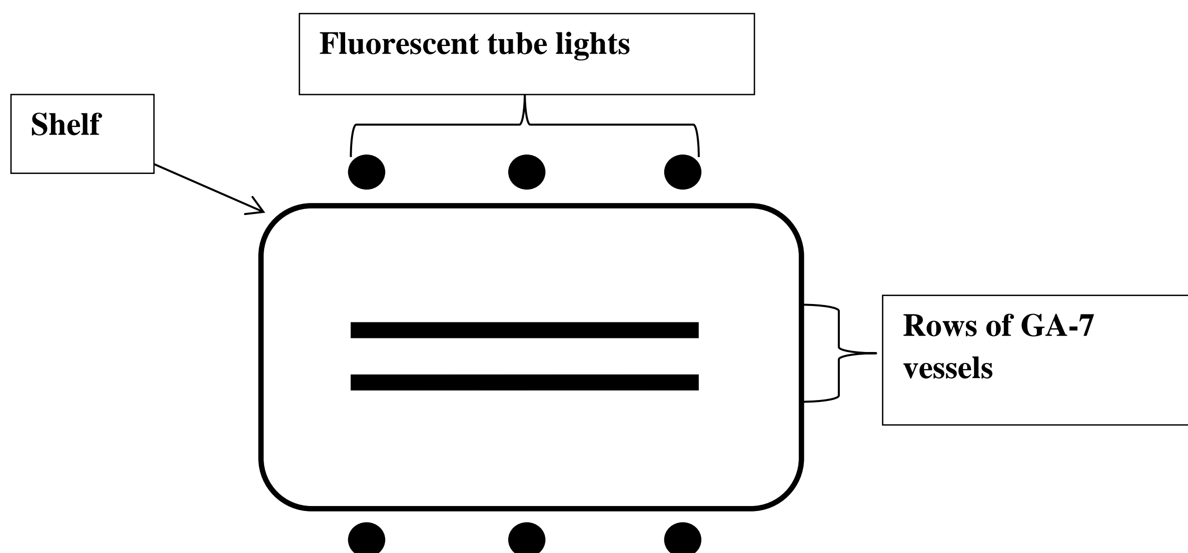

Figure 1.2. Top view of growth chamber shelf showing positioning of vessels for *Efficiency Experiment 1*. Two vertically adjacent shelves were used. Each shelf had 2 rows of 6 vessels.

Efficiency Experiment 2 was conducted with the purpose of determining whether explant density per GA-7 vessel impacted individual explant growth. The results of this experiment were intended to improve resource and cost efficiency of micropropagation. *Efficiency*

Experiment 2 design was a randomized complete block with 5 block replications. This experiment was blocked by row to account for light intensity (see Figure 1.1). The independent variable was explant density at 4, 6 and 8 explants per vessel, positioned as shown in Figure 1.3. The dependent variables were total shoot length, number of nodes per explant and dry weight of all plant tissue per vessel (refer to *standard data collection procedures,* aforementioned). Genotype 1 *stock explants* were used. Three vessels were used per block (3 treatments with 1 treatment replicate each). Only 1 subculture was implemented for this experiment, after which, data were collected. The subculture lasted 27 days for block 1 and 30 days for blocks 2 through 5. Unfortunately, exact specifications for explant length at experiment commencement were not documented. However, it is certain that they started between 5 and 20 mm (0.2 and 0.79 in) in length and had 2 to 3 leaves. All explants were nodal (non-apical) shoot explants.

The *standard medium* recipe (aforementioned) was used for the medium employed during this experiment. Autoclaved zeatin at 4 μM concentration was the growth regulator of choice.

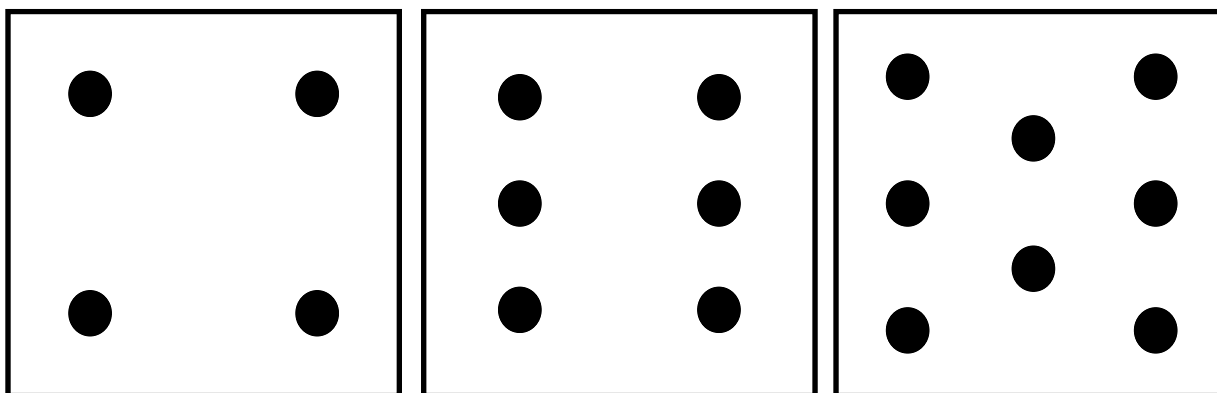

Figure 1.3. Top view diagram of explant positioning within GA-7 tissue culture vessels for the 3 explant densities tested in *Efficiency Experiment 1*. The squares represent the outline of the walls of the GA-7 vessel, while the solid, black circles denote explants and their approximate position in medium within the culture vessel. The left vessel is explant density 4 (this density and arrangement was also used in all other experiments except establishment and *Cytokinin Experiment 1*); the middle vessel is explant density 6; and right vessel is explant density 8.

Light Intensity

A light intensity experiment was conducted with the purpose of determining whether lower than normal light intensities would improve explant quality as seen in sugar maple (Singh and others 2017). The experimental design was a randomized complete block with 4 block replications. The experiment was blocked by row to account for light intensity (see figure 1.4). The independent variable was light intensity which was administered by 0, 1, or 2 layers of shade cloth which was wrapped around, placed on, and placed under, the GA-7 vessels. The dimensions of the shade cloth wrapped around the vertical faces of the vessel were roughly 11.4 x 35.6 cm (4.5 x 14.0 in) for 1 layer/wrap of shade cloth, and 11.4 x 71.1 cm (4.5 x 28.0 in) for 2 layers/wraps of shade cloth. One or 2, 7.6 x 7.6 cm (3.0 x 3.0 in) patches were also placed at the bottom and top faces of the vessel, as appropriate per treatment. The shade cloth was black in color, and reduced light intensity by roughly 55%. The actual PPF's for the treatments were 36.6 ± 1.7 $\mu mols \bullet m^{-2} \bullet s^{-1}$ (3.40 ± 0.16 $\mu mols \bullet ft^{2} \bullet s^{-1}$) (no shade cloth), 16.8 ± 1.5 $\mu mols \bullet m^{-2} \bullet s^{-1}$ (1.56 ± 0.14 $\mu mols \bullet ft^{-2} \bullet s^{-1}$) (1 layer of shade cloth), and 6.6 ± 0.7 $\mu mols \bullet m^{-2} \bullet s^{-1}$ (0.61 ± 0.07 $\mu mols \bullet ft^{-2} \bullet s^{-1}$) (2 layers of shade cloth). Four explants were used per vessel. Genotype 1 *stock explants* were used in this experiment. Specifications of explants were 8 to 16 mm (0.31 to 0.63 in), 2 leaves, and nodal explants only. The dependent variables were total shoot length per explant, number of nodes per explant, SPAD reading, and dry weight of all plant tissue per vessel (refer to *standard data collection procedure,* aforementioned). Three vessels were used per block (3 treatments with 1 treatment replicate per treatment). The duration of this experiment was 1 subculture of 32 days. Data were collected at the end of the subculture.

The *standard medium* recipe (aforementioned) was employed for this experiment. Autoclaved zeatin at 4 µM concentration was the growth regulator used.

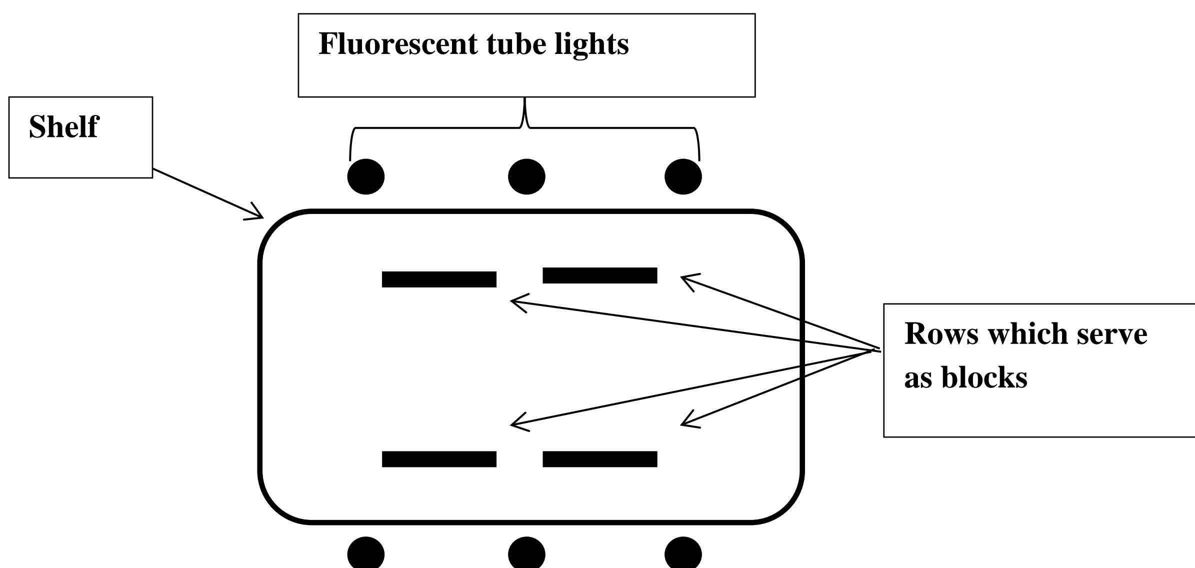

Figure 1.4. Top view of shelf in growth chamber showing blocking structure in the light intensity experiment.

Environmental Conditions of Culture: The establishment experiment was cultured on an open-air rack with above-lighting from General Electric brand Ecolux 34 W soft white fluorescent light tubes. All other experiments mention herein were cultured in a Hoffman Manufacturing Company (Albany, Oregon) growth chamber using side-mounted General Electric brand Ecolux 34 W soft white fluorescent light tubes. The PPF in the growth chamber ranged greatly from roughly 5 to 80 $\mu mols \bullet m^{-2} \bullet s^{-1}$ (0.46 to 7.43 $\mu mols \bullet ft^{-2} \bullet s^{-1}$) depending on position of vessel on the shelf, the number of rows, and the amount of in vitro vegetation blocking light to inside rows. This wide range of PPF in the growth chamber was addressed by blocking the experiments according to row upon the shelf, as seen in Figures 1.1, 1.2 and 1.4. Addressing this wide range of PPF by blocking was important, because some *Acer* species (e.g. *Acer saccharum*) are very sensitive to light intensity. Douglas maple also showed signs of light stress at just 37 $\mu mols \bullet m^{-2} \bullet s^{-1}$ (3.44 $\mu mols \bullet ft^{-2} \bullet s^{-1}$) PPF. The photoperiod in the growth chamber was kept at 16 h, and the temperature was kept constant at 22° ± 2° C (71.6° ±3.6° F). Lastly, no micropore tape was used around the lids of the tissue culture vessels to allow maximum gas exchange and minimize potential ethylene accumulation.

Statistical analyses of data from experiments were completed using the RStudio function ‘lmer’ to create linear mixed effect regression models. Treatments were the fixed effects,

whereas the blocks were the random effects within the models. Analysis of deviance was completed on the models using the 'Anova' function from the 'car' package. Type-II sums of squares were used for the calculation of analysis of deviance tables. The F-test and statistic were used in providing P-values in the analysis of deviances. The T-test and statistic were used in providing P-values for the Holm-Bonferroni pairwise comparisons of individual treatment means.

RESULTS

Establishment

Image 1.1. A shoot that had been successfully surface sterilized, and had broken bud, and was initiating shoot growth in the establishment stage of micropropagation.

Rates of explants free of contamination after 28 initial days of culture was 45.8%. The control (no supplemental cytokinin) had the highest ratio of bud break – 10 broken buds from 12 explants. The second best with 8 buds breaking of 12 explants, were those placed in a medium containing zeatin. Medium containing BA yielded 5 broken buds from 12 explants. After a second subculture, the zeatin containing medium produced 15 broken buds, whereas BA produced 7. Despite the control medium producing high numbers of broken buds after the first subculture, shoot elongation was slow relative to growth on zeatin containing medium, and was therefore considered to be inferior. Single node explants produced 10

broken buds from 18 explants, and double node explants produced 13 broken buds from 18 explants.

Cytokinin Experiment 1

Cytokinin Experiment 1 was considered to be a preliminary experiment due to its low sample size and large breadth of concentration levels. The residuals were far from normally distributed and the studentized residual by predicted plot showed patterning, not random scatter. The data failed to meet assumptions for statistical analysis. Nevertheless, average values provided a good estimate of performance of particular cytokinins. Kinetin, 2iP and BA all consistently performed poorly. None of the concentrations of those 3 cytokinins enhanced explant shoot length more than the control treatment containing 0 µM cytokinin. Zeatin at concentrations between 2 to 8 µM induced the highest average shoot lengths. The effects of the sterilization method on the effectiveness of zeatin were unclear.

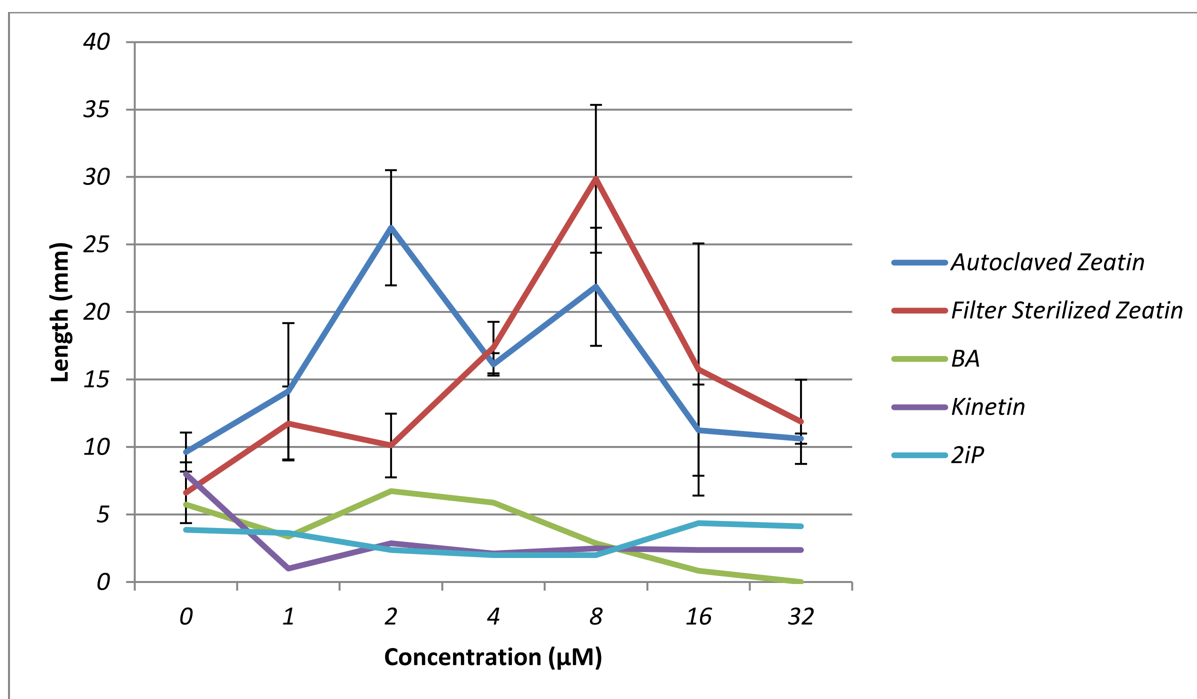

Figure 1.5. Average shoot length in *Cytokinin Experiment 1* for cytokinin treatments, including factors composed of sterilization methods, cytokinin compound, and cytokinin concentration. Error bars show the standard error for autoclaved zeatin and filter sterilized zeatin. Error bars for other cytokinins were omitted because of extensive overlapping of error bars that are difficult to decipher.

Table 1.1. ***Cytokinin Experiment 1*** **average shoot length, number of shoots, and average number of nodes produced by explants grown on filter sterilized or autoclaved zeatin at 0, 1, 2, 4, 8, 16 or 32 µM. The data for kinetin, 2iP and BA were omitted from this table due to lack of significance.**

Sterilization and Compound	Concentration	Average Shoot Length (mm)	Average Number of Shoots	Average Number of Nodes
Filter Sterilized Zeatin	0	6.6	0.9	1.0
Filter Sterilized Zeatin	1	11.8	1.1	2.8
Filter Sterilized Zeatin	2	10.1	1.3	2.1
Filter Sterilized Zeatin	4	17.4	1.8	4.0
Filter Sterilized Zeatin	8	29.9	2.3	5.8
Filter Sterilized Zeatin	16	15.8	1.4	3.9
Filter Sterilized Zeatin	32	11.9	1.5	3.0
Autoclaved Zeatin	0	9.6	1.3	1.1
Autoclaved Zeatin	1	14.1	1.5	3.5
Autoclaved Zeatin	2	26.3	1.4	4.5
Autoclaved Zeatin	4	16.1	1.4	3.6
Autoclaved Zeatin	8	21.9	1.6	5.4
Autoclaved Zeatin	16	11.3	1.5	3.4
Autoclaved Zeatin	32	10.6	1.6	2.4

Cytokinin Experiment 2

Average shoot length was transformed using a $\log_{10}$ function prior to analysis to meet the assumption of normality of residuals for regression. Shoot length was significantly affected by the sterilization method ($P = 0.002$) – autoclaved zeatin producing the longer shoots (see Table 1.2). The effect of concentration of zeatin was nearly significant ($P = 0.070$) in effecting shoot length. Average dry weight and average number of nodes were also significantly affected by the sterilization method of zeatin ($P = 0.019$ and $P = 0.038$, respectively). The concentration of zeatin had a statistically significant effect only on average

dry weight ($P = 0.003$). The interaction between sterilization method and zeatin concentration did not significantly affect any of the response variables.

Grouping average shoot length data by sterilization method and then applying separate regression procedures to each group showed that the concentrations significantly affected shoot length for autoclaved zeatin ($P = 0.018$). A Holm-Bonferroni pairwise comparison of means showed that the significant differences were between 4.0 µM to 11.5 and 14.0 µM zeatin. Four µM autoclaved zeatin produced the longest shoots. With filter sterilized zeatin, average shoot length was transformed using $\log_{10}$ to meet normality of residuals. An analysis of deviance of the regression model lacked a significant difference for the effect of concentration.

Using a Pearson test of correlation, all 3 response variables were significantly correlated ($P < 0.001$ for all 3 paired combinations). This result could mean that zeatin directly affected shoot length, yet indirectly affected the average number of nodes and average dry weight both via increased shoot growth.

Table 1.2. ***Cytokinin Experiment 2*** **average values for dry weight, number of nodes, and shoot length, as separated by treatment group of 2 factors (sterilization method and zeatin concentration). The abbreviation SE stands for standard error. The SE value corresponds to the average value directly to its left.**

Concentration (µM)	Sterilization Method	Average Dry Weight per Explant (mg)	SE	Average Number of Nodes	SE	Average Shoot Length	SE
4.0	Autoclaved	29.3	4.5	4.3	0.2	35.2	3.3
6.5	Autoclaved	28.2	2.5	4.9	0.2	29.5	1.2
9.0	Autoclaved	28.1	4.5	4.3	0.4	25.2	4.7
11.5	Autoclaved	34.6	0.7	4.4	0.5	23.6	4.9
14.0	Autoclaved	38.6	2.5	5.0	0.2	21.3	1.9
4.0	Filter Sterilized	31.4	2.2	4.1	0.5	20.0	3.2
6.5	Filter Sterilized	26.2	4.3	4.0	0.4	23.6	4.6
9.0	Filter Sterilized	29.0	4.3	4.4	0.4	21.8	3.1
11.5	Filter Sterilized	29.0	1.8	3.8	0.3	16.5	2.3
14.0	Filter Sterilized	28.2	3.2	3.9	0.7	16.9	3.3

Cytokinin Experiment 3

Shoot length was significantly affected by treatment ($P < 0.001$). A Holm-Bonferroni pairwise comparison of the treatment means showed that 2, 4 and 8 µM MT were not significantly different from 6.5 µM zeatin (the positive control), whereas 0 µM cytokinin (negative control), 16 µM MT, and 50, 100, 200, 400 nM TDZ resulted in significantly less shoot growth (Table 1.3). The treatment producing the longest shoots was 2 µM MT (39.1 mm (1.54 in)) (see Figure 1.6). An analysis of deviance of regression model for average dry weight showed a significant affect from treatment ($P < 0.001$). Shoot length required transformation using a $\log_{10}$ function in order to satisfy normality of residuals. Normality of residuals for average dry weight was not met, so an outlier was removed to satisfy this requirement. The normality of residuals for the average number of nodes was not satisfied by transformation or removal of outliers so no significance is noted.

Table 1.3. ***Cytokinin Experiment 3*** **average values of response variables (dry weight, shoot length and number of nodes) by treatment (cytokinin and concentration). Abbreviations are: MT – meta-topolin; TDZ – thidiazuron. Letters denote significance, where $P \leq 0.05$. Average number of nodes failed to meet assumptions for analysis so no significance is denoted.**

Growth Regulator and Concentration	Average Dry Weight (mg)	Average Shoot Length (mm)	Average Number of Nodes
Zeatin 6.5 µM	25.4 a	31.0 a	4.8
0 µM Cytokinin	22.8 a	9.3 b	1.8
MT 2 µM	24.7 a	39.1 a	5.1
MT 4 µM	18.1 a	24.8 a	4.9
MT 8 µM	16.0 b	18.7 a	3.6
MT 16 µM	14.5 b	10.2 b	2.3
TDZ 50 nM	16.6 a	16.2 b	2.7
TDZ 100 nM	21.2 a	13.8 b	2.6
TDZ 200 nM	12.4 b	9.9 b	2.2
TDZ 400 nM	12.1 b	9.7 b	1.9

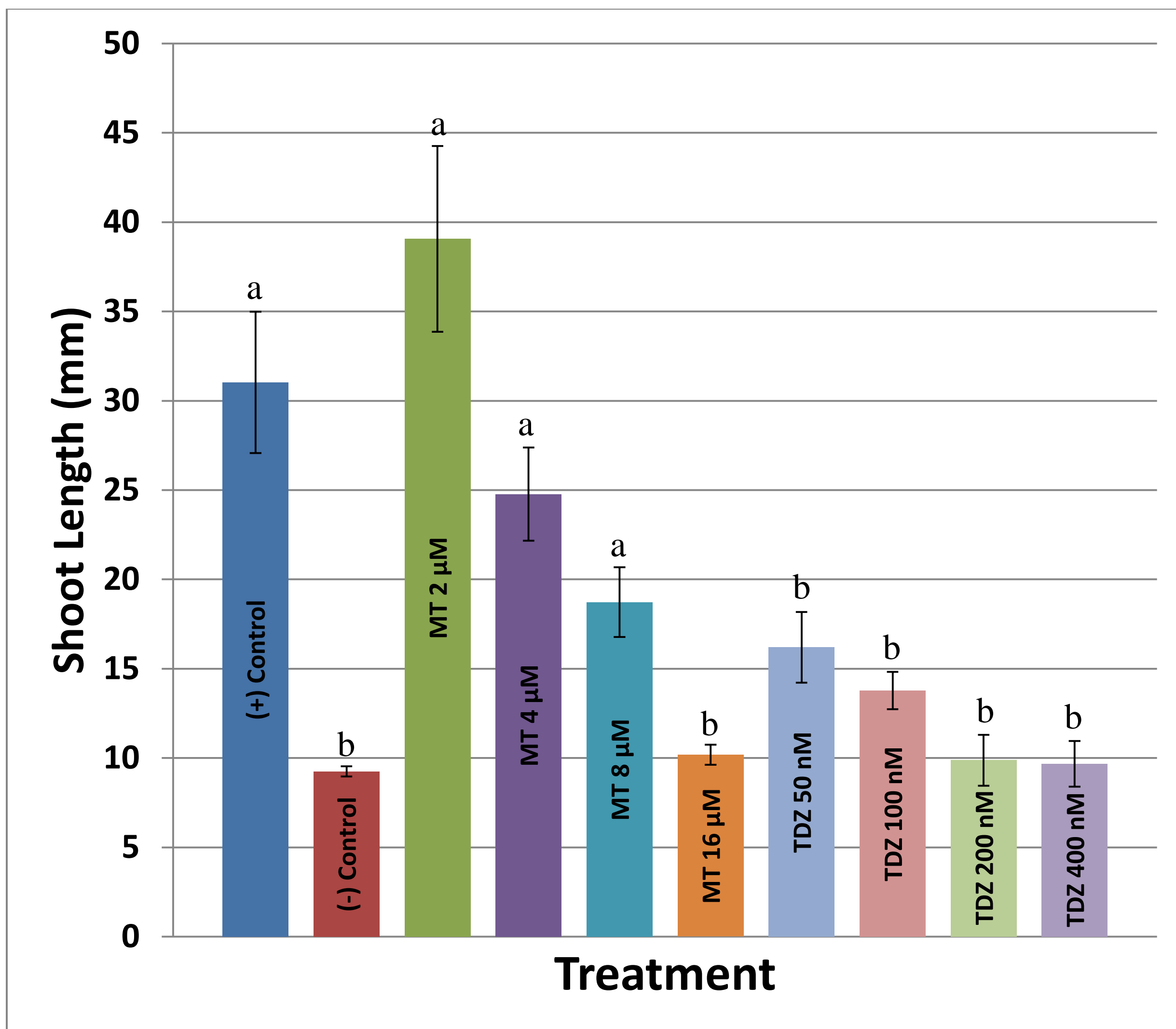

Figure 1.6. Average shoot length from *Cytokinin Experiment 3* as a result of treatment with a range of MT and TDZ concentrations. Error bars show standard error. Letters are provided to show significant differences with respect to the positive control with P ≤ 0.05.

Efficiency Experiment 1

Image 1.2. Explants from *Efficiency Experiment 1* after roughly 6 weeks of growth. The left picture is of 2 explants from G1 and the right picture is of 2 explants from G2.

Average shoot length was significantly affected by both the genotype ($P < 0.001$) and amount of time in culture (weeks in culture) ($P < 0.001$). Genotype by week interaction approached significance ($P = 0.074$). A Holm-Bonferroni pairwise comparison test showed the significant differences existed between week 3 to weeks 5 and 6, and between week 4 and week 6 – with longer times in culture resulting in higher week means.

Average dry weight was significantly affected by weeks in culture ($P < 0.001$). A Holm-Bonferroni pairwise comparison test showed the significant differences were between all week contrasts ($P < 0.020$) except 5 to 6.

Table 1.4. Means of dependent variables shoot length, number of nodes, dry weight and length per node by independent variables week and genotype from *Efficiency Experiment 1*. The abbreviation SE stands for standard error. The SE values are associated with the average value directly to the left.

Week	Genotype	Average Shoot Length Per Explant (mm)	SE	Average Number of Nodes Per Explant	SE	Average Dry Weight Per Explant (mg)	SE	Average Length (mm) Per Node	SE
3	G1	21.9	5.0	4.0	0.4	19.0	1.6	5.5	1.4
3	G2	17.0	2.6	4.2	0.2	16.9	1.1	4.1	0.4
4	G1	32.9	3.1	4.6	0.4	22.5	1.8	7.2	0.4
4	G2	25.3	6.3	5.2	0.8	31.8	3.4	4.9	0.5
5	G1	41.9	2.5	5.9	0.1	43.8	1.3	7.1	0.3
5	G2	29.8	4.4	6.8	0.7	42.6	2.8	4.4	0.4
6	G1	53.5	3.7	7.3	0.3	46.8	4.0	7.4	0.6
6	G2	27.9	0.9	6.5	0.1	51.5	4.1	4.3	0.1

The average number of nodes was significantly affected by the effect of week ($P < 0.001$). A Holm-Bonferroni pairwise comparison test showed the significant differences were between all week contrasts ($P < 0.020$) except 3 to 4 and 5 to 6 (Table 1.4). Average number of nodes was not affected by genotype, however, genotype did significantly influence length of internodes ($P < 0.001$). Genotype 1 had longer internodes.

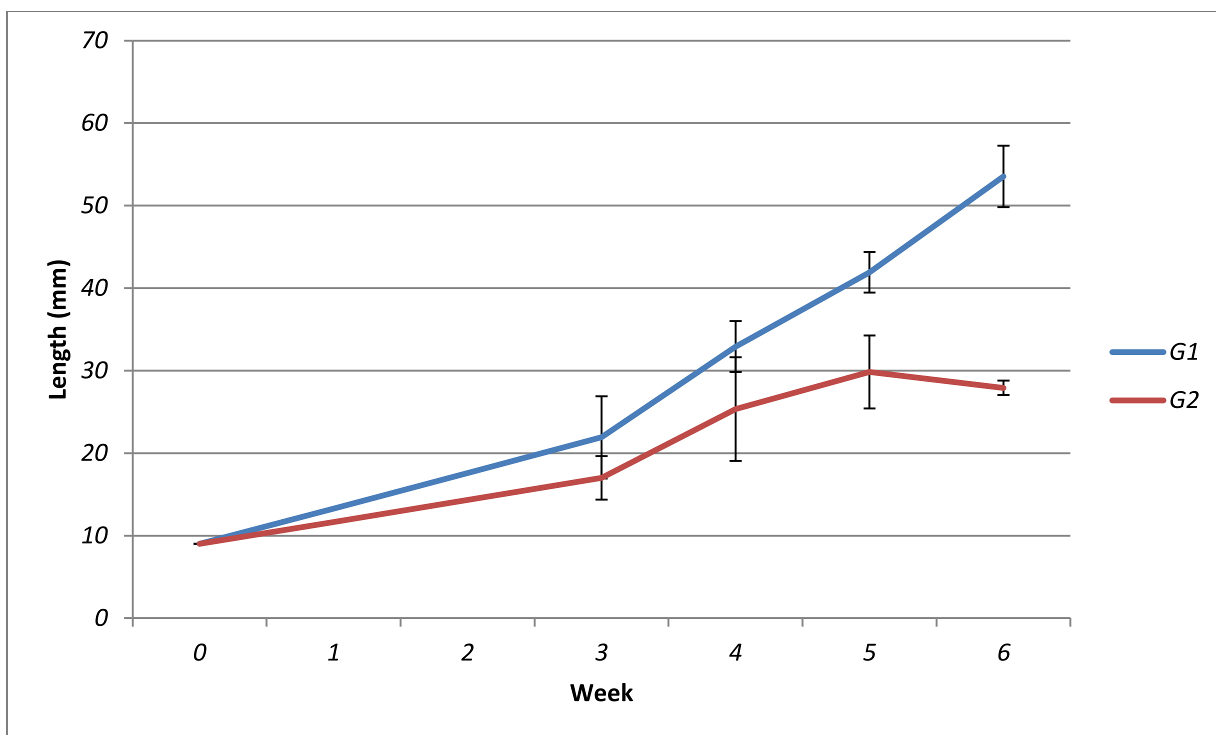

Figure 1.7. Graph of average shoot length progression over 6 weeks for 2 genotypes of Douglas maple, genotype 1 (G1) and genotype 2 (G2), in *Efficiency Experiment 1*. Error bars indicate standard error.

The growth rates between the two genotypes were very different (Figure 1.7). Genotype 1 appeared to have approximately linear growth from week 3 to week 6, whereas, G2 had a growth rate that decreased from over time. Genotype 2 had a high growth rate from week 3 to week 4 which was similar to the growth rate of G1, but each subsequent 1 week interval showed a degreasing rate of growth. The negative growth rate from week 5 to week 6 of G2 is indicative of variation likely due to varying degrees of explant stabilization. A spline fit of G2 shoot length by week yielded a low R-squared value (0.425), also indicating high variation. The linear equation of the regression for G1 shoot length is Y = (1.456) X – 7.937 (see Figure 1.8). The adjusted R-squared value of this line was 0.809, meaning this model explains 80.9% of the variation.

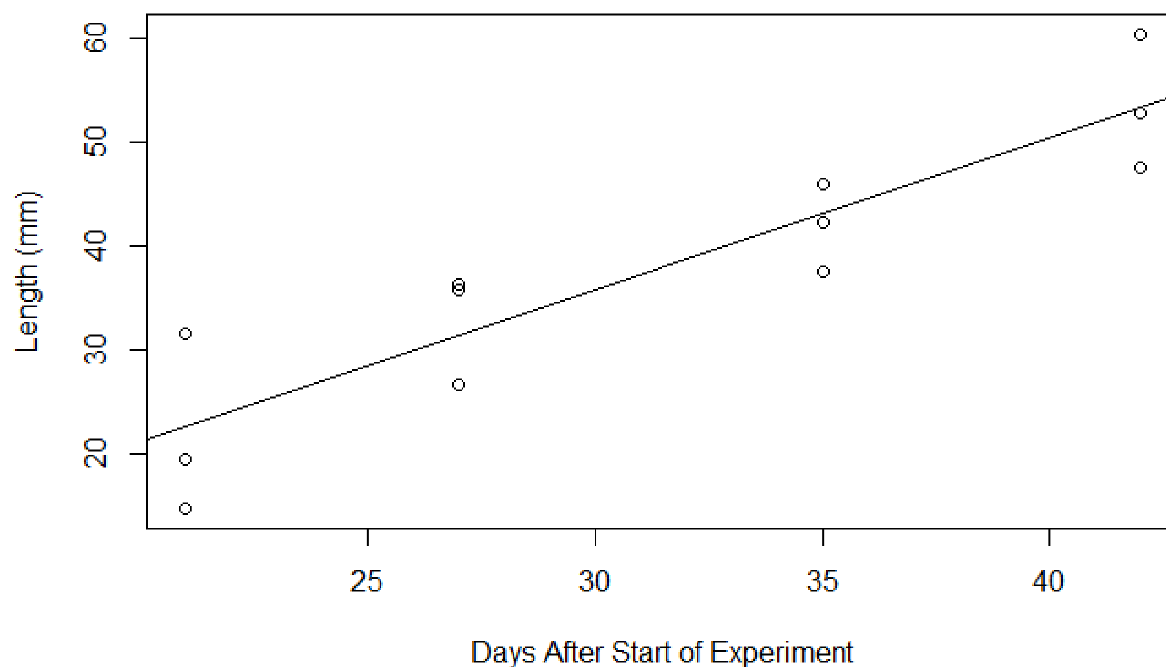

Figure 1.8. Best fit line of genotype 1 average shoot length by day after start of experiment (days in culture) of *Efficiency Experiment 1.* Equation of line is Y = (1.456) X – 7.937. Adjusted R-squared = 0.81.

Efficiency Experiment 2

Dry weight data was transformed using a $\log_{10}$ function to allow acceptable conformation to the rules of normality. The other regression models of the other 2 response variables met assumptions of analysis of deviance. Explant density (4, 6 or 8 explants per GA-7 vessel) lacked a significant effect on plantlet dry weight, number of nodes, or shoot length (Table 1.5). The lowest P-value came from the analysis of deviance of the regression model of average dry weight (P = 0.296).

Table 1.5. Mean responses of shoot length, number of nodes and dry weight of Douglas maple too explant density in *Efficiency Experiment 2*. Treatments failed so significantly affect response variables. The abbreviation SE stands for standard error. The SE value corresponds to the average value directly to the its left.

Explant Density	Average Shoot Length (mm)	SE	Average Number of Nodes	SE	Average Explant Dry Weight per Explant (mg)	SE
4 explants	61.2	7.5	8.2	0.7	34.2	4.3
6 explants	55.3	3.5	7.5	0.6	32.1	3.8
8 explants	59.3	7.6	7.6	0.3	30.7	2.0

Light Experiment

Image 1.3. Visual effects of light intensity exhibited from treatments of the *Light Experiment*. The left vessel was treated with 36.6 ± 1.7 µmols•m^{-2}•s^{-1} (3.40 ± 1.7 µmols•ft^{2}•s^{-1}) PPF (no shade cloth), the middle vessel was treated with 16.8 ± 1.5 µmols•m^{-2}•s^{-1} (1.56 ± 0.14 µmols•ft^{-2}•s^{-1}) PPF (1 layer of shade cloth), and the right vessel was treated with 6.6 ± 0.7 µmols•m^{-2}•s^{-1} (0.61 ± 0.07 µmols•ft^{-2}•s^{-1}) PPF (2 layers of shade cloth).

Dry weight accumulation was the only significant dependent variable ($P = 0.026$). A Holm-Bonferroni pairwise comparison showed the difference was between no shade cloth and 2 layers shade cloth treatments (Figure 1.9). In general, dry weight tended to decrease as the amount of available light decreased.

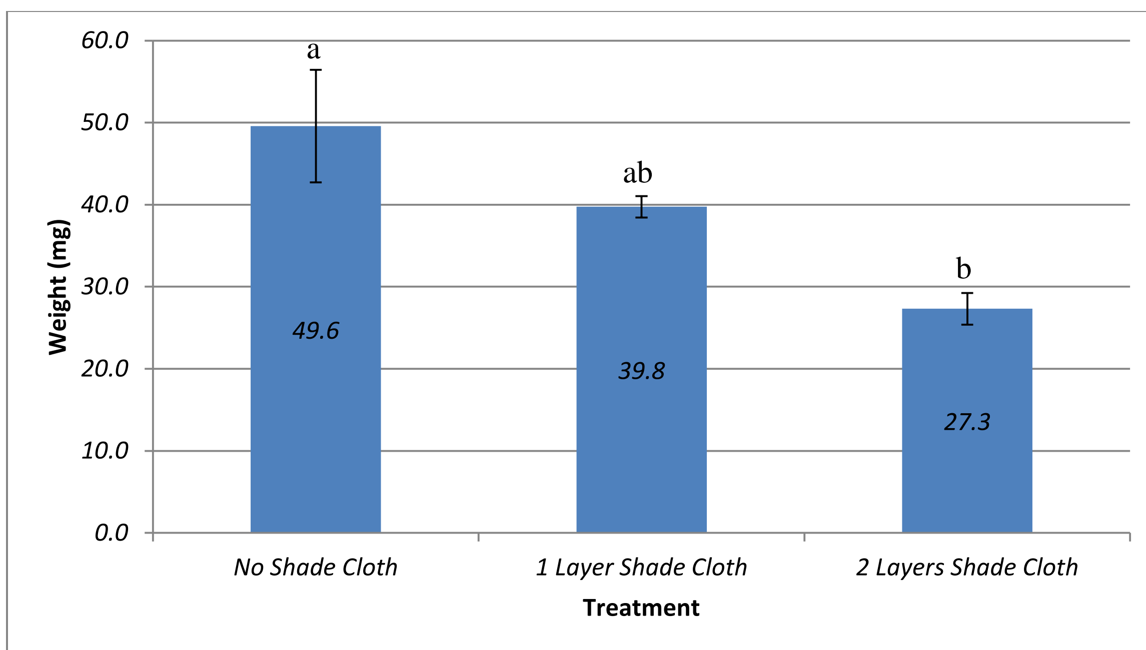

Figure 1.9. Average dry weights by treatment (layers of shade cloth) of the *Light Experiment*. Letters denote statistical significance with P ≤ 0.05 of associated F-statistic.

DISCUSSION

Establishment was accomplished easily on zeatin containing medium, though contamination rates were moderately high. Contamination may have been reduced if whole shoots had been sterilized prior to cutting into 1 or 2 node treatment lengths. Thakur and Sood (2006) documented an improved surface sterilization method whereby whole, multinodal stem sections were sterilized and then cut into nodal explants. This method of sterilization resulted in 20% contamination. In contrast, explants cut to size prior to sterilization had 52% contamination. This method also improved culture success, possibly by minimizing exposure of cut, exposed tissues to sterilization compounds.

The body of maple micropropagation literature suggested that 2iP and kinetin may not be highly successful in enhancing multiplication; still, these compounds were used successfully in a few instances, or in combination with TDZ (Ďurkovič and Andrea 2008). Results from *Multiplication Experiment 1* demonstrated shoot development and elongation in the presence of BA, kinetin and 2iP were poor. In fact, on the basis of shoot length, all 3 compounds appeared to adversely affect shoot development and elongation. In the literature, zeatin

enhanced multiplication of 1 maple species – *Acer grandidentatum* (Ďurkovič and Andrea 2008) and in my study zeatin was clearly effective on *Acer glabrum*.

On the website of laboratory supply company, Sigma Aldrich, zeatin is categorized as, "Autoclavable with other media components, however, some loss of activity may occur" (https://www.sigmaaldrich.com/technical-documents/protocols/biology/growth-regulators.html). Filter sterilized zeatin appeared to have a higher concentration optimum (8 μM) for shoot length than autoclaved zeatin (2 μM), and also produced longer shoots. Given the suggestions by Sigma Aldrich, this was an unexpected result. However, results demonstrated that filter sterilization, a practice which adds expense and is more prone to medium contamination, is unnecessary. Stability of zeatin after 1 autoclave cycle is supported by findings in Hart and others (2016), where, in comparison to non-autoclaved zeatin, no statistically significant difference in recovery of zeatin was found.

Multiplication Experiment 2 showed that the best concentration of autoclaved zeatin for promoting shoot elongation was roughly 4 μM (Table 1.2); however, this level was the lowest concentration tested and optimum may have been somewhere between 1 to 4 μM. Initial results from the first multiplication experiment supported this conclusion, wherein the longest shoots resulted from treatment of 2 μM autoclaved zeatin. As potential replacements for expensive zeatin, TDZ and MT showed strong potential. Unfortunately, in my experiments concentration levels chosen for TDZ and MT were too high and didn't reveal an optimum. Meta-topolin and TDZ both promoted the longest shoot lengths at their lowest concentration levels. In order to better determine the optimum concentration of TDZ for shoot elongation, I would evaluate concentration levels of 0.15, 0.75, 3.75, 18.75, 93.75 nM. Likewise, I would test MT at adjusted concentration levels of 0.65, 1.30, 1.95, 2.60 and 3.25 μM.

Multiplication was optimized for Douglas maple by use of zeatin or MT. Examination of molecular structure of these 2 compounds reveal a commonality in that they possess a hydroxyl group, a functional group missing in the less effective kinetin compounds. Zeatin and 2iP share the exact same molecular structure except for the presence of a hydroxyl group within the structure of zeatin (see Figure 1.10). Likewise, MT and BA share the exact same structure except for the presence of a hydroxyl group as part of the structure of MT (see

Figure 1.10). Under an appropriate pH, hydroxyl groups will deprotonate leaving the oxygen with a negative charge. A charge from this hydroxyl group may be essential in binding to proteins and/or enzymes involved in cytokinin metabolism and/or response.

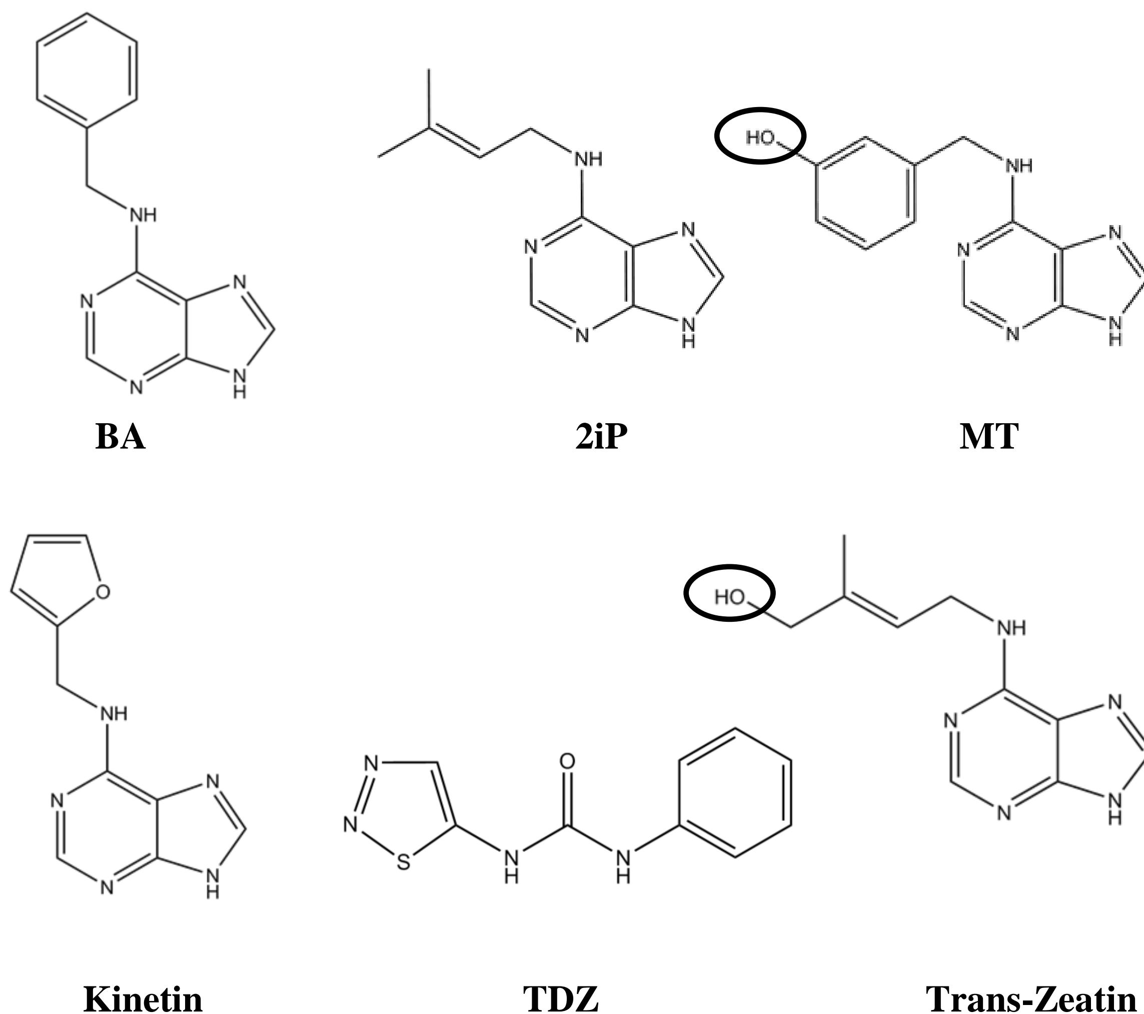

Figure 1.10. Molecular structures of cytokinin compounds evaluated at least once in *Cytokinin Experiment 1, Cytokinin Experiment 2* or *Cytokinin Experiment 3.* Black ovals denote hydroxyl groups. Abbreviations are: BA – benzyladenine; 2iP – 6-(γ,γ-dimethylallylamino)purine; MT – meta-topolin; and TDZ – thidiazuron.

Results from the multiplication efficiency studies resulted in a difference in response for the 2 Douglas maple genotypes evaluated. Explants from genotype 2 may not have stabilized in culture and this may have impacted the decrease in shoot length from week 5 to week 6. Genotype 1 showed signs of stabilization in tissue culture after roughly 11 months in culture. Among other characteristics, one indicator of stabilization might be spontaneous rooting.

Spontaneous rooting of genotype 1 was observed on media lacking supplemental cytokinin, which first occurred at roughly 10.5 months during the *Cytokinin Experiment 2*. An additional sign of lack of stabilization may be compacted internodes. Genotype 2 had significantly shorter internodes than Genotype 1. Shorter internodes, however, could simply be a genetic trait rather than lack of stabilization.

Genotype 1 had very linear growth model from 3 to 6 weeks of culture. The linear equation was Y = (1.456) X – 7.937, where X = the number of days, and Y = the length in mm. Assuming this growth rate, continuing a subculture through a minimum of 6 weeks, or longer, would make sense (Figure 1.7). The first 14 days of a subculture produced very little growth – around 4 to 6 mm (0.16 to 0.24 in). Thereafter, growth accelerated quickly. Minimizing the number of subcultures would reduce the periods of slow growth and increase the proportion of time when rapid growth is occurring. A 6 week subculture would result in 53 mm (2.09 in) of shoot length. Also, the average number of nodes produced after 6 weeks (for G1) was about 7. Each node could serve as an explant, thus providing 7 explants which average 7.6 mm (0.30 in) in length. However, the growth rate experiment inducted only apical explants. If non-apical explants were used, a large increase in explant material would be likely. *Efficiency Experiment 2* used only non-apical explants, and the medium and cytokinin were the same as in *Efficiency Experiment 1*. The average shoot length of *Efficiency Experiment 2* for the 4 blocks with a 30 day subculture was 56.9 mm (2.24 in). A 30 day subculture using the linear model from *Efficiency Experiment 1* predicts a shoot length of just 35.7 mm (1.41 in). Assuming the 2 averages are comparable, that is a 58.9% increase in shoot length by use of non-apical explants. This supports visual observations that vessels with non-apical (nodal) explants appeared to produce more biomass. If this increase could be applied to the linear model from *Efficiency Experiment 1* as Y = ((1.456) X – 7.937)*1.589, then at 42 days, shoot lengths would be a predicted 84.6 mm (3.33 in). Assuming a length of 7 mm per internode, that would equal roughly 12 explants from a single explant. Using these figures, a projection of population growth can be made. With a multiplication constant of 12, 1 explant could multiply to nearly a quarter million plantlets in 6 subcultures of 6 weeks each (36 weeks). Longer subcultures, although appearing to be beneficial, may result in nutrient deficiencies after many subcultures. If a 6+ week subculture

is used, increased vigilance will be necessary to spot potential deficiencies that could arise in plant tissues. No other growth rate experiments have been done on maple to my knowledge.

Efficiency Experiment 2 demonstrated that a higher explant density in culture vessels could save resources without compromising propagation ratios (Table 1.5). However, because this experiment lasted only through 1 subculture, a density of 6 or 8 explants per vessel might not be nutritionally sustainable for repeated subcultures due to increased nutrient demand per vessel.

The light experiment yielded results very similar to those of Singh and others (2017) in that plant weights were significantly affected by the light intensity. Based on visual observation, stems appeared thicker and leaves appeared larger at the highest light intensity, which would explain the higher observed dry weights. Singh and others (2017) also showed that chlorophyll content decreased in leaves exposed to 40 $\mu mols \bullet m^{-2} \bullet s^{-1}$ (3.72 $\mu mols \bullet ft^{-2} \bullet s^{-1}$) PPF. A SPAD meter was used in my experimentation to test relative chlorophyll content, but no differences were detected at statistically significant levels. The SPAD readings were often taken from central portions of the leaf to avoid any non-leaf or leaf margin readings. By sampling from the middle sections, the samples were probably mostly taken from portions of the leaf that were minimally chlorotic. In spite of the negative SPAD results, photographs show visually marked interveinal and marginal chlorosis in the highest PPF treatment.

CONCLUSION

Establishment of Douglas maple stem explants in culture was accomplished without major complications. I would recommend following the procedures herein with autoclaved zeatin at a range of concentrations from 4 to 16 µM. However, contamination rates could be reduced by using additional disinfecting compounds such as PPM, or by using methods similar to that described by Thakur and Sood (2006). Explants recently established in vitro lack stabilization, thus, lower and more variable multiplication ratios may be observed during the first several subcultures.

Multiplication can be accomplished on what I have described as *standard medium* (including the boron fortification) supplemented with either 4 µM autoclaved zeatin, or with 2 µM MT.

To minimize costs, I would recommend using 2 µM MT. Further optimization of MT concentration may improve the multiplication ratios.

Utilizing nodal (non-apical) explants will likely improve multiplication ratios. In addition, longer subcultures of about 6 weeks appear to be most efficient due to the long lag period at the beginning of the subculture when minimal shoot growth occurs. Long subculture intervals will require vigilance to ensure plantlets do not develop nutritional deficiencies. An explant density of 6 or 8 explants per vessel may also be helpful in efficient use of space and minimizing resource expenditures.

Finally, high light intensities have a tendency to negatively impact explant quality as evidenced by the interveinal chlorosis. More experimentation may reveal that this light-caused chlorosis can be remediated by nutrient modification, so that higher explant weight can be reaped as a benefit.

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CHAPTER 2 – Rooting and Acclimatization of Douglas Maple

ABSTRACT

Douglas maple (*Acer glabrum* Torr. var. *Douglasii* (Hook.) Dipple [Aceraceae]) is a potentially valuable landscape plant that lacks proven asexual propagation protocols. Micropropagation has potential as an efficacious method for asexually propagating Douglas maple. Rooting and acclimatization are the final stages of micropropagation, and are crucial to the overall success of a micropropagation protocol. In vitro and ex vitro rooting procedures were tested for their ability to root in vitro grown microcuttings. The in vitro rooting factors tested were: auxin type (IAA, IBA or both in combination), light vs dark, apical vs nodal explants, half vs full strength DKW medium salts, IBA concentration (0, 1, 2, 4 and 8 µM), and the length of the explant (2.0 to 3.0 cm (0.79 to 1.18 in), or 3.5 to 4.5 cm (1.38 to 1.77 in)). Ex vitro rooting factors tested included explant length as a covariate, and concentrations of IBA (0.1 and 0.3%) in talcum based rooting powders. The success of the rooting methods and treatments was primarily assessed by measuring the overall survival rate of plantlets after rooting and acclimatization. The most successful procedure was ex vitro rooting with a 0.1% IBA talcum powder treatment, which yielded a 92% survival rate after rooting and acclimatization. In comparison, the best in vitro rooting treatment (1 µM IBA regardless of explant length) yielded just 75% survival after rooting and acclimatization. A second ex vitro experiment involving a second Douglas maple genotype further validated the effectiveness of ex vitro rooting using 0.1% IBA talcum powder. Rooting and acclimatization of in vitro grown shoots was most efficiently and effectively accomplished using ex vitro rooting procedures.

KEY WORDS: micropropagation, in vitro, ex vitro, *Acer glabrum*, method evaluation.

NOMENCLATURE:
USDA NRCS (2019)

Abbreviations: DKW – Driver Kuniyuki Walnut, IAA – indole-3-acetic acid, IBA – indole-3-butyric acid, PPF – Photosynthetic Photon Flux.

INTRODUCTION

Douglas maple, also known as Rocky Mountain maple, (*Acer glabrum* Torr. var. *Douglasii* (Hook.) Dipple [Aceraceae]) possesses excellent landscape plant characteristics, including high adaptability, bright red or yellow fall foliage, attractive red young stems, and a moderate degree of drought tolerance. In spite of excellent landscape potential, use of this species is limited, in part, due to a lack of published protocols for asexual propagation. Rupp and Wheaton (2014) state in their publication on native plant propagation that, "There are no published techniques for its [*Acer glabrum*] vegetative propagation."

Propagation of Douglas maple is most commonly accomplished by seed, but due to poor natural seed quality and recalcitrant dormancy characteristics, this process is not efficient. Also, seed propagation does not allow conservation of superior traits that make selected Douglas maple genotypes desirable as urban landscape plants. Propagation by cuttings is considered to be difficult, and a tendency for overwintering death of cuttings may also be a detriment (Hartmann and others 2011, Nesom 2006). A high throughput asexual propagation protocol for Douglas maple is needed such that superior genotypes can be genetically conserved and rapidly propagated. High landscape potential combined with a lack of established protocols for asexual propagation provides ample justification for micropropagation research on *Acer glabrum.* A positive attribute of micropropagation is that it typically causes a partial reversion of cultured plant tissue to a more juvenile state (Hartmann and others 2011). Tissue juvenility is important because juvenile stems are easier to root (Hartmann and others 2011). Overall, micropropagation via nodal culture, or axillary shoot culture, is a good technique for maintaining true-to-type plantlets, and also potentially achieving high propagative increase ratios (Hartmann and others 2011).

Within this paper the last 2 stages of micropropagation – rooting and acclimatization – of Douglas maple are addressed.

Root Formation: In vitro, adventitious rooting is often accomplished by the addition of some form of the plant growth regulator, auxin, to the growth medium. Auxin is naturally occurring in plants and is a crucial plant growth regulator for root formation and development in vitro (Hartmann and others 2011). Although no studies of in vitro

adventitious rooting of *Acer glabrum* have been reported in the literature, studies have been completed on several other *Acer* species. These other *Acer* species were often rooted by adding IBA to the growth medium. The best treatments from across several *Acer*-specific experiments yielded rooting percentages between 65 and 100% (Ďurkovič and Andrea 2008). A common treatment for in vitro adventitious rooting was the use of 1 mg/l IBA (Ďurkovič and Andrea 2008). Bigtooth maple (*Acer grandidentatum* Nutt. [Aceraceae]) rooted successfully without any exogenous auxin, but this response appeared to be highly dependent on genotype (Bowen-O'Connor and others 2007). Various salt concentrations in the medium were employed during the rooting stage, and often applied at half-strength (Ďurkovič and Andrea 2008).

Ex vitro rooting procedures were also successfully implemented. Preece (1991) showed that *Acer saccharinum* L. (Aceraceae) microcuttings could be rooted by applying a liquid IBA treatment to the base of microcuttings – resulting in a rooting rate of 70%. Overall, successful rooting was not a big research challenge for most *Acer* species.

Acclimatization: Acclimatization was easily accomplished by researchers studying several *Acer* species by transferring rooted plantlets to soilless potting media, and keeping environmental humidity high (Bowen-O'Connor and others 2007, Wilhelm 1999). Survival rates after acclimatization across several *Acer*-specific experiments ranged from 77 to 100% (Ďurkovič and Andrea 2008).

The research documented in this chapter is focused on identifying the most important factors in rooting, and the optimum treatment for inducing successful rooting and acclimatization. In addition, 2 different methods of rooting (in vitro and ex vitro methods) were evaluated. The success of rooting methods and treatments were primarily evaluated upon their survival after a defined acclimatization period, but the number of roots produced was important too.

MATERIALS AND METHODS

A total of 4 rooting and acclimatization experiments were conducted on Douglas maple. Acclimatization experiments were a continuation of, or congruent with, the rooting experiments (Figure 2.1). No acclimatization-specific factors were tested.

Two accessions of Douglas maple were used in the rooting and acclimatization experiments. Stem tissue of the first accession, herein called genotype 1 (G1), was collected from the University of Idaho Arboretum in Moscow, Idaho. The plant was obtained by the arboretum staff from Plants of the Wild nursery in Tekoa, Washington. The nursery personnel from Plants of the Wild was unable to provide precise provenance information, but said the seed used in propagating the specimen was likely from Bonner County in northern Idaho. The accession material was ontologically immature, having yet to produce seeds. The plant material used in this experiment was collected and inducted into tissue culture on August 9, 2017, established on DKW medium salts with zeatin, and multiplied on DKW medium salts with zeatin and supplemental iron.

The second accession, herein called genotype 2 (G2), was obtained from Dr. Stephen Love, extension/research professor at the University of Idaho Aberdeen Research and Extension Center. The source plant was located on the research station in Aberdeen. The accession was originally collected in the Albion Mountains, near the community of Oakley in southern Idaho. Ontologically immature stem cuttings were used as a source of propagation material and inducted into tissue culture July 30, 2018. Tissue was established on DKW medium salts with zeatin and added iron, and was multiplied on DKW medium with zeatin, added iron and boron.

Explants were grown in a Hoffman Manufacturing Company (Albany, Oregon) growth chamber using General Electric brand Ecolux 34 W soft white fluorescent light tubes. The photosynthetic photon flux (PPF) ranged from roughly 5 to 80 $\mu mols \bullet m^{-2} \bullet s^{-1}$ (0.46 to 7.43 $\mu mols \bullet ft^{-2} \bullet s^{-1}$) depending on the explant and vessel density, the position of vessels on the shelf, and the size of explants in culture. This wide range of PPF was addressed by blocking of experiments according to row of vessels upon the shelf, as seen in Figure 2.2. Addressing this wide range of PPF by blocking was important, because some *Acer* species like sugar maple (*Acer saccharum* Marshall [Aceraceae]) are very sensitive to light intensity (Singh and others 2017). During the process of culture establishment, Douglas maple also showed signs of light stress due to a PPF of just 37 $\mu mols \bullet m^{-2} \bullet s^{-1}$ (3.44 $\mu mols \bullet ft^{-2} \bullet s^{-1}$). The photoperiod in the growth chamber was kept at 16 h, and the temperature was kept constant at 22° ± 2° C (71.6° ± 3.6° F). Greenhouse environmental conditions (concerning the acclimatization and

ex vitro experiments) were a 14 h photoperiod from a combination of high pressure sodium lamps and natural light, and a minimum night to maximum day temperature range of 17.7° to 25.6° C (62° to 78° F).

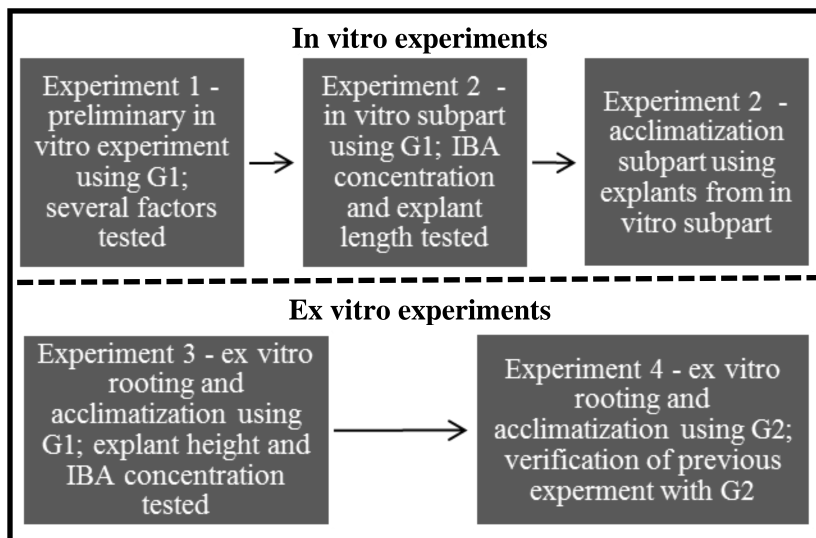

Figure 2.1. Relative chronology and origin of explant materials for rooting and acclimatization experiments shown visually; arrows indicating the chronological order. Genotype 1 is denoted by G1, genotype 2 is denoted by G2, and indole-3-butyric acid is abbreviated as IBA.

Experiment 1 was a preliminary experiment and was conducted in order to determine the most influential factors of in vitro rooting. An unreplicated factorial split plot design was utilized to test several independent variables and levels thereof, which included: light vs dark (darkness being implemented by placing vessels in a cardboard box for the first 2 weeks of culture in the growth chamber), DKW medium salts strength (half or full), auxin concentration and type(s) (0 µM auxin, 6 µM IAA, 6 µM IBA, or 12 µM of IAA and IBA (6 µM of each)), apical vs nodal explants (2 of each per vessel). Genotype 1 explants were inducted into this experiment by cutting in vitro grown, stock microshoots to a length of 2.0 to 3.0 cm (0.79 to 1.18 in) with 3 to 5 leaves. Dependent variables measured were: the number of rooted explants per vessel, the number of roots per explant, number of roots > 2.0 cm (0.79 in) in length per vessel, whether the rooted explants were apical or nodal, callus presence or absence, and explant survival. Data on dependent variables were collected 9 times over the duration of the experiment (39 days). Results herein are based on data after 28

days in culture, except for the explant survival data, which was taken at 39 days. Dependent variable data were collected while the explants were still within the vessels by visual approximation and determination. Explant survival was defined as having at least 1 living, green leaf. The media for this experiment was made with the following constituents per liter (0.26 gal): 5.2 or 2.6 g (0.18 or 0.09 oz) of DKW medium salts (full-strength and half-strength respectively), 45 mg (0.0016 oz) NaFeEDTA, 30.0 g (1.06 oz) D-sucrose, 5.0 ml (0.17 oz) Stock G vitamin solution (20 mg (0.00070 oz) thiamine-HCl, 10 mg (0.00035 oz) nicotinic acid, 10 mg (0.00035 oz) pyridoxine HCl and 40 mg (0.0014 oz) glycine in 100 ml (3.38 oz) of reverse osmosis (RO) water), 5.0 ml (0.17 oz) Stock H vitamin solution (2.0 g (0.071 oz) myo-inositol in 100 ml (3.38 oz) of RO water), addition of appropriate amount of IBA and/or IAA for treatment, and 8.0 g (0.28 oz) agar. The pH of the media was adjusted to 5.8 ± 0.02 using HCl and NaOH. Roughly 50 ml (1.69 oz) of medium were aliquoted per vessel. Media were autoclaved for 25 minutes at 121° C (250° F) and 103 kPa (15 psi). One replicate of each treatment was used in the experiment. Each replicate (the tissue culture vessel (Magenta® GA-7)) had 4 pseudo-replicates (explants) within. The dimensions of the GA-7 were 7.7 x 7.7 x 9.7 cm (3.0 x 3.0 x 3.8 in).

Experiment 2 was a replicated in vitro experiment that was conducted with the purpose of identifying the best IBA concentration for rooting, and whether explant length plays a role. *Experiment 2* also incorporated an acclimatization subpart, which was included for the purpose of determining if the best rooting treatments successfully produced acclimatized plantlets. The experiment was designed as a factorial, randomized complete block with independent variables of IBA concentration at 5 levels (0, 1, 2, 4 or 8 μM) and apical explant size at 2 levels (small (2.0 to 3.0 cm (0.79 to 1.18 in)) or large (3.5 to 4.5 cm (1.38 to 1.77 in))). Genotype 1 explants were inducted into the experiment by cutting in vitro grown, stock microshoots to length using a scalpel, and then placing them in the media. Evaluated dependent variables were number of rooted explants, number of roots per explant, longest root per explant, and explant shoot length. Four block replicates were arranged by row to account for light intensity (Figure 2.2). Each treatment replicate consisted of 4 explants within a GA-7 vessel. Each block had 1 treatment replicate. Data collected per explant were averaged between explants within the GA-7 to provide a single data point. Data were collected 30 days after the start of the experiment on blocks 1, 2 and 3, and 32 days after the

start of the experiment for block 4. Data collection was accomplished by carefully removing the rooted explants from the medium and measuring and counting rooting characteristics for the dependent variables. For shoot length, the basal callus formation was excluded from the measurement by excising the basal callus. Media were made with the following constituents on a per liter (0.26 gal) basis: 2.6 g (0.09 oz) DKW medium salts (half strength), 30.0 g (1.06 oz) sucrose, 23 mg (0.00081 oz) NaFeEDTA, 5.0 ml (0.17 oz) Stock G vitamin solution, 5.0 ml (0.17 oz) Stock H vitamin solution, addition of appropriate amount of IBA for treatment, and 8.0 g (0.28 oz) agar. The pH of the media were adjusted to 5.8 ± 0.02 using HCl and NaOH. Roughly 50 ml (1.69 oz) of medium were aliquoted per vessel. Media were autoclaved for 25 minutes at 121° C (250° F) and 103 kPa (15 psi).

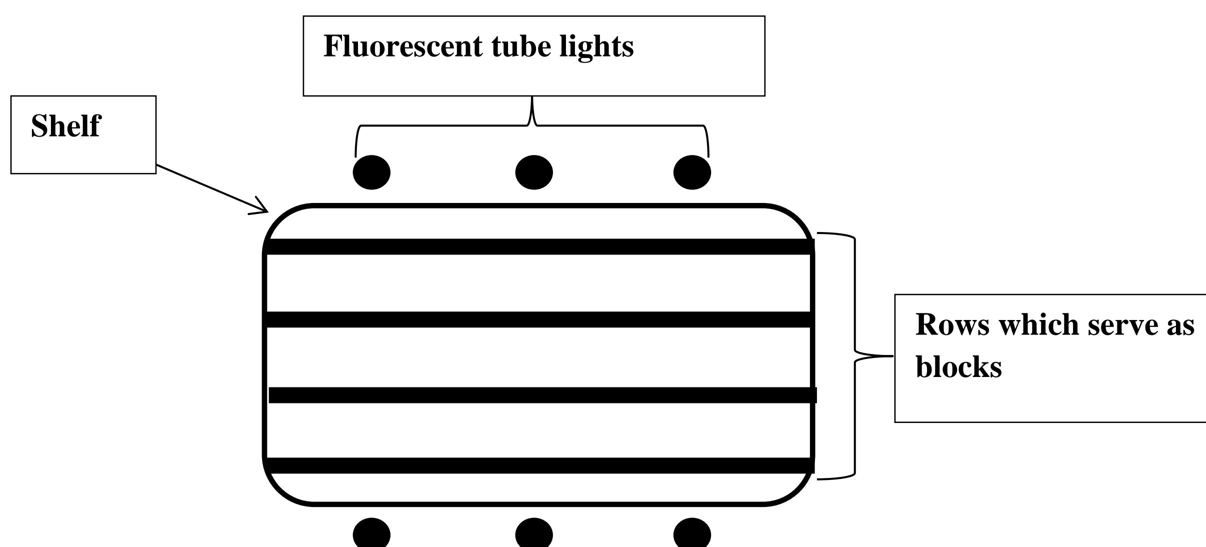

Figure 2.2. Top-view diagram of the blocking structure used for in vitro methods of *Experiment 2*. Rows contain GA-7 tissue culture vessels.

To evaluate rooting treatments for their effect on acclimatization, plantlets from *Experiment 2* were transferred from the agar based media of the in vitro environment, to pots filled with soilless peat-based potting medium. This transfer was completed 30 days after starting *Experiment 2*. All explants from blocks 1 through 3 - including those without roots - were transferred. The potting medium was Sungrow® Horticulture Professional Growing Mix, the pots were 4 x 4 in (10.2 x 10.2 cm), and 1 plantlet was placed in each pot. These pots were stored in a greenhouse. The explants were kept under conditions of high humidity and low light. These conditions were provided by placing empty GA-7 vessels over each plantlet and

then placing a large sheet of plastic over the entire group of experimental plants. Plants were watered only when soil looked dry. Fung-onil fungicide by Bonide® was applied once to minimize leaf decay/rot issues that had arisen. Plantlets from block 1 were ex vitro for 35 days prior to post acclimatization data collection, and plantlets from blocks 2 and 3 were ex vitro for 34 days prior to post acclimatization data collection. Dependent variables were: plantlet survival and numbers of green leaves at the end of the experiment. Plants with at least 1 green leaf and a green apical meristem were considered to be alive.

Experiment 3 was designed to determine the efficacy of ex vitro rooting methods using rooting powders. Microshoots of G1 were used for this experiment. The design was a randomized complete block testing 3 rooting treatments: tap water (control), Botone®[II] Rooting Powder (0.1% IBA), and Hormex® #3 (0.3% IBA). A covariate, length of explant, was included. The treatments were applied by cutting the callus tissue from the bottom of the microshoot using a scalpel, measuring the shoot length, and then dipping the cut end in water. If the explant was destined for a rooting powder treatment, then it was dipped in powder to a depth of approximately 1 cm (0.39 in). If there were leaves at, or below, the bottom 1 cm (0.39 in) of the shoot, they were removed with a scalpel. The minimum length of shoot inducted into this experiment was 3.5 cm (1.38 in), and no maximum length. The dependent variables for which data was recorded were: number of roots per explant, survival (yes or no), and whether the plant had at least 5 living leaves. An alive explant was defined as having at least 1 green leaf that had a portion of non-desiccated blade and a visibly green apical meristem. A living leaf was defined as having at least some portion of the blade that was green and not desiccated. Three rows of 4 microcuttings were contained within a 32.4 cm x 22.9 cm x 4.7 cm (12.75 in x 9.0 in x 1.84 in) aluminum Reynolds baking tray. The position of treatments within the tray was randomized. Each baking tray served as a block, and 6 were used in total. The baking trays were filled with soilless peat-based potting medium (Sungrow® Horticulture Professional Growing Mix). This experiment was stored in a growth chamber until the final stages of acclimatization. Within the growth chamber, clear plastic lids were used to retain humidity during the process of acclimatization. Over the course of the experiment, humidity within the tray was gradually reduced by adding holes to the plastic lids. The lids started with 2 holes of 1.0 to 1.5 cm (0.39 to 0.59 in) in diameter. Every few days, a few more holes were added to each lid until finally the lids were taken off.

Approximately 3 days in the growth chamber without a lid, was followed by approximately 4 days without a lid in the greenhouse, and then data were collected 30 days after the start of the experiment for blocks 1-5, and 29 days after the experiment started for block 6. The roots were counted by carefully removing the plantlet from the potting mix and counting the roots. If the roots were dense and held soil that could not easily be removed without breakage, the roots were dunked repeatedly in water to dislodge the potting mix.

Experiment 4 was a small experiment with the goal of verifying the rooting and acclimatization response of *Experiment 3* by repeating with a different Douglas maple genotype. This experiment used microshoots from genotype G2. *Experiment 4* duplicated the conditions and procedures of *Experiment 3* except for a few exceptions outlined next. The experimental design was a completely randomized design. The entire experiment was contained within just 1 Reynolds brand baking tray. Two treatments (water and Botone® II Rooting Powder (0.1% IBA)) were applied to 20 microcuttings – 10 microcuttings per treatment. A fungus gnat infestation of the tray, at about 2 weeks from the start of the experiment, required that the tray be removed from the growth chamber to prevent further infestation into other plant material. The tray was transferred to the greenhouse, where it remained until data collection. An increasing number of holes were added to the lid every few days until roughly 3 days before data collection, when the lid was removed. Data collection was completed 30 days after commencing the experiment. Data collection mimicked that of *Experiment 3*.

Statistical analysis was conducted using RStudio. The function 'lmer' was used to create linear mixed effect regression models of data from *Experiment 2*. The 'lmer' and 'glmer' functions were used for creating linear mixed effect regression and generalized linear mixed effect regression models of the data from *Experiments 3* and *Experiment 4*. Analysis of deviance was conducted on the models using the 'Anova' function from the 'car' package. Type-II sums of squares were used for the analysis of deviance. The F test and statistic were used in providing P-values, unless otherwise stated or shown.

RESULTS

Experiment 1

Experiment 1 was an unreplicated, preliminary experiment, providing data inappropriate for analysis using standard statistical methods. Nonetheless, the study showed that the number of roots produced per explant was over twice as many in half-strength salts, relative to full strength salts. Shoots on treatments inclusive of IBA and IAA – having a total auxin concentration of 12 µM - produced 2 times as many rooted explants, 6 times as many roots, and 3 times as many roots > 2.0 cm (0.79 in), relative to the auxin-free media. Shoots treatmented with IBA produced 4 times as many roots and 4 times as many roots > 2.0 cm (0.79 in), relative to the auxin-free media. Shoots treatments with IAA produced 3 times as many roots > 2.0 cm (0.79 in) relative to the auxin-free media. Callus formation (determined by visual approximation) was observed to some degree on most explants regardless of the treatment. Shoot treatments with IBA and IAA together produced the most callus, shoots treated with IBA alone produced the second most callus, and shoots treated with IAA appeared to produce slightly less callus than IBA treated shoots. Shoots on auxin-free medium produced plantlets with very little callus.

Exogenous auxin improved rooting ratios; however, at the 12 µM concentration (treatments with IBA and IAA together), explants tended to exhibit excessive callogenesis. Twelve µM auxin also caused the formation of very thick roots, and some root formation occurred on petiole tissue above the medium. All of these growth and rooting responses also occurred in the 6 µM IAA and 6 µM IBA containing treatments, but at lesser degree, visually. These responses are indicative of an overdose response. As a result of these overdose-like responses observed in *Experiment 1*, the highest auxin concentration used in the subsequent experiments was 8 µM IBA. Also, based on the findings from this preliminary study, IBA became the auxin of choice in *Experiment 2* because this compound appeared to promote rooting at a slightly higher rate (based on averages) when compared to IAA.

Experiment 1 also indicated that the effect of apical or nodal explants on rooting responses was minimal; 61.3% of apical explants rooted, and 57.6% of nodal explants rooted, when averaged across all treatments. Also, this experiment demonstrated that darkness was

detrimental to cultured explants. At the end of 2 weeks in the dark, leaves of explants were chlorotic, some leaves were abscising, and plantlets lacked vigor relative to the light treatment. Because of these results, *Experiment 2* was conducted without a dark treatment.

Experiment 2

The shoot length response of explants was examined by making a subset of small and large sized explants. Shoot length was significantly affected by IBA concentration for both large and small sized explants. A Holm-Bonferroni pairwise comparison showed that large sized explants exposed to 8 µM IBA were significantly shorter than those grown on 0 µM IBA. No other comparisons were significant for large explants. For small sized explants, every comparison was significantly different except for 0 compared to 1 µM IBA. Regardless of original explant size, the higher the concentration of IBA, the shorter the shoots were. See table 2.1 for analysis of deviance table of small explants and Table 2.2 for analysis of deviance table of large shoots. Higher concentrations of IBA were associated with larger callus formation on the explant stems, which may have deducted from shoot length measurements.

Table 2.1. Analysis of deviance table of the shoot length of small sized explants as affected by IBA (indole-3-butyric acid) concentration in *Experiment 2*. Abbreviations are: F – F statistic; Df – degrees of freedom; Df.res – degrees of freedom for residual term; Pr(>F) – probability of getting that F statistic value.

Analysis of Deviance Table (Type II Wald F tests with Kenward-Roger df)

Response: average shoot length for small sized shoots

	F	Df	Df.res	Pr(>F)
IBA	76.377	4	12	1.974e-08 ***

Significance codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Table 2.2. Analysis of deviance table of the shoot length of large sized explants as affected by IBA (indole-3-butyric acid) concentration in *Experiment 2*. Abbreviations are: F – F statistic; Df – degrees of freedom; Df.res – degrees of freedom for residual term; Pr(>F) – probability of getting that F statistic value.

Analysis of Deviance Table (Type II Wald F tests with Kenward-Roger df)

Response: average shoot length for large sized explants

	F	Df	Df.res	Pr(>F)
IBA	4.2285	4	12	0.02305 *

Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

The ratio of rooted explants was significantly affected by both initial explant size (P = 0.010) and concentration of IBA (P < 0.001) (see Table 2.3). Large sized explants had a higher average rooting ratio than small sized explants (0.70 and 0.54, respectively) (see Table 2.6). The concentration of IBA with the highest rooting ratio was 1 µM, which had a ratio of 0.78 (see Table 2.6). A Holm-Bonferroni pairwise comparison showed that the significant differences existed between 0 µM IBA and all other concentrations, but no other comparisons were significant. The highest rooting ratio was observed within treatment combination large explants x 8 µM IBA – exhibiting a 0.94 rooting ratio. The lowest rooting ratio was observed within treatment combination consisting of small sized explants x 0 µM – exhibiting a 0.19 rooting ratio.

Table 2.3. Analysis of deviance table of the rooting ratio by main effects of explant size and indole-3-butyric acid (IBA) concentration, and the interaction of these in *Experiment 2*. Abbreviations are: L – size (length) of explant; F – F statistic; Df – degrees of freedom; Df.res – degrees of freedom of residual; Pr(>F) – probability of getting that F statistic value.

Analysis of Deviance Table (Type II Wald F tests with Kenward-Roger df)

Response: ratio of rooted explants

	F	Df	Df.res	Pr(>F)
L	7.7734	1	27	0.009594 **
IBA	9.1303	4	27	8.445e-05 ***
L:IBA	1.9089	4	27	0.137828

Significance codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Image 2.1. Visible root growth of explants from in vitro *Experiment 2* showing the influence of indole-3-butyric acid (IBA) at 0, 2 and 8 µM, respectively, from left to right.

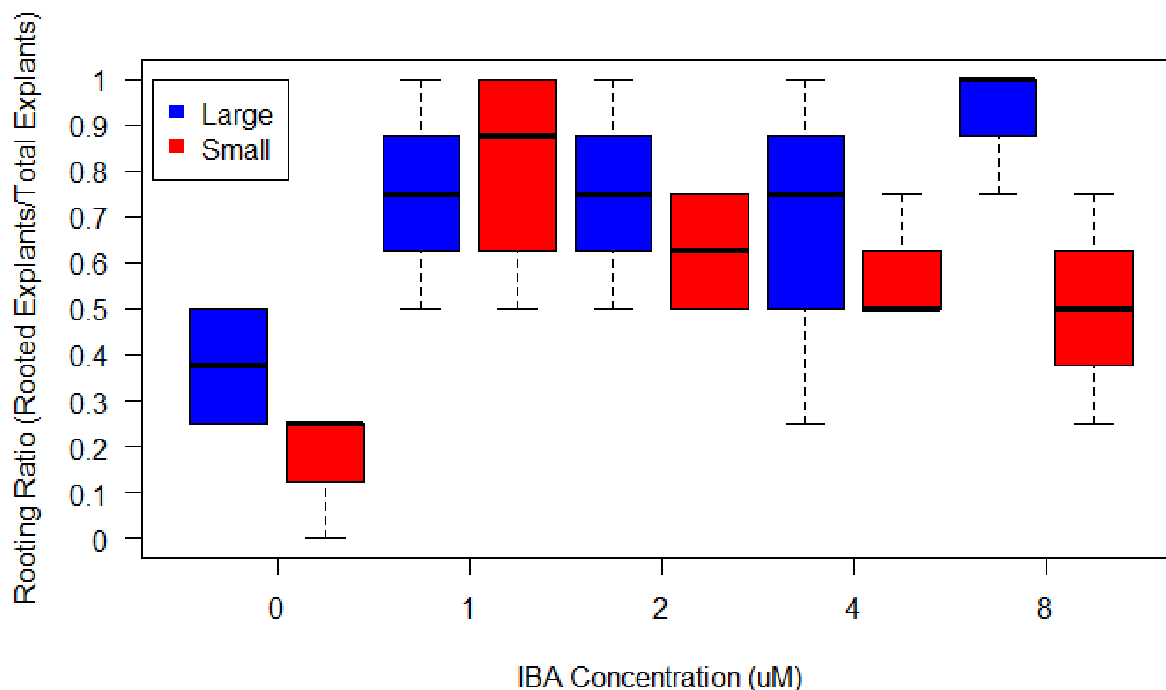

Figure 2.3. Vertical boxplots showing the effect of indole-3-butyric acid (IBA) concentration and size of explant (small (2.0 to 3.0 cm (0.79 to 1.18 in)) or large (3.5 to 4.5 cm (1.38 to 1.77 in)) on the rooting ratio within each treatment combination in *Experiment 2*.

The average number of roots was significantly affected by explant size (P = 0.013), IBA concentration (P < 0.001), and the interation of these main effects (P = 0.005) (Table 2.4). On average, large sized explants produced more roots than small sized explants – 2.2 per explant, and 1.5 per explant respectively. A Holm-Bonferroni pairwise comparison of all levels of IBA demonstrated that the significant effect of IBA exists between auxin-free media and all other concentrations of IBA. The significant interaction of the main effects can be visualized in figure 2.5.

Table 2.4. Analysis of deviance table of the average number of roots by the main effects of explant size and indole-3-butyric acid (IBA) concentration, and their interaction of them in *Experiment 2*. Abbreviations are: L – size (length) of explant; F – F statistic; Df – degrees of freedom; Df.res – degrees of freedom of residual; Pr(>F) – probability of getting that F statistic value.

Analysis of Deviance Table (Type II Wald F tests with Kenward-Roger df)

Response: average number of roots

	F	Df	Df.res	Pr(>F)
L	7.0834	1	27	0.0129409 *
IBA	8.0087	4	27	0.0002157 ***
L:IBA	4.7304	4	27	0.0050497 **

Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

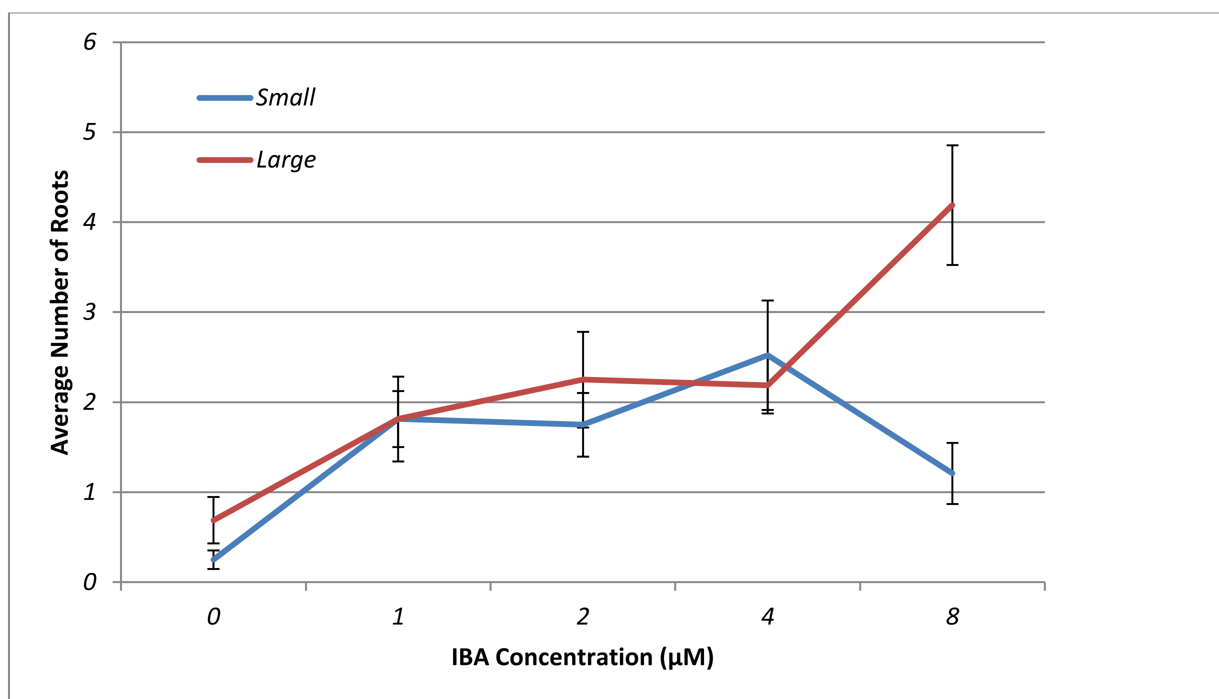

Figure 2.4. Average number of roots as a result of the interaction between explant size and IBA concentration in *Experiment 2*. Small explants are 2.0 to 3.0 cm (0.79 to 1.18 in) and large explants are 3.5 to 4.5 cm (1.38 to 1.77 in) in length.

Data of the average longest root was not normally distributed due to 2 data point outliers. These 2 outliers were removed from the data set in order to meet the Shapiro-Wilk normality

test; however, normality was still poorly met even though statistically satisfied (P = 0.051). The average length of longest root was affected by the concentration of IBA (P < 0.001) and the interaction of explant size and IBA concentration (P = 0.008) (see Table 2.5). A Holm-Bonferroni pairwise comparison of all levels of IBA showed that concentrations 0 and 1 µM were different from concentrations 2, 4 and 8 µM; 0 and 1µM treatments produced the longer root lengths.

Table 2.5. Analysis of deviance table of the average number of roots by main effects of explant size and indole-3-butyric acid concentration, and the interaction of these in *Experiment 2*. Abbreviations are: L – explant size (length); F – F statistic; Df – degrees of freedom; Df.res – degrees of freedom of residual; Pr(>F) – probability of getting that F statistic value.

Analysis of Deviance Table (Type II Wald F tests with Kenward-Roger df)

Response: average length of longest root

	F	Df	Df.res	Pr(>F)
L	1.0025	1	25.190	0.3262200
IBA	8.8584	4	25.250	0.0001304 ***
L:IBA	4.3163	4	25.332	0.0084894 **

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Table 2.6. Average length of longest root, number of roots, and rooting ratio as affected by indole-3-butyric acid (IBA) concentration and explant size (small 2.0 to 3.0 cm (0.79 to 1.18 in) explants, and large 3.5 to 4.5 cm (1.38 to 1.77 in) explants) in *Experiment 2*. Response variables categorized with distinct letters denote significance at $P \leq 0.05$. Letters denoting significance among IBA concentrations are not relevant to letters denoting significance between explant sizes. Average length of longest root reflects the removal of the 2 outlier data points. Removal of these outliers inflated the average length of longest root for 0 and 1 µM IBA. This table includes data from all 4 blocks.

Main Effect	Average Length of Longest Root (mm)	Average Number of Roots per Explant	Average Rooting Ratio
0 µM IBA	79.2 a	0.5 a	0.3 a
1 µM IBA	75.7 a	1.8 b	0.8 b
2 µM IBA	55.5 b	2.0 b	0.7 b
4 µM IBA	55.2 b	2.4 b	0.6 b
8 µM IBA	46.3 b	2.7 b	0.7 b
Small Explant	*60.5 a*	*1.5 a*	*0.5 a*
Large Explant	*64.3 a*	*2.2 b*	*0.7 b*

***Experiment 2* - Acclimatization of cultured explants**

No additional post-culture treatments were imposed on explants following completion of the in-vitro portion of *Experiment 2*. Acclimatization response was related to the influence of in vitro conditions. Acclimatization was not entirely dependent on roots being present at out-planting (Figure 2.7). In fact, 26.3% of non-rooted explants acclimatized. Conversely, 14.5% of rooted explants did not acclimatize. The treatment with the highest proportion of acclimatized explants with 5+ leaves was the large explant x 1 µM IBA combination (see Figure 2.8).

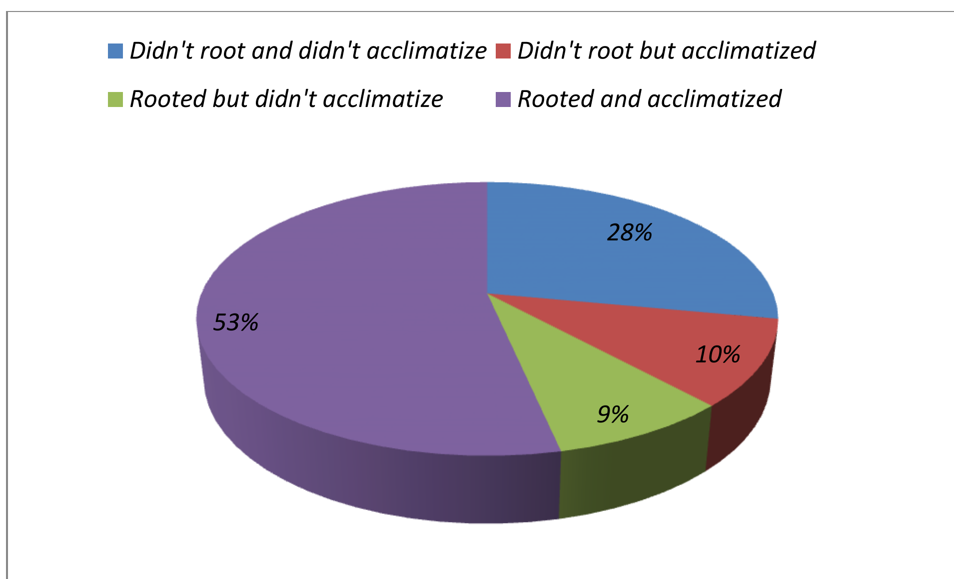

Figure 2.5. Acclimatization of explants that either rooted in culture or were planted without roots in *Experiment 2*. The term 'rooted' is defined as any explant having at least 1 clearly recognizable root of any length. The term 'acclimatized' is defined as any explant that survived until the end of the acclimatization period of 34 to 35 days; survival was evidenced by at least 1 non-desiccated, green leaf and green apical meristem.

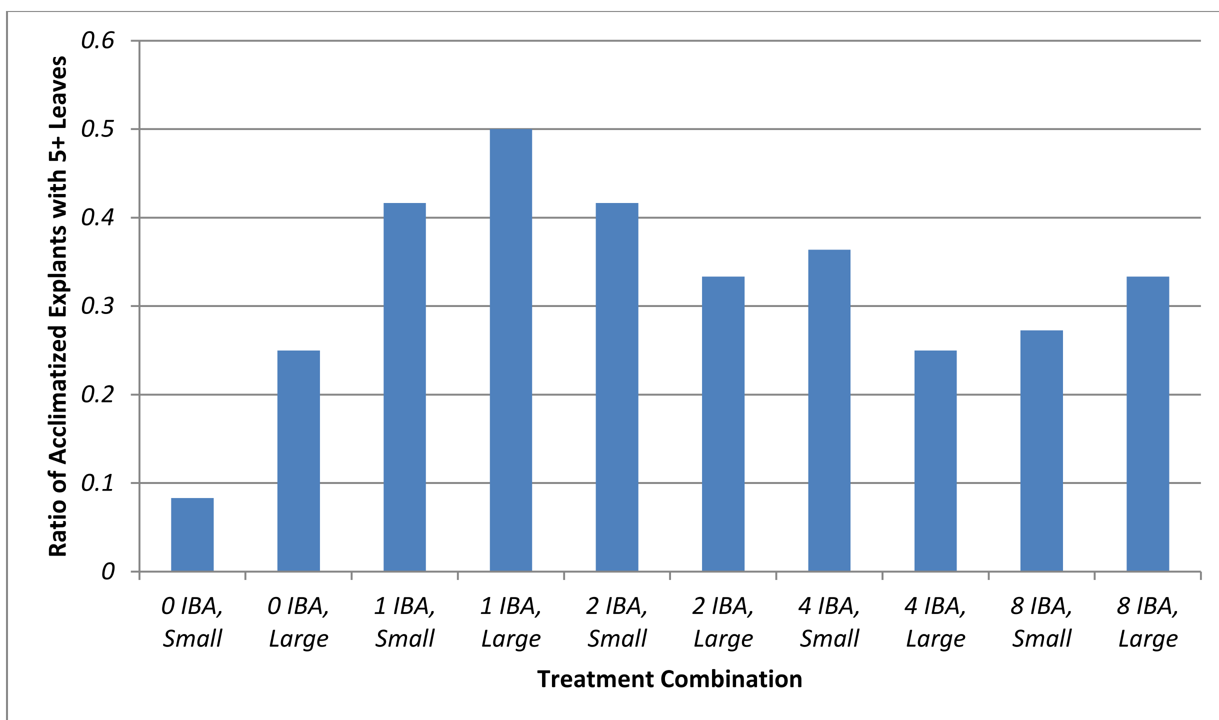

Figure 2.6. Proportion of acclimatized explants with 5+ leaves with at least some portion being non-desiccated and green in *Experiment 2*. Ratio of acclimatized explants is on the y-axis and treatment combination is on the x-axis. The number associated with the indole-3-butyric acid (IBA) is the concentration of IBA in µM. Small explants were 2.0 to 3.0 cm (0.79 to 1.18 in) and large explants were 3.5 to 4.5 cm (1.38 to 1.77 in).

Treatment combination 8 µM IBA x large explant rooted 92% of explants, but only 58% acclimatized (survived). In contrast, the auxin-free x small explant treatment combination rooted 17% of the explants, but 50% acclimatized (see Figure 2.9).

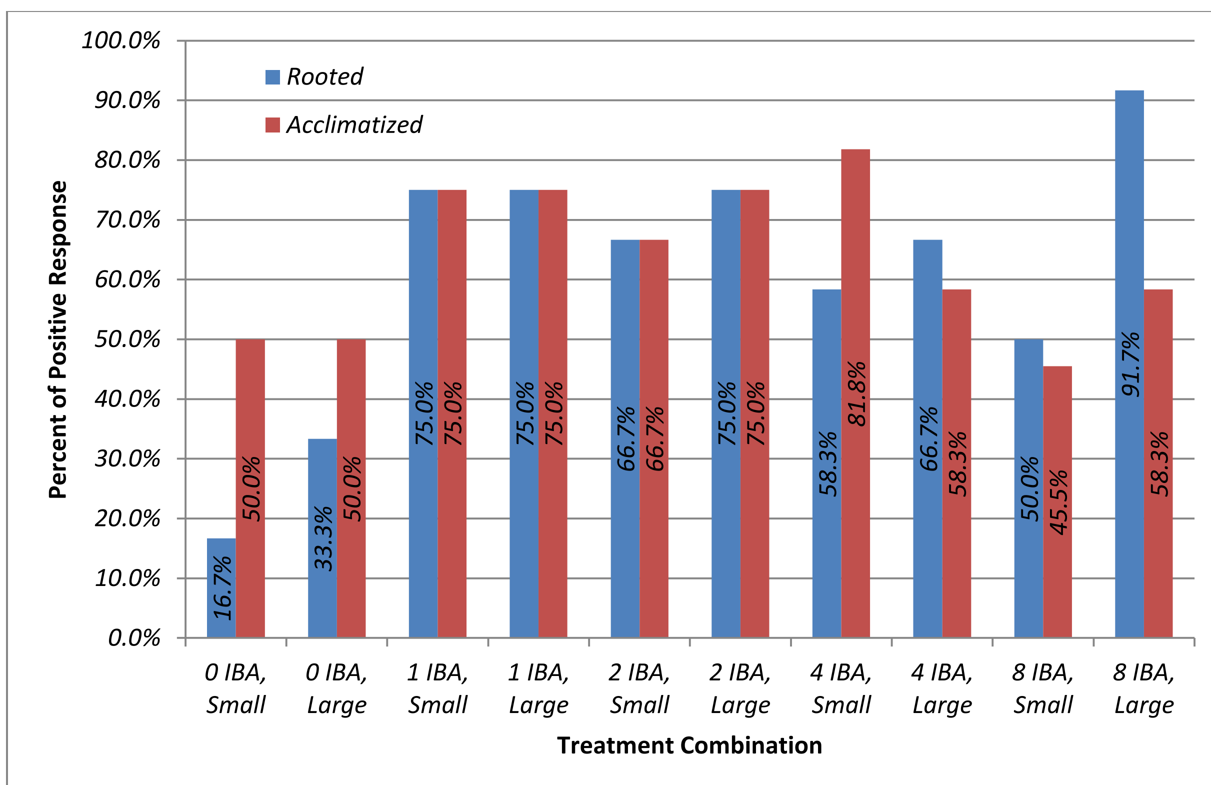

Figure 2.7. Percent in vitro rooting and ex vitro acclimatization as influenced by indole-3-butyric acid (IBA) concentration x explant size in *Experiment 2*. The numbers associated with the IBA is the concentration of IBA in µM. Small explants are 2.0 to 3.0 cm (0.79 to 1.18 in) and large explants are 3.5 to 4.5 cm (1.38 to 1.77 in).This data is from blocks 1, 2 and 3.

Experiment 3

The number of roots produced ex vitro wasn't significantly affected by the covariate of explant length, but was significantly affected by rooting treatment ($P < 0.001$). A Holm-Bonferroni pairwise comparison showed that the significant differences were exhibited between the water treatment and both of the auxin-containing rooting powders ($P \leq 0.001$ for both comparisons). An average of 5.8 roots per explant for 0.1% IBA, 5.2 roots for 0.3% IBA, and 2.2 roots for the water (control) treatment were produced (see Figure 2.10).

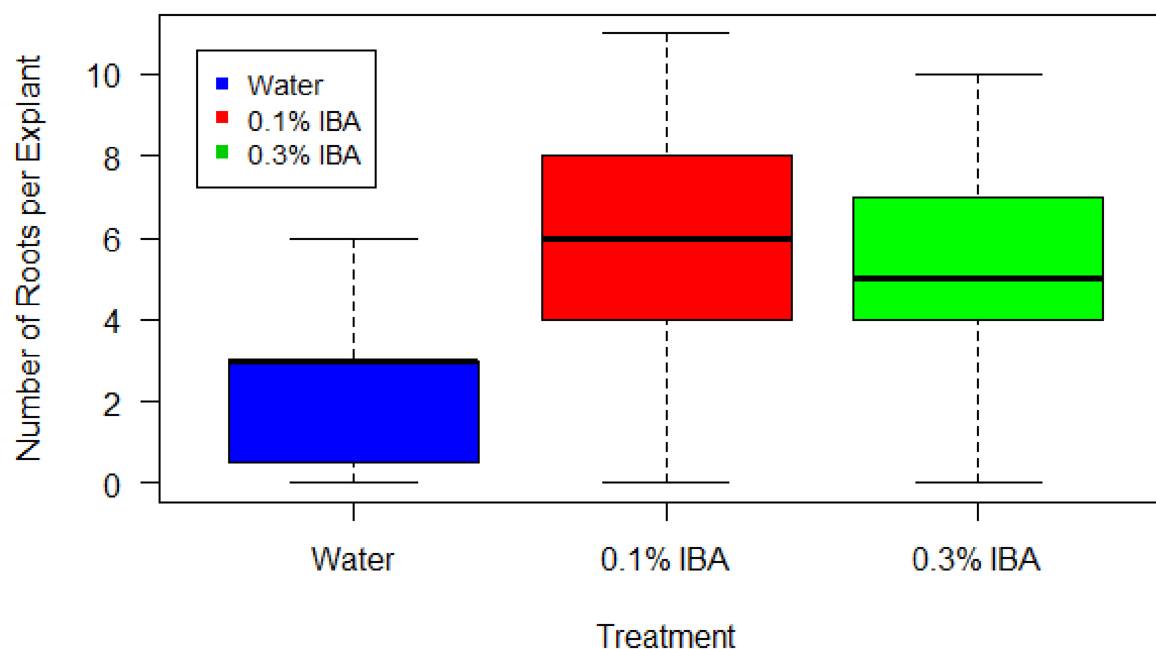

Figure 2.8. Number of roots per ex vitro genotype 1 (G1) stem explant as affected by rooting treatment in *Experiment 3*. The treatment was a microcutting dip into tap water (control treatment), 0.1% indole-3-butyric acid (IBA) talcum powder or 0.3% IBA talcum powder.

Both IBA containing treatments produced acclimatized plantlets at a rate of 91.7%, whereas the control (water) produced 83.3% acclimatized plantlets. The rooting treatment significantly impacted the number of explants with 5 or more leaves ($P = 0.025$ with associated chi squared value). A Holm-Bonferroni pairwise comparison showed that the significance was between the water treatment and both of the IBA treatments ($P \leq 0.010$ for both). Only 16.7% of plantlets treated with water had 5 or more leaves, whereas 62.5% of plantlets treated with IBA had 5 or more leaves.

Experiment 4

The average number of roots was significantly affected by treatment ($P < 0.001$ chi-square value) for G2 microcuttings. The average number of roots per ex vitro microcutting for the 0.1% IBA treatment was 10.5, while the water treatment produced an average 2.5 roots per

microcutting. Of microcuttings treated with 0.1% IBA, 9 of 10 had 5 or more leaves and 10 of 10 acclimatized. For the water treated microcuttings, 3 of 10 had 5 or more leaves and 7 of 10 acclimatized.

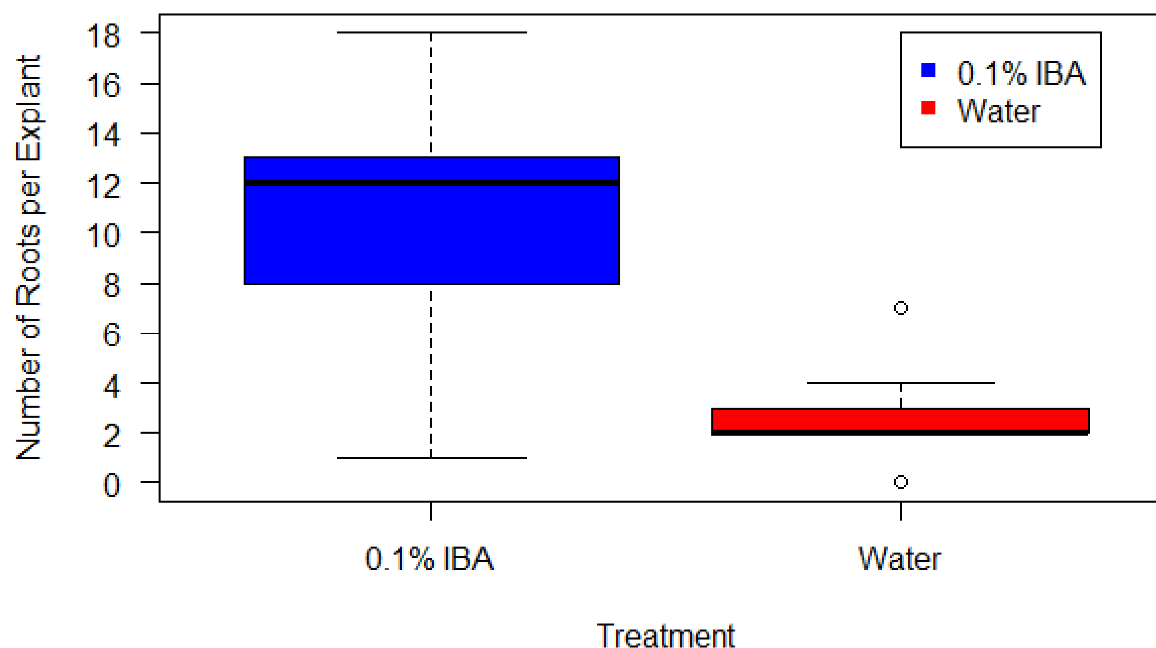

Figure 2.9. Number of roots per ex vitro genotype 2 (G2) microcutting as affected by rooting treatment in *Experiment 4*. The treatment was a microcutting dip into tap water (control treatment), or 0.1% indole-3-butyric acid (IBA) talcum powder.

DISCUSSION

A pre-treatment consisting of holding plants in the dark to enhance rooting has been shown to be efficacious in some species. For example, *Acacia mangium* Willd. (Leguminosae), displayed a statistically significant improvement in rooting with a dark pre-treatment (Monteuuis and Bon, 2000). Despite the low light intensity requirement of Douglas maple (experimentation suggested that roughly 17 µmols•m^{-2}•s^{-1} (1.58 µmols•ft^{-2}•s^{-1}) PPF is ideal), a 2-week dark pre-treatment ultimately resulted in the death of half of all dark treated explants. However, the death of explants may be partly due to the lack of photodegredation of auxin. Auxin at 6 and 12 µM may have been acceptable for a light treatment due to rapid photodegredation, but without this degradation, these levels may have been toxic. A study on

the degradation of auxin in the in vitro environment showed that filter sterilized IBA in an agar based MS medium after 10 days exposed to light at 74 $\mu mols \bullet m^{-2} \bullet s^{-1}$ (6.87 $\mu mols \bullet ft^{-2} \bullet s^{-1}$) PPF for 16 hrs per day was reduced to roughly 10% of the original IBA concentration (Nissen and Sutter, 1990). The dark treatment within this same experiment was reduced to roughly 71% of the original IBA concentration (Nissen and Sutter, 1990). This big difference in percent of original concentration demonstrates the effectiveness of light in degrading IBA. Evidence for toxic levels of IBA is present in *Experiment 1*, in which, 0 of 8 auxin-less x dark treated explants died, but 16 of 24 auxin-containing-media x dark treatment explants died. In addition, auxin treated explants sometimes exhibited root growth on above-agar petiole tissue. Nonetheless, Monteuuis and Bonn (2000) mention degradation of explant quality starting at 2 to 3 weeks after start of dark treatment, thus it is possible that the explants were simply left in the dark treatment for too much time. In order to test this hypothesis of toxic IBA levels, a new experiment with a dark treatment and lower levels of IBA would need to be included in order to discern if death was due to auxin toxicity, the dark treatment, or an interaction of these two factors.

Experiment 2 showed that Douglas maple is highly sensitive to exogenous IBA. Just 1 µM IBA statistically increased the rooting ratio and the number of roots per explant. Furthermore, Nissen and Sutter (1990) found that autoclaving IBA can reduce IBA recovery by roughly 20%. Exposure to 74 $\mu mols \bullet m^{-2} \bullet s^{-1}$ (6.87 $\mu mols \bullet ft^{-2} \bullet s^{-1}$) PPF for 2 days can result in roughly 80% reduction of filter sterilized IBA recovery in solid MS agar medium. Using this data, I speculate that after 2 days of culture, the concentration of IBA in the 1 µM treatment of *Experiment 2* contained just 0.16 µM IBA, which further demonstrates how sensitive Douglas maple is to exogenous IBA.

Auxin-free treated explants from *Experiment 2* produced 28% rooted explants. This result may be evidence of reversion of tissue to a more ontologically juvenile state.

Large explants in *Experiment 2* had better rooting ratios and root numbers than did small explants. This differential was clearly evident at the highest concentration of IBA (8 µM). The interaction (see Figure 2.3) between explant size and IBA concentration on root number may indicate that explants with higher mass are better able to tolerate and respond to higher concentrations of IBA than explants of less mass. Although the combination of large explants

and high IBA concentration produced the highest rooting ratio, signs of auxin overdose effects were evident, typified by formation of very thick roots.

Although the higher auxin concentrations benefited rooting ratios and rooting numbers in large sized explants, I demonstrated that shoots with high rooting ratios and root numbers do not equal the best treatments for acclimatization. Many large explants that were rooted in vitro with 8 µM IBA failed to acclimatize once transferred to the ex vitro environment. In contrast, many non-rooted explants treated with auxin-less medium, rooted successfully during the ex vitro acclimatization process. This result suggests that even though explants treated with high levels of IBA produced a high number of rooted explants, the roots resulting from this treatment were maladapted to the ex vitro environment and/or were predisposed to damage during transplantation. Roots formed under high levels of IBA were more brittle and tended to be easily damaged during the transplant process. In general, as concentrations of IBA increased, the diameter of the roots increased (visual observation), number of roots per explant increased (Table 2.6), length of roots decreased (Table 2.6), and branching of roots decreased (visual observation). On the other hand, roots formed without supplemental IBA, though slender, were more resilient and flexible during physical disturbance. Abnormal morphology of in vitro formed roots was previously documented for red maple (*Acer rubrum* L. [Aceraceae]) – specifically that the cortical cells were enlarged and vascular tissue was poorly developed compared to an ex vitro formed roots (McClelland and others 1990). Additionally, the ex vitro environment may have provided a stimulus that promoted rooting in ways different from the in vitro environment. This suggestion is based on the fact that rootless in vitro explants not treated with IBA eventually rooted and acclimatized in the ex vitro environment. However, this ex vitro rooting may have alternatively been a function of time, instead of a response to ex vitro environmental stimuli.

Research on bigtooth maple (*Acer* grandidentatum Nutt. [Aceraceae]) – a maple species native to many of the same regions as Douglas maple - showed that etiolated stem cuttings treated with a liquid solution of 0.4% IBA and 0.2% NAA rooted between 85 (year 1) and 89% (year 2) of cuttings averaged across 5 different genotypes (Richards and Rupp 2012). Cuttings from many other species of maple rooted best with a treatment of 0.5% to 0.8% IBA by either a quick liquid dip or an application with talcum powder (Hartmann and others

2011). In our study of *Acer glabrum* in *Experiments 3* and *Experiment 4,* explants rooted remarkably well when treated with 0.1% IBA rooting powder. This lower concentration of IBA needed for Douglas maple microcuttings in comparison to other species, may again be evidence of restored juvenility of tissue during culture. The percentages achieved by rooting microcuttings of Douglas maple was 92 to 100%, depending on genotype.

Experiment 3 showed that ex vitro rooting and acclimatization appear to be far more efficient than in vitro rooting and acclimatization (*Experiment 2*). This result supports Hartman and others (2011) who stated that most commercial propagation operations commonly use ex vitro rooting because of its ease and improved adaptation of adventitious roots to the ex vitro environment.

Verification of *Experiment 3* rooting efficacy was confirmed by using a different genotype in *Experiment 4*. Although *Experiment 4* lacks G1 as a control for reference, comparing these 2 comparable experiments showed a similar rooting response, which was enhanced by using 0.1% IBA rooting powder. One response difference was that G2 microcuttings produced nearly twice as many in vitro roots as compared to G1 microcuttings, on average. Though this experiment was limited with regard to rooting response due to genetic background, the study indicates that the propagation protocols developed as a result of this research will be effective across a range of *Acer glabrum* genotypes.

CONCLUSION

Experiment 1 made it clear that 2 wks of dark treatment designed to promote rooting was ineffective. In addition, minimal difference between the rooting responses of apical vs nodal explants allowed the conclusion that usage of apical explants is preferable for creating uniformity and superior form of clonal saplings intended for use in urban landscapes.

In vitro root growth, as indicated by rooting ratio and number of roots was strongly influenced both by initial explant size and presence of supplemental IBA. The highest acclimatization rates could be observed at 1 μM IBA regardless of explant size. Overall, 1 μM IBA was near optimum for in vitro treatments because it produced the highest rates of survival after rooting and acclimatization, and the most similar root morphology to the auxin-less medium.

When comparing ex vitro with in vitro procedures for rooting and acclimatization, ex vitro was superior in many ways. First, ex vitro rooting had the highest survival rate after rooting and acclimatization at 92%. In vitro procedures resulted in a maximum of 75%. Also, ex vitro rooting and acclimatization was more time and resource efficient. In comparison to in vitro methods, using the ex vitro methods outlined in this paper will reduce the rooting and acclimatization time by about 1 month, deduct 1 subculture worth of material and labor costs, and produce a higher percentage of rooted and acclimatized explants (≥ 92%).

Based on this research, I would recommend using ex vitro rooting methods with a talcum-based rooting powder containing 0.1 to 0.3% IBA for optimal rooting and acclimatization of micropropagated *Acer glabrum* plants.

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CHAPTER 3 – Douglas Maple Nutrient Optimization and Related Experiments

ABSTRACT

Douglas maple (*Acer glabrum* Torr. var. *Douglasii* (Hook.) Dipple [Aceraceae]) is a potentially valuable landscape plant that lacks proven asexual propagation protocols. Micropropagation has potential as an efficacious asexual method for propagating Douglas maple. Research regarding all stages of micropropagation have presented elsewhere in this thesis, but without addressing the topic of nutrition. Medium nutrition is very important for developing a successful micropropagation protocol. Deficiency symptoms such as chlorosis and shoot tip necrosis of Douglas maple plantlets grown on Driver Kuniyuki Walnut (DKW) medium salts prompted this nutrient optimization research. Preliminary experimentation with 2x concentration of iron (relative to Fe content in DKW medium salts) remediated the chlorosis, and iron was thus determined to be the most probable causal factor of the observed chlorosis. To extend this line of investigation, nutrition experimentation was designed to evaluate performance of standard commercial salt mixtures (DKW, Murashige and Skoog (MS) and Woody Plant Medium (WPM)) with supplemental iron were tested for their ability to rapidly grow shoots, increase node number, and increase dry weights of Douglas maple plantlets in culture. The DKW medium was statistically superior to the other nutrient formulations for promoting shoot growth and enhancing node production. Another experiment tested several nutrient supplementation treatments to DKW medium salts for remediating the shoot tip necrosis. The shoot tip necrosis was resolved by the supplementation of 12.0 mg/L boric acid to DKW medium salts. A final experiment tested a hypothesis that nutrient ratios within sap of *Acer glabrum* could be used as a basis for formulating salt concentrations in culture media. Shoot explants were placed on a medium based on ratios of natural sap nutrient ratios performed slightly worse as measured by average shoot length than those on a comparative commercial medium, but were statistically similar. Sap-based medium showed potential as a guiding principle for nutrient management in vitro, though much experimentation is still needed to improve the methods and strategies of sap-based nutrient optimization.

KEY WORDS: micropropagation, in vitro, tissue culture, *Acer glabrum*, accelerated nutrient optimization, sap nutrient analysis, boron deficiency, iron deficiency, maple endophyte, maple syrup carbon source, medium pH, nutrient precipitation.

NOMENCLATURE:
USDA NRCS (2019)

Abbreviations: zeatin – (E)-2-methyl-4-(1H-purin-6ylamino)-2-buten-1-ol or trans-zeatin, DKW – Driver and Kuniyuki Walnut, WPM – Woody Plant Medium, MS – Murashige and Skoog, LS – Linsmaier and Skoog, G-B5 – Gamborg B-5, KM – Kao and Michayluk, SNRF – sap-based nutrient ratio formulation, PPF – photosynthetic photon flux, SPAD – soil plant analysis development, PPM – Plant Preservative Mixture™.

INTRODUCTION

Douglas maple, also known as Rocky Mountain maple, (*Acer glabrum* Torr. var. *douglasii* (Hook.) Dipple [Aceraceae]) possesses excellent landscape plant characteristics, including high adaptability to temperature and soil types, bright red or yellow fall foliage, attractive red young stems, and a moderate degree of drought tolerance (USDA NRCS Plants Database 2019). In spite of excellent landscape potential, use of this species is limited, in part, due to a lack of published protocols for asexual (vegetative) propagation. Rupp and Wheaton (2014) state in their publication on native plant propagation that, "There are no published techniques for its [*Acer glabrum*] vegetative propagation."

Propagation of Douglas maple is most commonly accomplished by seed (Rupp and Wheaton 2014), but due to poor natural seed quality and recalcitrant seed dormancy characteristics, this process is inefficient. Also, seed propagation fails to maintain superior traits that make selected Douglas maple genotypes desirable as urban landscape plants. Propagation by stem cuttings is usually difficult and a tendency for overwintering death of cuttings may also be a detriment (Hartmann and others 2011, Nesom 2006). A high throughput asexual propagation protocol for Douglas maple is needed such that superior genotypes can be genetically conserved and rapidly propagated. High landscape potential and a lack of established

protocols for asexual propagation provide ample justification for micropropagation research on Douglas maple.

Micropropagation by nodal microcuttings (nodal culture) provides important advantages in propagating a species like Douglas maple. This method minimizes potential genetic variability induced by somaclonal variation (mutations), which is important because plants must be true-to-type to be marketable. In comparison to nodal culture, adventitious shoot proliferation methods would likely achieve higher propagative increase ratios, but somaclonal variation is considered to be more likely with this method (Hartmann and others 2011).

Research reported in this chapter was initiated to address specific nutritional problems that emerged during the early stages of this Douglas maple micropropagation project. During the first few subcultures after placing Douglas maple explants into tissue culture, interveinal chlorosis symptoms appeared on the youngest leaves and progressed in severity. This issue quickly became a hindrance to efficient plant multiplication in culture. Based on reports from earlier research, the chlorosis was hypothesized to be an iron deficiency; a problem somewhat common in maple species (Bowen-O'Connor and others 2007, Heidari and Safarnejad 2015, Taiz and Zeiger 2010).

Image 3.1. Douglas maple plantlet exhibiting iron deficiency as evidenced by leaf chlorosis.

Heidari and Safarnkjejad (2015) found that doubling iron content relative to standard MS medium salts was needed for optimal growth of Montpellier maple (*Acer monspessulanum* L.

[Aceraceae]). Bowen-O'Connor and others (2007) found that DKW was superior to other medium salt types for promoting shoot growth and hypothesized this superiority was due to the increased iron content and in other minerals that aid in iron uptake, which are present in DKW but absent in WPM, LS and MS medium salts.

Preliminary experiments were conducted to test the Douglas maple iron deficiency theory. A doubling of the iron content (supplemented as NaFeEDTA) provided a rapid improvement in leaf color and general plant health. Comparison plants without supplemental iron continued to decline in health and retained severe symptoms of chlorosis. As the iron deficiency issue was resolved, additional nutritional deficiency symptoms emerged. The uppermost unexpanded leaves and shoot tips were sporadically, but increasingly, becoming necrotic. The newest leaves were also exhibiting some hooking. Based on earlier reported work, this necrosis was hypothesized as being due to deficiencies of calcium, boron or silicon (Taiz and Zeiger 2010).

Image 3.2. Douglas maple microshoots exhibiting boron deficiency evidenced by necrosis of new leaves and shoot tip.

Such deficiencies demanded solutions. But standard methods of comprehensive nutrient optimization would be much too time-consuming and labor-intensive considering constraints and overall goal to develop an efficacious and efficient micropropagation protocol.

Nutrient deficiency issues in Douglas maple cultured on a commercial medium salt mixture was somewhat unexpected. Individual species - and even genotypes within a species - often

have unique nutritional requirements (Nas and Read 2004). Frequently, nutrient optimization for new species or genotypes in tissue culture is accomplished by modifying (i.e. supplementing with specific nutrients) commercially available media formulations such as DKW, WPM and MS using a trial-and-error process (Nas and Read 2004).

Most formulas for the commercially available salt mixtures were developed by sequentially testing levels of single nutrients and finding the optimum concentrations of that nutrient for a subject species (Halloran and Adelberg 2011). Because of this, culture medium nutrient optimization represents a time-consuming and laborious process due to the large numbers of nutrients involved. For example, the MS medium salt formula took 5 years to finalize (Nas and Read 2004). Sequential testing of individual nutrients, however, may not be best because relative ratios of nutrients are also important – such as NO_3^- with NH_4^+ and K^+ (Halloran and Adelberg 2011). Testing for these interactions between nutrients can further complicate the experimental design and process of medium optimization.

An alternative to using commercially available salt mixtures is to customize media formulations using individual salt constituents. To account for interactions among nutrients, a fractional factorial or Plackett-Burman experimental design could be used to test many nutrient combinations at once and reduce the number of treatments needed (Nas and others 2004). However, some interactions will inevitably be confounded by using these experimental designs. Sequential or fractional factorial experiments are still laborious when considering the 16 plant-essential elements that are obtained from the soil and may need to be included and optimized (Taiz and Zeiger 2010). Another alternative is to formulate media holistically based on nutrient concentrations present in plants of a subject species. This method relies on the assumption that nutrient ratios found in biological samples should be present in the same ratios in culture medium (Spaargaren 1996). Moderate success in media development has been achieved by using media formulations based on nutrient content of seeds (Nas and Read 2004); however, some nutrient concentrations in the seed were not appropriate to media formulation. Copper and nitrogen levels, for example, were at toxic concentrations in proportion to other nutrients. Leaf tissue nutrient analysis has been used to guide and optimize concentrations of inorganic media components in tissue culture for passion fruit with good success (Monteiro and others 2000).

Sap samples may be a better indicator of nutrient requirements than tissue samples because a sap sample would show the nutrients in solution that are supplied from an external source to the cells, whereas, a tissue sample is indicative of what the cells have accumulated. I have hypothesized that accumulated nutrient ratios (in seed or leaves) may be askew from ratios needed for maximizing growth and development rates in vitro.

Historically, researchers have collected sap for constituent analysis by using excised aphid stylets in order to minimize the alteration of in vivo sap constituents (Gaupels and others 2008). Companies such as Advancing Eco Agriculture have adopted sap analysis as a commercial agronomic service. They claim it to be a very effective way of identifying near-deficiencies before they fully develop. In addition, sugar maple sap has been analyzed in-depth by researchers from the Centre ACER (Center for Maple Syrup Research, Development and Technology Transfer Inc. in Canada) for purposes relevant to the maple syrup industry (Lagacé and others 2015). Nevertheless, to my knowledge, the application of sap analysis for in vitro medium nutrient optimization is a concept never attempted or discussed in published literature.

The purpose of the research herein is to identify nutrient issues by remediation of deficiency symptoms, and to test SNRF efficacy.

MATERIALS AND METHODS

Three nutrient experiments were conducted: the first *(Experiment 1)* to compare efficacy of 3 commercially available medium salts formulations, a second *(Experiment 2)* to test the impact of specific nutrient fortifications designed to resolve negative symptomology in culture, and a final experiment *(SNRF Experiment)* to assess the usefulness of sap nutrient levels and ratios in creating an optimal custom media salts formulation.

Materials and methods pertinent to all experiments and referenced as part of descriptions of methods within each experiment are:

Standard Data Collection Procedures:

- Total number of shoots was recorded by counting the number of shoots. The main apical shoot was included in the count. A minimum of 5 mm (0.20 in) stem length was required to be considered a shoot.
- Shoot length was recorded by measuring from the base of the explant (excluding basal callus tissue which was excised with a scalpel) to the apical meristem of the shoot. In order to qualify for a recorded length, the shoot must have been at least 5 mm (0.20 in) in length. A sum was recorded for explants with multiple shoots of ≥ 5 mm (0.20) length.
- Number of nodes was counted only on explants that were ≥ 5 mm (0.20 in) in length. Visible buds, or any 2 oppositely arranged leaves originating from the same position on the stem counted as a node. The uppermost node was not included if leaves associated with it were still crinkled due to their lack of development. The goal was to include only nodes that could be cut and used as an explant.
- Dry weights were recorded by removing basal callus tissue and then placing all shoot and leaf material into an envelope. The envelope was put in a drying oven for a minimum of 3 days at ~ 77° C (171° F). The dried plant material was then weighed.
- SPAD chlorophyll meter readings were conducted by choosing the biggest 2 leaves from each explant and recording 2 readings from each leaf. The 4 SPAD data points were then averaged among all data points from explants in a single culture vessel.

Standard Medium:

A culture medium (*standard medium*) was common to all experiments. Deviations from *standard medium* specifications are included in the descriptions of respective experimental materials and methods. The *standard medium* recipe per liter (0.26 gal) was: 5.2 g (0.18 oz) DKW medium salts, 45.4 mg (0.0016 oz) NaFeEDTA, 30.0 g (1.06 oz) D-sucrose, 5.0 ml (0.17 oz) stock G vitamin solution (20 mg (0.00071 oz) thiamine-HCl, 10 mg (0.00035 oz) nicotinic acid, 10 mg (0.00035 oz) pyridoxine HCl and 40 mg (0.0014 oz) glycine in 100 ml (3.38 oz) of reverse osmosis (RO) water), 5.0 ml (0.17 oz) stock H vitamin solution (2.0 g (0.07 oz) myo-inositol in 100 ml (3.38 oz) of RO water), 0.877 mg (0.000031 oz) trans-zeatin for 4 µM concentration, 1 M or 0.1 M HCl and/or NaOH to adjust the pH of the medium to 5.80 ± 0.02, and 8.0 g (0.28 oz) agar. The ingredients were added to a flask with RO water at

roughly half of the final medium volume (e.g. 0.5 l (0.13 gal) RO water for medium of 1.0 l (0.26 gal) final volume). A stir bar was used to accelerate dissolution of constituents. The constituents were added individually, and allowed to partially/mostly dissolve before the next constituent was added. Solid constituents were weighed in plastic weigh boats. Residual constituent on weigh boat was rinsed with RO water into the flask except for agar. Once all constituents listed up until pH adjustment were dissolved, medium was brought to volume with RO water. Hydrochloric acid (HCl) and sodium hydroxide (NaOH) were then used to adjust pH. Then the agar was added. Medium was microwaved to melt the agar, which usually entailed bringing the solution to a moderate-to-rolling boil 3 times. Roughly 50 ml (1.69 oz) of media were aliquoted per 7.7 x 7.7 x 9.7 cm (3.0 x 3.0 x 3.8 in) tissue culture vessels (Magenta™ GA-7). The autoclave cycles ran for 25 minutes at 121° C (250° F) and 103 kPa (15 psi).

Stock Explants:

The term *stock explant* used herein refers to explants that have been cultured on *standard medium*. *Stock explants* were usually grown at a density of 5 explants per Magenta™ GA-7 vessel. *Stock explants* were subcultured about every 5 weeks. *Stock plant* tissue was from a genotype called genotype 1 (G1), which was collected from the University of Idaho Arboretum in Moscow, Idaho. The plant was obtained by the arboretum staff from Plants of the Wild nursery in Tekoa, Washington. Plants of the Wild staff were unable to provide precise provenance information, but said the seed used was likely from Bonner County in northern Idaho. The explant material was ontologically immature, having not yet produced reproductive organs. Genotype 1 was collected and inducted into tissue culture on August 9th, 2017. Genotype 1 was in culture for 10.5 months, 13 months and 15 months prior to the start of *Experiment 1*, *Experiment 2*, and the *SNRF Experiment*, respectively. The plant material in all experiments in this chapter used G1 *stock plant* explants, except when stated otherwise.

All explants for experiments and for *stock explants* were grown in a Hoffman Manufacturing Company (Albany, Oregon) growth chamber using General Electric brand Ecolux 34 W soft white fluorescent light tubes. The photosynthetic photon flux (PPF) ranged from roughly 5 to 80 µmols•m^{-2}•s^{-1} (0.46 to 7.43 µmols•ft^{-2}•s^{-1}) depending on the explant and vessel density, the position of vessels on the shelf, and the size of explants in culture. This wide range of

PPF was addressed by blocking of experiments according to row of vessels upon the shelf, as seen in Figure 3.1. Addressing this wide range of PPF by blocking was important, because some *Acer* species like sugar maple (*Acer saccharum* Marshall [Aceraceae]) are very sensitive to light intensity (Singh and others 2017). During the process of culture establishment, Douglas maple also showed signs of light stress due to a PPF of just 37 $\mu mols \cdot m^{-2} \cdot s^{-1}$ (3.44 $\mu mols \cdot ft^{-2} \cdot s^{-1}$). The photoperiod in the growth chamber was kept at 16 h, and the temperature was kept constant at 22° ± 2° C (71.6° ± 3.6° F). Greenhouse environmental conditions (concerning the acclimatization and ex vitro experiments) were a 14 h photoperiod from a combination of high pressure sodium lamps and natural light, and a minimum night to maximum day temperature range of 17.7° to 25.6° C (62° to 78° F).

Experiment 1

The goal of *Experiment 1* was to identify the commercial medium salts formulation that was most appropriate for supporting rapid growth of Douglas maple. The experimental design was a randomized complete block. The experiment had 4 blocks which were arranged by shelf within the growth chamber for the first subculture, but shortly after the start of the second subculture, the experiment was blocked by row on a shelf in the growth chamber to account for light intensity (see Figure 3.1).The independent variable was the nutrient salt mixture (DKW, MS or WPM). The dependent variables were number of nodes per explant, average dry weight per explant, and total shoot length per original explant. Six treatment replicates were used in total (2 replicates per treatment), per block. A replicate was the averaged explant data from within a single GA-7 vessel. Four explants were placed within each vessel. *Standard medium* and *standard data collection procedures* (aforementioned) were used for this experiment with the exception that 1.315 mg/l (6 µM) trans-zeatin was used instead of 4 µM. Recommended concentrations of the salt mixtures were used – 4.33 g/l (0.578 oz/gal) for MS and 2.3 g/l (0.310 oz/gal) for WPM medium salts.

The experiment was conducted for a duration of 3 subcultures: the first of 28 days, the second of 29 days, and the third of 28 days. Explant specifications for the first subculture were undocumented but between 5 and 20 mm (0.20 and 0.79 in) with 2 to 3 leaves, the second subculture was 7 to 13 mm (0.28 to 0.51 in) and 2 to 3 leaves, and the final subculture between 5 and 15 mm (0.20 to 0.59 in) and 2 to 3 leaves. Explants for subcultures were

prepared by using a scalpel to segment plant material as needed. For the final subculture, apical explants were used whenever possible. If apical explants were unavailable due to poor growth and development on these treatments, a nodal explant was used. After the final subculture, data were collected on a block by block basis.

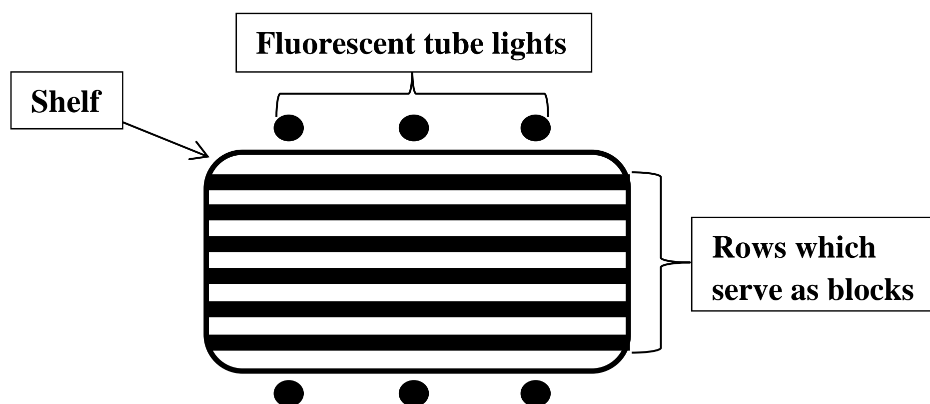

Figure 3.1. Top-view diagram of the blocking structure used in *Experiment 1, Experiment 2*, and the *SNRF Experiment*. Rows are of GA-7 tissue culture vessels.

Experiment 2

The primary goal of *Experiment 2* was to evaluate nutrient fortifications to DKW medium salts with intent to remediate necrotic symptoms of shoot tips and young leaves. The experimental design was a randomized complete block. The experiment was blocked by row on a shelf in the growth chamber to account for light intensity (see Figure 3.1). Six blocks were used in total. The independent variable was the nutrient fortification treatment. The treatments (fortifications) were: no fortification (control), 100 mg/l (0.013 oz/gal) calcium silicate (Ca_2SiO_4), 12 mg/l (0.0016 oz/gal) boric acid (H_3BO_3), 180 mg/l (0.24 oz/gal) magnesium sulfate ($MgSO_4$), 400 mg/l (0.53 oz/gal) calcium chloride ($CaCl_2$), 45 mg/l (0.0060 oz/gal) ferric sodium EDTA (NaFeEDTA, or $C_{10}H_{12}FeN_2NaO_8$), or 554 mg/l (0.074 oz/gal) mesos nutrients (consisting of 398 mg/l (0.053 oz/gal) calcium chloride, 102 mg/l (0.014 oz/gal) dibasic potassium phosphate (K_2HPO_4), 54 mg/l (0.0072 oz/gal) magnesium sulfate).

Dependent variables were the number of nodes per explant, the average dry weight per explant within each vessel, average SPAD value and the total shoot length per original explant. One treatment replicate was used per block for a total of 7 experimental units per

block. The tissue culture vessel (GA-7) was the experimental unit, and 4 explants were placed within each GA-7. *Standard medium* and *standard data collection procedures* (aforementioned) were used except for the addition of the specific fortifications to form treatments.

The experiment was conducted over the duration of 2 subcultures: the first of 34 days, and the second of 35 days. Explant specifications for the first subculture were between 8 and 15 mm (0.31 and 0.59 in) with 2 to 3 leaves, and for the second subculture they were between 8 and 14 mm (0.31 and 0.55 in) with 2 to 3 leaves. Explants were prepared by using a scalpel to segment plant material as needed. The first subculture used 3 nodal explants and 1 apical explant per GA-7, whereas the second subculture contained only apical explants in all GA-7 vessels. After the final subculture, data were collected on a block by block basis.

SNRF Medium Experiment

Sap samples were collected from *Acer glabrum* trees found in a nature preserve called Idler's Rest, near Moscow, Idaho, on March 12 to 13, 2018. The preserve is found on the south aspect of the Palouse mountain range. The trees varied in size and maturity. Samples were collected by drilling 0.31 in (7.9 mm) diameter holes to a depth of approximately 1.0 in (2.5 cm) in the bottom 0.31 to 0.61 m (1.0 to 2.0 ft) of the trunk. Handmade wooden spiles with an inner hole diameter of approximately 0.25 in (6.4 mm), an outer diameter of approximately 0.31 in, and lengths between 2.25 and 3.25 in (5.7 and 8.3 cm), were firmly tapped into the drilled hole. Samples were retrieved after roughly 24 hours of collection time and then stored in a refrigerator for a day prior to submitting the samples to the Holm Research Center Analytical Sciences Laboratory at the University of Idaho, Moscow, ID.

Image 3.3. Sap collection from tapped *Acer glabrum* trees in Idler's Rest nature preserve near Moscow, Idaho.

Nutrients in sap were analyzed by inductively coupled plasma atomic emissions spectroscopy, which quantified the following elements: Ca, K, Mg, Na, P, S, Ba, Cd, Co, Cr, Cu, Fe, Mn, Mo, Ni, Vd and Zn. Of these elements, Ca, K, Mg, P, Ba, Mn and Zn were detected in all 4 samples, and Cu and S were detected in 2 of the 4 samples. Total nitrogen (NO_2 + NO_3 + TKN (Total Kjeldahl Nitrogen)) was also analyzed by Anatek Labs Inc. in Moscow, Idaho, but no nitrogen was detected.

For the development of a sap-based nutrient medium, the molar concentration of each element in sap was averaged between samples. Next, molar concentration for each element in DKW medium was determined using ARS Media for Excel (https://www.ars.usda.gov/research/software/). Each element in sap was then expressed as a percentage relative to the concentration of that same element in DKW medium.

Concentrations of sulfur and copper, both of which lacked 2 data points, were each given 2 dummy data points, which were just below the detection limit. This provided an approximate value for the elemental molar concentration. Percentages of individual elements relative to DKW individual elements ranged from roughly 1 to 40%. The percent of sulfur in sap relative to DKW was around 1%. This concentration was deemed too low for supplying rapid growth and was therefore excluded from the medium formulation process. Barium was also excluded because it is not considered a plant beneficial or essential element. The remaining elements were between 8 and 40% of the molar concentration of the respective elements in DKW medium. The sum of the ion concentration from the 7 remaining elements was 14.3% as concentrated as the totaled ion concentration of those same elements in DKW medium salts at standard dilution. Each of the 7 elemental concentrations in sap was multiplied by the constant, 6.999, which was derived by dividing the sap total molar concentration (for the 7 elements) by the DKW medium total molar concentration (for the 7 elements). By doing this, DKW and sap nutrient molar concentrations for these 7 elements were equalized, while retaining the relative ratios found in sap. All other elements undetected in sap adopted molar concentrations equal to that found in DKW medium; therefore, total molar concentrations of elements were equalized, but ratios between the 7 elements in sap were maintained for the SNRF medium. This sap-based nutrient ratio medium was finally tested against standard DKW medium which served as the control.

The SNRF medium was made by mixing individual stock solutions of each constituent and then adding the appropriate amount of stock to a RO water/salt stock mixture. Acidic stock solutions were added first. Compounds and amounts used for making the SNRF medium are listed in Table 3.1.

In combination, some of the salts precipitated upon addition of liquid (Image 3.4). Several solutions were trialed to eliminate the precipitate, including: making and mixing stock solutions comprised of individual salts, employing alternative salts, adding the acidic constituents first, vigorously stirring the precipitate-laden solution overnight, and varying the temperature of solution. None of these methods notably decreased the precipitate in solution.

Explants were placed onto precipitate-laden SNRF medium to determine if plant tissues would acidify the medium and help the precipitate dissolve in the agar matrix, but this strategy failed. Selecting strong acids to supply nutrients resolved the precipitate problem, but the pH of final solution was too basic. The solution was acidified to a pH of 5.7 using HCl (hydrochloric acid) and HNO_3 (nitric acid). This procedure changed the medium elemental concentrations such that DKW medium was no longer a completely appropriate control for testing the efficacy of the SNRF medium. Additional control treatments were added to the experiment based on changes to the *standard medium*: DKW with added NaCl (sodium chloride) to test effect of added chlorine to the *standard medium*, DKW with added $NaNO_3$ (sodium nitrate) to test effect of added nitrate to *standard medium*, and DKW with added NaCl and $NaNO_3$ (most appropriate control for SNRF medium). For treatments including sodium salts, the same concentration of sodium nitrate and/or sodium chloride as in SNRF medium was used for these treatments.

Image 3.4. Bottom view of a tissue culture vessel containing SNRF medium with constituent precipitate(s) from preliminary SNRF formulations. The abbreviation SNRF stands for sap-based nutrient ratio formulation.

The SNRF medium was created using *standard medium* recipe and procedures, except DKW salts were replaced with salts listed in Table 3.1, and pH was adjusted to 5.7 instead of 5.8. Control treatments were also adjusted to a pH of 5.7 and used *standard medium* recipe and procedures.

Table 3.1. Constituents and amounts used in SNRF medium of the *SNRF Experiment.* The abbreviation SNRF stands for sap-based nutrient ratio formulation.

Constituent	Amount (mg/l)
HNO_3	2057.1
H_3PO_4	198.1
HCl	73.9
H_3BO_3	4.8
$NaH_2PO_4 \cdot H_2O$	17.3
NaFeEDTA	90
NH_4NO_3	140.3
$(NH_4)_2SO_4$	1052.9
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.28
$Ca(OH)_2$	970.3
KOH	831.1
$NiSO_4 \cdot 6H_2O$	0.007
$Zn(NO_3)_2 \cdot 6H_2O$	9.8
$MnSO_4 \cdot H_2O$	20.7
$MgSO_4 \cdot 7H_2O$	1029.3
$CuSO_4 \cdot 5H_2O$	0.71
NaNO3	335.5
NaCl	77.1

Stock explants were employed in this experiment. Experimental design was a randomized complete block with 4 replications. Each replicate was a tissue culture vessel (GA-7), and

there were 4 explants (pseudo-replicates) per GA-7. Culture vessels were blocked by row on a shelf in the growth chamber to account for variable response to light intensity (see Figure 3.1). The independent variable was the medium formulation. The dependent variables were the number of nodes per explant, the average dry weight per explant, and the total shoot length. *Standard data collection procedures* (aforementioned) were utilized.

The experiment was maintained over 2 subcultures: the first of 30 days for blocks 1 through 3, and 29 days for block 4 and the second subculture of 32 days for all blocks. Explant length specifications for the first subculture were between 5 and 20 mm (0.20 and 0.79 in) with 2 to 3 leaves, and for the second subculture, apical explants of length 9 to 17 mm (0.35 to 0.70 in) with 2 to 3 leaves. Explants were prepared by using a scalpel to segment plant material as needed. After the final subculture, data was collected on a block by block basis.

Another experiment, called the *Syrup Experiment*, involved the use of maple syrup as a sucrose replacement in DKW medium. The amount of syrup used was based on the amount of sugar in the syrup. *Standard medium* contains 30.0 g (1.06 oz) sucrose, so 30.0 g (1.06 oz) sugars from Great Value™ maple syrup were used. There was 51 g (1.80 oz) sugar per 60 ml (2.03 oz) maple syrup, thus 35.3 ml (1.19 oz) per l (0.26 gal) of medium were used. Maple sap sugar is predominantly sucrose (Lagacé and others 2015), so it was assumed that maple syrup sugars were also predominately sucrose. *Standard medium* recipe and preparation procedures were used, except sucrose being replaced by syrup.

Statistical analysis of data from experiments was conducted using the RStudio function ‘lmer’ to create linear mixed effect regression models. Treatments were the fixed effects, while the blocks were the random effects within the models. Analysis of deviance was conducted on the models using the ‘Anova’ function from the ‘car’ package. Type-II sums of squares were used for the calculation of analysis of deviance tables. The F-test and statistic were used in providing P-values in the analysis of deviances. The T-test and statistic were used in providing P-values for the Holm-Bonferroni pairwise comparisons of individual treatment means.

RESULTS

Experiment 1

An analysis of deviance detected significance for average number of nodes and average shoot length as affected by the medium salt type (P-values < 0.001). A Holm-Bonferroni pairwise comparison showed that DKW medium was superior to both MS and WPM media in producing longer shoots (Figure 3.2) and more nodes (Table 3.2).

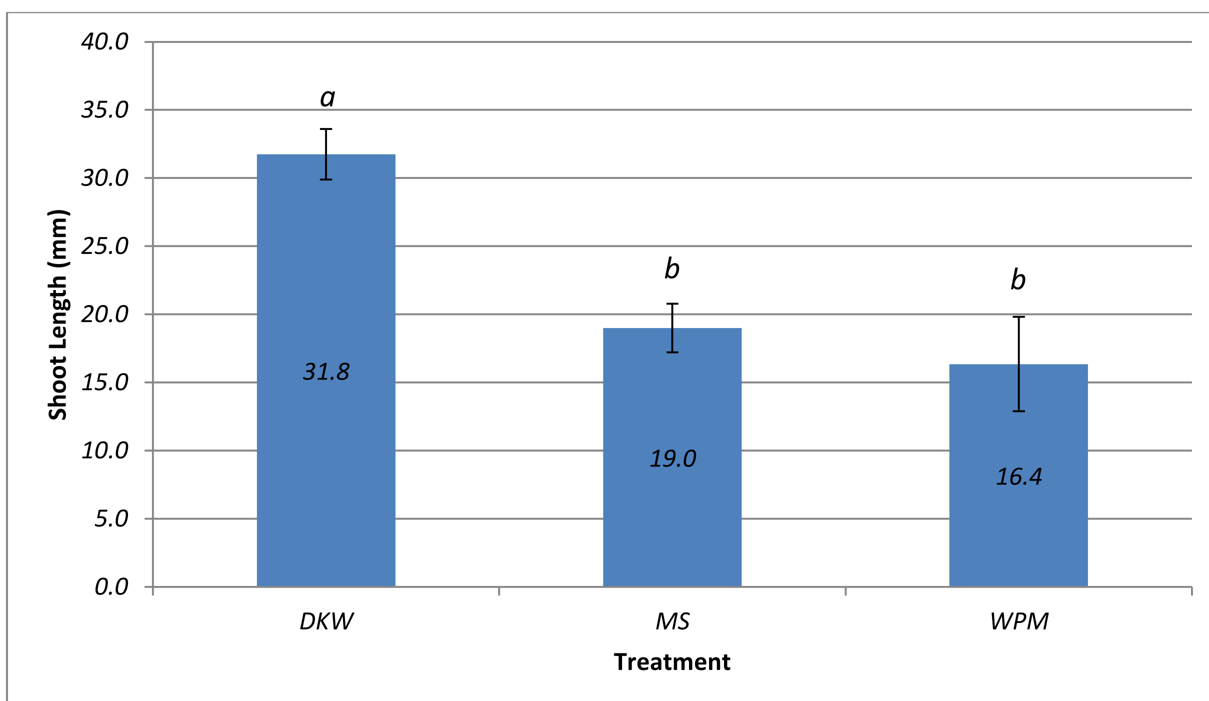

Figure 3.2. Average shoot length as affected by commercially available medium salt formulations (treatment) in *Experiment 1.* Letters denote significance with P ≤ 0.05. Vertical bars signify standard errors. The treatment abbreviations are: DKW – Driver Kuniyuki Walnut medium; MS – Murashige and Skoog medium; and WPM – Woody Plant Medium.

Table 3.2. Independent variable (shoot length, number of nodes and dry weight) averages as affected by treatment (commercially available medium salt formulations) in *Experiment 1.* The treatment abbreviations are; DKW – Driver Kuniyuki Walnut medium; MS – Murashige and Skoog medium; and WPM – Woody Plant Medium. Letters within columns denote significance of F test with P-value ≤ 0.05.

	Average Shoot Length (mm)	Average Number of Nodes	Average Dry Weight Per Explant (mg)
DKW	**31.8 a**	**5.4 a**	**45.1 a**
MS	**19.0 b**	**3.5 b**	**31.6 a**
WPM	**16.4 b**	**2.5 b**	**29.8 a**

Experiment 2

Average shoot length was the dependent variable that showed significant differences based on analysis of deviance (P = 0.011). All other dependent variables were statistically insignificant. A Holm-Bonferroni pairwise comparison of means showed that the addition of boron produced a greater shoot growth response than 2x iron, $CaCl_2$, and the control treatment (Table 3.3 and Figure 3.3).

Table 3.3. Average values of response variables (shoot length, SPAD (soil plant analysis development) value, number of nodes, number of shoots and dry weight) in *Experiment 2.* Treatments: control – *standard medium* (DKW medium with supplemental iron), CaSi – *standard medium* + Ca_2SiO_4, $MgSO_4$ - *standard medium* + $MgSO_4$, MESOS - *standard medium* + $CaCl_2$ + $MgSO_4$ + K_2HPO_4, B - *standard medium* + H_3BO_3, 2xFe - *standard medium* + NaFeEDTA (2 times the supplemental iron used in *standard medium*).

	Average Shoot Length (mm)	Average SPAD Value	Average Number of Nodes	Average Number of Shoots	Average Dry Weight Per Explant (g)
Control	28.8	32.1	5.2	1.2	43.8
CaSi	35.5	31.3	5.7	1.6	38.1
MgSO4	38.0	32.1	5.8	1.3	47.5
MESOS	33.5	30.6	5.2	1.3	40.1
CaCl2	25.6	31.2	4.7	1.4	42.5
B	50.5	29.5	6.8	1.1	43.3
2xFe	30.3	31.9	5.1	1.3	38.6

Image 3.5. Rows from left to right show arrangements of blocking structure for *Experiment 2.* Treatment position was randomized within blocks, so black squares indicate vessels containing medium with the boron fortification. Three other blocks were omitted from this image.

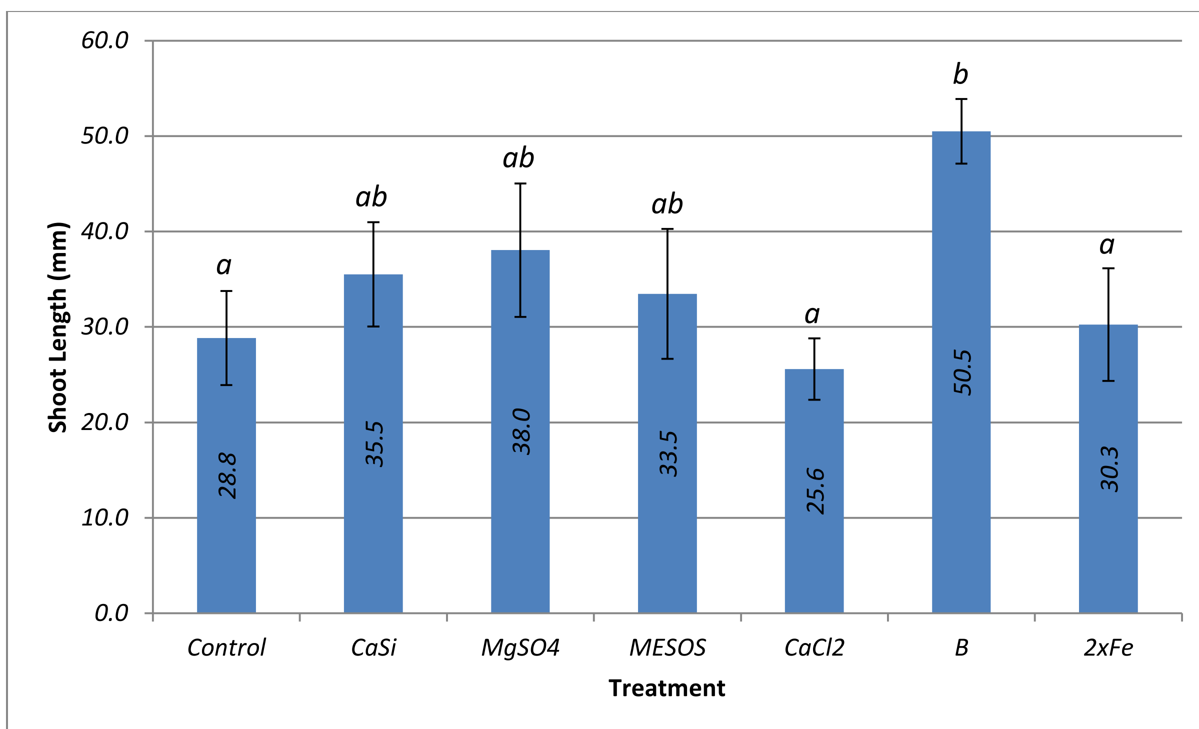

Figure 3.3. Shoot length as affected by nutrient fortification (treatment) in *Experiment 2*. Treatments: control – *standard medium* (DKW medium plus supplemental iron), CaSi – *standard medium* + Ca_2SiO_4, $MgSO_4$ - *standard medium* + $MgSO_4$, MESOS - *standard medium* + $CaCl_2$ + $MgSO_4$ + K_2HPO_4, B - *standard medium* + H_3BO_3, 2xFe - *standard medium* + NaFeEDTA (2 times the supplemental iron used in *standard medium*). Bars show standard error. Letters denote significance from F-test with $P \leq 0.05$.

SNRF Medium Experiment

An analysis of deviance of average dry weight showed that at least 1 treatment was different from the control (P = 0.019). No other growth responses were significantly affected by the treatment (Image 3.5). A Holm-Bonferroni pairwise comparison of means showed that the significant differences were with SNRF medium in comparison with *standard medium* plus sodium nitrate, *standard medium* plus sodium chloride, and DKW with sodium nitrate and sodium chloride. Shoots on the SNRF medium had the lower dry weights in these comparisons (Figure 3.4 and Table 3.3).

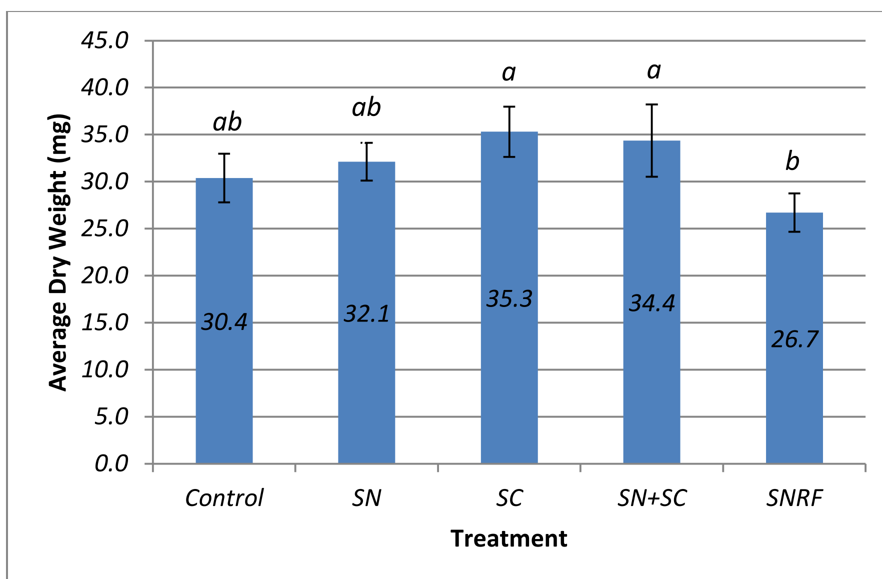

Figure 3.4. Average shoot length as affected by treatment in the *SNRF Experiment*. Treatments were: Control – *standard medium* (DKW medium plus supplemental iron); SN – *standard medium* + $NaNO_3$; SC – *standard medium* + NaCl; SN+SC – *standard medium* + $NaNO_3$ + NaCl; and SNRF – sap-based medium formulation. Letters denote significance with $P \leq 0.05$. Error bars represent standard error.

Table 3.4. Average values of response variables (shoot length, number of nodes and dry weight) of shoots in the *SNRF medium experiment.* Treatments were: control – *standard medium* (DKW medium plus supplemental iron); SN – *standard medium* + $NaNO_3$; SC – *standard medium* + NaCl; SN+SC – *standard medium* + $NaNO_3$ + NaCl; and SNRF – sap-based medium formulation. Letters denote significance from an F-test with $P \leq 0.05$.

Treatment	Average Shoot Length (mm)	Average Number of Nodes	Average Dry Weight per Explant (mg)
Control	**32.9 a**	**5.3 a**	**30.4 ab**
SN	**29.9 a**	**5.5 a**	**32.1 ab**
SC	**31.7 a**	**5.3 a**	**35.3 a**
SN+SC	**41.5 a**	**5.5 a**	**34.4 a**
SNRF	**30.6 a**	**5.1 a**	**26.7 b**

Image 3.6. Vessels of 2 treatments from *SNRF Experiment.* The left 2 vessels are of the control treatment, which was *standard medium* (DKW medium with iron supplementation), and the right 2 vessels are of the sap-based medium formulation.

The *Syrup Experiment* had to be discontinued due to the presence of contamination in all vessels (Image 3.6). Also, using syrup as a carbon source made medium pH much higher prior to pH adjustment. Based on this observation, a separate trial of pH adjustment of a DKW solution adjusted pH to 7.0 without any visible precipitation. Without syrup, precipitation of DKW constituents happens at a pH near 6.1.

Image 3.7. *Standard medium* (DKW medium supplemented with iron) enriched with maple syrup in place of D-sucrose revealed a bacterial endophyte that had been inconspicuously surviving in vitro.

DISCUSSION

My findings with respect to optimal nutrient salt formulation were similar to that of Bowen-O'Connor and others (2007) in that medium utilizing Driver Kuniyuki Walnut salts was best

for promoting shoot growth of Douglas maple. The DKW medium salts appeared to be the best available commercial formulation as a basal salt mixture for Douglas maple. Possible explanations for the superior performance of DKW medium salts is a Douglas maple preference for the higher total nutrient content in DKW; or possibly preference for the higher calcium content.

Remediation of chlorosis by iron fortification suggests that iron was the causal agent of chlorosis exhibited after induction. Iron has been shown to be crucial for proper chlorophyll structure and function, and is also needed in proper function of the electron transport chain of aerobic respiration (Rout and Sahoo 2015). Most maple species tend to be susceptible to iron deficiency (Kuhns and Koenig 2003), and Douglas maple, from an in vitro standpoint, appears to fit this tendency.

Positive growth response in the presence of additional boron during the nutrient additive experiment suggests that deficient levels of this nutrient are responsible for tip necrosis and leaf malformation. These symptoms are characteristic of boron deficiency in tissue culture (Eaton 1940, Taiz and Zeiger 2010). Because boron affects the apical meristem, a deficiency would hinder growth rates and reduce the efficiency of micropropagation. Supplementation of boron to a more optimal level would logically lead to better growth rates and have a positive impact on the economic feasibility of using tissue culture as a routine propagation method.

A negative result in the *SNRF Experiment* was actually an encouraging result. No significant differences between means of the shoot length and number of nodes between medium developed from sap ratios and the DKW formulations suggests that this concept could prove to be a valuable tool for developing culture medium for plant species new to tissue culture. In order for sap-based nutrient ratio methodology to be fully effective, additional research is essential.

Sap-based methodology needs to be evaluated in the context of all findings from this research project, such as optimal levels of medium iron and boron. Also, timing of sap collections needs to be studied. Since, February/March sap collection via tapping may not be the best timing and method for collection. In my study, sulfur was detected at very low levels and

nitrogen was not even detectible. Absence of these plant essential nutrients in sap suggests that either the plants sampled were deficient in these nutrients (though plants didn't exhibit symptoms of this), or that these nutrients are stored in physical matrix and were still immobilized for translocation to a sink site. If the latter, then sampling sap at a different time in the season could capture the translocation of these nutrients in sap. Unfortunately, sap collection by tapping trees at later times isn't possible.

As an alternative, companies such as Advancing Eco Agriculture and Crop Health Labs have developed a commercial leaf-derived sap analysis. This method would allow sap collection during spring or summer. Actively growing foliage and shoots could create the nutrient sink needed to detect nutrients such as nitrogen and sulfur. An electronic document called Plant Sap Analysis Guide provided by Advancing Eco Agriculture reports being able to detect the following in leaf-derived sap: "…all the standard macronutrients and micronutrients, as well as multiple forms of nitrogen, pH, EC, sugar content, and extra traces such as molybdenum, selenium, cobalt, silicon, and others" (https://docs.wixstatic.com/ugd/237db0_c5e14f9baf0d469d9bc8cc937cffca18.pdf). The detection of nitrogen in the leaf-derived sap samples supports a hypothesis that a sink (active growth) may be needed for nutrient mobilization and translocation of nutrients such as N and S. The ability to detect all plant-essential elements in sap through this method may mean that comprehensive sap-based medium optimization is feasible.

Another proposed method of improving detection of micronutrients in February/March collected sap is by concentrating the sap by boiling off water. Elements such as Na, Co, Fe, Cr, Mo and Ni might have been detected if this method had been implemented.

Finally, maple syrup was found to have chelating or supersaturation-promoting properties. When syrup was added in place of sucrose to DKW salts, the pH could be raised to 7.0 without precipitation. Without syrup and just sucrose, precipitation starts as pH is raised to around 6.1. If syrup is added to medium at the beginning of the medium making process, it may prevent precipitation. This result may mean that normal salts could be used for formulation in place of the strong acids and bases and simplify the medium formulation process.

The *Syrup Experiment* yielded no results because of contamination on/in the medium. Colony morphology of the contaminant was fairly round, minimally raised, and off-white in color (see Image 3.6). The syrup revealed the presence of an endophyte that had been growing inconspicuously within the maple explants. The use of maple syrup in medium was replicated to see if the endophyte would grow conspicuously in a second occurrence using new *stock explants*, and conspicuous bacterial colonies grew again. Nearly all explants that were placed on syrup enriched medium showed growth of the endophyte either on the medium surface, or within the medium where the explant had been inserted into the medium. A second Douglas maple genotype, called G2, was placed on maple syrup enriched medium to see if it too had the endophyte, but no endophytic organisms were discovered.

The bacteria were streaked for isolation and cultured until individual colonies grew. The bacteria were gram negative. The 16S gene of this bacteria was amplified by polymerase chain reaction (PCR) using the 27f primer. The PCR product was sent to a lab for sequencing. The 16S sequence revealed that the bacterium was a member of the Alcaligenaceae family. Identification to genus was difficult because the 16S sequence had ≥ 99% homology with species from several different genera within the family. Bacteria from this family can be found as beneficial endophytes to plants (Jha and Kumar, 2009). Whether or not this particular bacterium was beneficial to plant health is unknown.

CONCLUSION

Douglas maple growth and development were optimized on DKW medium with supplemental iron and boron. Supplementation with these 2 nutritional elements also resolved serious nutrient deficiencies that were problematic to Douglas maple micropropagation. Sap-based nutrient ratios for development of species-specific culture medium showed significant potential as a routine research method for species with complicated nutritional requirements unmet by commercial salt mixtures. This concept and method needs further refinement. A comparison of nutrient ratios of sap between trunk-collected and leaf-collected sap may be beneficial. Regardless, nutrient ratios of sap collected prior to a spring growth flush does not appear to be optimal because of the absence of some critical macro and micro nutrients. In conducting media formulation experiments, use of the

ARS Media for Excel is extremely valuable for creating reliable and timely medium recipe formulations.

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CONCLUSION TO THESIS

A comprehensive nodal micropropagation protocol was developed for Douglas maple, a potentially valuable native landscape species. Many experiments were designed to optimize multiplication efficacy and economic efficiency during each of the 4 stages of micropropagation. Conclusions derived from experiments include:

- Establishment: juvenile stem tissue of healthy looking trees collected in August can be effectively established on Driver Kuniyuki Walnut medium with a range of zeatin concentrations from 7 to 21 µM. Surface sterilization of stem explants can be accomplished with 20% (v/v) bleach and subsequent sterile water soaks.
- Cytokinin: 2 µM meta-topolin and 4 to 6.5 µM zeatin were very effective cytokinin concentration x compound combinations for increasing shoot length. True optimums were not obtained for these cytokinins because of improper concentrations employed in experimentation. Kinetin and 2iP can be disregarded as effective cytokinins for micropropagation of Douglas maple when used alone, whereas, thidiazuron may have potential even though performing statistically worse to 2 µM meta-topolin at the levels tested.
- Efficiency: no statistically significant difference in shoot length, number of nodes or dry weight was observed between of 4, 6 or 8 explants per GA-7 tissue culture vessel. This result suggests that the most efficient use of resources would take place at 8 explants per vessel. Genotype 1 showed no decrease to its average growth rate through 6 weeks, which suggests that 6 weeks or longer (until a marked decrease in growth rate) is ideal for maximizing time efficiency. Nevertheless, this may not be true for all genotypes or levels of tissue stabilization, as shown by the difference between the two genotypes evaluated.
- Light intensity: high light intensity could negatively impact explant quality as indicated by interveinal chlorosis. A photosynthetic photon flux of about 17 $\mu mols \cdot m^2 \cdot s^{-1}$ (1.58 $\mu mols \cdot ft^2 \cdot s^{-1}$) was optimal for growing non-chlorotic explants with cool white fluorescent light. Higher light intensity did statistically increase dry weight, which was likely due to increased leaf area and thicker stems which were observed visually.

- Rooting: the highest survival rate after rooting and acclimatization of in vitro rooted explants was observed from explants treated with 1 µM indole-3-butyric acid (IBA), regardless of explant length. Suggested IBA concentration optimum for in vitro rooting is 1 µM IBA, because this concentration produced the highest rate of explant survival after acclimatization and rooting. Plantlets also rooted using ex vitro methods. Treating explants with a talc-based rooting powder with 0.1 or 0.3% IBA resulted in 92% rooted and acclimatized explants. When comparing ex vitro procedures to in vitro procedures for rooting and acclimatization, ex vitro was superior in many ways. First, ex vitro rooting had the highest survival rate (92%), whereas, in vitro procedures resulted in a maximum of 75% survival after rooting and acclimatization. Also, ex vitro rooting and acclimatization required less time and resources than in vitro rooting and acclimatization. Ex vitro rooting and acclimatization procedures deduct around 1 month of time needed to produce fully-adapted, potted plants.
- Nutrition: Douglas maple should be cultured on a Driver Kuniyuki Walnut medium with supplemental iron (45.4 mg/l (0.0060 oz/gal) NaFeEDTA) and boron (12.0 mg/l (0.0016 oz/gal) H_3BO_3). The sap-based nutrient ratio formulation medium showed potential, but needs to be developed further. Nutrient ratios of sap collected prior to a spring growth flush do not appear to be optimal because some important macronutrients are not yet mobilized. In addition, using maple syrup in sap-based nutrient ratio formulation medium could help with precipitation issues when making medium. The sap-based nutrient ratio formulation method of nutrient optimization may be invaluable to establishing some species that are recalcitrant to micropropagation.

Altogether, the information gained from the experimentation described in this thesis has led to the construction an efficacious, efficient, cost-effective protocol with the potential to propagate healthy plants at a multiplication ratio of 1 explant in, to 12 explants out, per 6 week subculture. Furthermore, only around 8% of multiplied plantlets are expected to be lost after rooting and acclimatization when ex vitro methods are used.

Proposed Protocol for Optimal Douglas Maple Nodal Micropropagation

1. Establishment
    a. Collection
        i. Late spring to late summer seem to be ideal time periods for collection, though not necessarily limited to this timeframe
        ii. Choose a healthy looking specimen that has copious ontologically juvenile (evidenced by no present or past reproductive organ formation) stem tissue
        iii. Cut the newest stem growth of the desired specimen
        iv. Keep the plant material cool and moist with a wet paper towel
    b. Sterilization
        i. With a scalpel or razor blade, remove all leaves including petioles from the stems and then cut stem into single node segments
        ii. In a laminar flow, or biosafety hood, place stem segments into a 20% (v/v) bleach plus Tween® 20 (1-2 drops per 100 ml (3.38 oz)) solution for 20 min, followed by 3 sterile water baths of 2 minutes each
    c. Induction into in vitro environment (use aseptic techniques)
        i. Use *standard medium** in culture tube vessels (about 10 ml (0.34 oz) per tube) and *standard culture conditions***
        ii. With a scalpel, cut 2 to 3 mm (0.08 to 0.12 in) of length from the exposed ends of the nodal segments, and place the proximal end into the medium
        iii. Place only one explant per vessel
        iv. Check every 3 to 5 days for phenolic compound accumulation, and if these compounds are prominently present, transfer explants to fresh medium,
        v. Explants that are contaminant free can be subcultured until bud break or death of the explant – transfer explants to new medium every 4 to 6 weeks
        vi. Non-contaminated explants with shoots can be cut and multiplied onto fresh medium in GA-7 vessels

vii. Stabilization of plant material may take several months. Stabilization is evidenced by predictable growth rates, consistent phenotype, and possibly, spontaneous rooting of explants on plant growth regulator free medium

2. Multiplication (use aseptic techniques)
    a. Use *standard medium** and *standard culture conditions***
    b. Place up to 8 explants per GA-7 vessel for improved resource efficiency
    c. Use 6 week subcultures or possibly longer for improved multiplication rates
    d. When subculturing, cut explants such that each stem segment contains 1 node with both leaves
    e. Maximize nodal explants by cutting-off the apical meristem from the most distal explant segment
    f. Explant length will vary, but generally try to keep explants ≥ 5 mm (0.20 in) in length
    g. If both buds of the nodal explant break and grow, the basal section of the explant can be split in half vertically to maximize multiplication rate
3. Rooting and Acclimatization
    a. Root explants ex vitro by dipping the proximal end in water followed by 0.1% indole-3-butyric acid talcum based rooting powder
    b. Place treated explants into potting soil with covering to keep humidity high
    c. Keep potting mix moist
    d. After a week at a constantly high humidity, gradually expose the plantlets to drier air until equalized with the ex vitro growing conditions

**Standard medium:*

The *standard medium* recipe per liter (0.26 gal) was: 5.2 g (0.18 oz) DKW medium salts, 45.4 mg (0.0016 oz) NaFeEDTA, 30.0 g (1.06 oz) D-sucrose, 5.0 ml (0.17 oz) stock G vitamin solution (20 mg (0.00071 oz) thiamine-HCl, 10 mg (0.00035 oz) nicotinic acid, 10 mg (0.00035 oz) pyridoxine HCl and 40 mg (0.0014 oz) glycine in 100 ml (3.38 oz) of reverse osmosis (RO) water), 5.0 ml (0.17 oz) stock H vitamin solution (2.0 g (0.07 oz) myo-inositol in 100 ml (3.38 oz) of RO water), 0.877 mg (0.000031 oz) trans-zeatin for 4 μM

concentration or 0.483 mg (0.000017 oz) meta-topolin for 2 µM concentration, 1 M or 0.1 M HCl and/or NaOH to adjust the pH of the medium to 5.80 ± 0.02, and 8.0 g (0.28 oz) agar. The ingredients are added to a flask with RO water at roughly half of the final medium volume (e.g. 0.5 l (0.13 gal) RO water for medium of 1.0 l (0.26 gal) final volume). A stir bar should be used to accelerate dissolution of constituents. The constituents should be added individually, and allowed to partially/mostly dissolve before the next constituent is added. Once all constituents listed up until pH adjustment are dissolved, medium should be brought to volume with RO water. Hydrochloric acid (HCl) and sodium hydroxide (NaOH) are then used to adjust pH. Then the agar is added. Roughly 50 ml (1.69 oz) of medium is aliquoted per 7.7 x 7.7 x 9.7 cm (3.0 x 3.0 x 3.8 in) tissue culture vessel (Magenta™ GA-7). The autoclave cycle should run for 25 minutes at 121° C (250° F) and 103 kPa (15 psi).

***Standard Environmental Conditions*

Though many environmental conditions may be acceptable, the conditions used in developing this protocol were as follows:

- General Electric brand Ecolux 34 W soft white fluorescent light for in vitro culture
- A photosynthetic photon flux of roughly 17 $\mu mols \bullet m^{-2} \bullet s^{-1}$ (1.58 $\mu mols \bullet ft^{-2} \bullet s^{-1}$) for in vitro culture
- A growth chamber photoperiod of 16 h
- A growth chamber temperature of 22° ± 2° C (71.6° ± 3.6° F)
- Greenhouse conditions of 14 h photoperiod and a minimum night to maximum day temperature range of 17.7° to 25.6° C (62° to 78° F)