PROPAGATION OF AMERICAN HOPHORNBEAM (*OSTRYA VIRGINIANA* (MILL.) K. KOCH.)

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

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ABSTRACT

American Hophornbeam (*Ostrya virginiana* (Mill.) K. Koch) is an underutilized ornamental landscape tree with limited species improvement partially as a result of a lack of a clonal propagation protocol and its slower growth rate as compared to other commercially produced trees such as maple (*Acer* spp.) and ash (*Fraxinus* spp.). A recent decline in ash and the potential decline in maple have increased American Hophornbeam's importance. Currently, American Hophornbeam is seed propagated (sexual), with no clonal propagation (asexual) reported within the species. The objective of this research was to reduce the precise germination requirements of seed propagation as well as developing clonal propagation methods for commercial production. Seed treatments were examined to determine if precise stratification requirements could be simplified. Results indicated that scarification eliminated the precise stratification requirements for seed germination. Asexual propagation was successful with respect to tissue culture (micropropagation and organogenesis) but not with rooting cuttings.

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CHAPTER 1. LITERATURE REVIEW

American Hophornbeam (*Ostrya virginiana* (Mill.) K. Koch) is a member of the family Betulaceae (Birch Family). The family includes other important genera including *Betula* (Birch), *Alnus* (Alder), *Carpinus* (Hornbeam), and *Corylus* (Hazel). *Betulaceae* is an economically important family; *Betula* had ornamental nursery crop yearly sales in the United States of about $29,480,00 in 2007 (USDA-NASS, 2009). American Hophornbeam has potential itself to become an economically important ornamental tree in North America, especially in the Northern Great Plains. According to Dirr (2009), American Hophornbeam is an attractive tree that the American nursery industry has never pursued. The potential planting range of American Hophornbeam is reasonably extensive, including all 48 states in the continental United States (Gilman and Watson, 1994).

Ostrya is derived from the Greek word 'ostrua', which means 'a tree with very hard wood' (Fehrenbach, 1980). The genus *Ostrya* includes species, Zhejiang Hophornbeam (*O. rehderianaI)*, European Hophornbeam (*O. carpinifolia* Scop.), Chisos Hophornbeam or Big Bend Hophornbeam (*O. chinosensis* Corell), Central American Hophornbeam (*O. guatemalensis* (Winkler) Rose), Japanese Hophornbeam (*O. japonica* Sarg.), Knowlton Hophornbeam, or Western Hophornbeam (*O. knowltonii* Coville), Central Chinese Hophornbeam (*O. multinervis* Rehd), Yunnan Hophornbeam (*O. yunnanesis* Hu.), and *O. oregoniana* (extinct). There are only three species native to the United States and Canada: American Hophornbeam, Knowlton Hophornbeam, and Chisos Hophornbeam (*O. Chisosensis*) (Dirr, 1978). The species *O. knowltonii* and *O. chisosensis* are extremely rare specimens that are only found at high altitudes around 1500 to 2200 m in the mountains of Texas, New Mexico, Arizona, and southern Utah (Fehrenbach, 1980).

American Hophornbeam is endemic to the eastern half of the United States, usually as an understory tree on the drier slopes of woodlands (Dirr, 2009). It is a widespread species that is usually found in association with oaks (*Quercus spp.*) and mockernut hickory (*Carya alba* (L.) Nutt.) (Fehrenbach, 1980).

The elegance of the American Hophornbeam comes from the form, texture, and versatility of its canopy, which gives it potential for ornamental landscaping. The habit is varied, ranging from conical, to oval, to irregularly rounded (Dirr, 1978). It is often overlooked as an ornamental tree because of its slow growth rate however, once established has excellent growth (Dirr, 2009). There is disagreement in the mature height that American Hophornbeam can reach. It is anywhere between 7.5-18 m in the understory and 7.5-9 m for open grown or cultivated trees (Fehrenbach, 1980). The spread of the canopy is roughly two thirds of the height. The new growth is shiny and smooth with older growth developing a fluted or 'muscle-like- appearance similar to American Hornbeam (*Carpinus caroliniana* Walter). The bark forms vertical fissuring which exfoliates at each end providing an additional seasonal ornamental characteristic (Dirr, 2009).

Figure 1. American Hophornbeam tree courtesy of Paul Wray.

American Hophornbeam is a short to medium lived tree at 150 years (Fehrenbach, 1980). It is monoecious, with male staminate catkins grouped in threes beginning to be visible in late summer on the tips of previous year's twigs and lasting through the following spring. Catkins open in late April to pollinate the flowers. The flowers are not showy or ornamental (Dirr, 2009). The fruit is unique and has some ornamental appeal. The fruit of American Hophornbeam are nutlets borne in clusters of bladdery sacs that are similar of true hop fruit (*Humulus spp.*). Each sac contains one ovoid nutlet that is about 6 mm long. The fruit begins to be conspicuous in July and can be seen on the trees well into winter. The fruit ripens from August to October, at which time the sacs turn pale green to brown. The sacs are covered with fine, stiff hairs that can be irritating when they come into contact with the skin (Fehrenbach, 1980). Another ornamental characteristic of American Hophornbeam is the foliage. Leaves are alternate, simple, between 5-13cm long and half as wide (Bailey, 1976). They are doubly serrated with an acuminate tip. The leaves are a quality dark green in the summer and yellow in the fall. They typically color quite early (Dirr, 2009). Although it will grow in partially shaded areas, it achieves its best form in full sun, where it develops its most desirable branching. The wood of American Hophornbeam is light brown tinged with red and white. Where the wood is available, it is used for tool handles, golf clubs, mallets, fence posts, and other miscellaneous wood ware. American Hophornbeam is known as a very tough and durable tree that is extremely resistant to wind, snow and ice damage. The hardiness of this species is especially important for the Northern Great Plains, as trees that are aesthetically pleasing and hardy for this zone are not easily found. It is winter hardy to USDA hardiness zone 3 and is found growing in its native range down to zone 9.

American Hophornbeam is a rugged tree, tolerant of the urban environment including poor soil conditions (poor drainage, compacted soils) and drought conditions (Gillman and

Watson, 1994). It is a versatile ornamental tree, which can be used as a boulevard tree since they do not damage the sidewalk with surface roots (Dirr, 2009). They can be used as a shade tree as they have a thick canopy that forms a rounded outline.

According to Dirr (2009) it does not have any serious insect or disease issues, which is a very desirable landscape tree trait. Scale (*Coccoidea spp.*), neonectria canker (*Neonectria galligena* Bres.), leaf blister (*Taphrina virginica*), leaf spot, powdery mildew, and the two-lined chestnut borer *Agrilus bilineatus* (Weber) have all been found on American Hophornbeam. The limited pests, which infect American Hophornbeam, are a result of high concentrations of phenolic compounds in the stems and leaves (George et al., 2008). Phenolics are compounds, which carry one or more hydroxyl group on an aromatic ring. The high concentration of phenolic compounds in American Hophornbeam serve as protein binding agents that are able to reduce the amount of ingestion and efficacy of insect herbivores (Barbehenn et al., 2008). With the limited pests that affect American Hophornbeam, the nursery industry is reviewing its status as an alternative for ash trees (Iles, 2012). Ash trees (*Fraxinus* spp.) in the United States have been ravaged by emerald ash borer (EAB) (*Agrilus planipennis* Fairmaire), a beetle native to Asia and eastern Russia, in the last ten years costing $10.7 billion in control and removals (Kovacs et al., 2009). The need for clonal propagation of American Hophornbeam will be essential to increase potential selections and improvement of the species for viable commercial production.

The potential selections and improvement of this tree have been hindered by the method of production that is currently in place. In the ornamental nursery trade, American Hophornbeam is seedling propagated. Limited improvement of the species has been performed (Dirr, 2009). There is one cultivar of American Hophornbeam selected for the ornamental nursery trade, *Ostrya virginiana* ‘Camdale’ – Sun Beam® American Hophornbeam. Sun Beam® was released

by the North Dakota State University Woody Plant Improvement Program in 2011. This selection has a branch habit more upright than typical for the species. It is currently unavailable in the commercial trade because no clonal propagation protocol has been developed for the species.

Sexual Propagation Efforts

American Hophornbeam seed requires stratification to break dormancy for germination. A seed is dormant when it fails to germinate under normally favorable conditions for germination. There are different methods to overcome the natural dormancy requirements of seed such as stratification and scarification. Stratification is a pretreatment of moist conditions coupled with either warm or cool temperatures to simulate natural conditions that seeds require to germinate. American Hophornbeam seed stratification protocol is a warm stratification period of 60 days (30°C for eight h and 20°C for 16 h), followed by a period of cold stratification of 120-140 days (4-5°C) (Pitel et al., 1984). This process requires precise timing for commercial seedling production and requires detailed management from a commercial propagator to ensure good germination.

There is another method of removing the minimum dormancy requirements needed by seed called scarification. Scarification is any process for which the seed coat is broken, scratched, or altered using chemical, mechanical, or temperature methods to make the seed permeable to water and gases (Hartmann et al., 2011). Pitel et al., (1984) reported that scarification of American Hophornbeam assists in removing the physical dormancy of the seed and increases germination rates. The study compared three different scarification treatments sulfuric acid to split the pericarp (physical barrier that can affect dormancy) and weakening of the seed coat (testa), clipping the seed, and complete removal of the pericarp) in combination

with the phytohormone gibberellic acid (GA_3) to break dormancy. The results showed that the best treatment was the complete removal of the pericarp in combination with GA_3. Further research was necessary to obtain a treatment for commercial use that would be less cumbersome. This lead to this study, which tested scarification using sulfuric acid in combination with GA_3, or potassium nitrate (KNO_3) as an imbibition priming agent. Although seedlings can be produced with ease with the current protocols, sexual propagation cannot provide growers with clones of superior selections. Currently there is no asexual propagation method for American Hophornbeam.

Asexual Propagation Efforts

Asexual propagation or vegetative propagation is when a plant population is multiplied by any means other than seed. This process produces a clone, which is a plantlet that is genetically identical to the mother or stock plant. Vegetative tissues commonly used are stem, leaf, root and even single cells. There are different types of asexual propagation including cuttings, grafting, layering, and tissue culture. Studies on propagation of American Hophornbeam have been limited to sexual propagation (seed) (Pitel et al., 1984). There are no published reports of cuttings, grafting or tissue culture for asexual propagation of American Hophornbeam. However there have been reports of asexual propagation with other species in *Ostrya*. European Hophornbeam has been reported to be propagated successfully by cuttings (Galopin et al., 2010). Grafting has been reported on Zhejiang Hophornbeam (Ruohui et al., 1991) and European Hophornbeam (Gualaccini, 2009). Grafting is the joining of a root system, or rootstock, and a shoot system, or scion, and aligning the vascular bundles within each to make a composite plant.

Plant Tissue Culture

Plant tissue culture is known as the science of growing plant tissues or organs in an artificial media in a test tube (*in vitro*) (George et al., 2008). There are different types of tissue culture such as micropropagation, somatic embryogenesis, and organogenesis. Tissue culture within the *Ostrya* genus is very limited. Yang et al. (1991) reported germinating pollen grains of Zhejiang Hophornbeam *in vitro*. There are no reports of micropropagation in *Ostrya*. Micropropagation is a form of tissue culture in which plant organs and plant tissues are used to regenerate new plants under aseptic conditions. It is commonly used in the commercial nursery industry as a way to increase clonal numbers quickly.

Organogenesis is a process in which adventitious shoots and/or roots are formed from somatic cells. There are changes that take place in the cells depending on the hormones and media they are exposed to, leading to the development of root or shoot primordium. Shoot organogenesis is the production of unipolar structures such as adventitious shoots from somatic cells. The rates of multiplication in shoot organogenesis are usually higher than those of axillary shoot proliferation (Ahuja, 2003). This is a very efficient method for propagation as you can produce a large number of explants with very little material, space, and time.

Research Objectives

1. To determine if scarification in combination with GA_3 or KNO_3 can be used as a substitute for stratification to overcome seed dormancy.
2. To determine if shoot cuttings can be utilized for clonal propagation.
3. To determine if micropropagation is a viable method of clonal propagation by evaluating nutrient salts and hormone concentrations for establishment, shoot proliferation, and root initiation *in vitro*.

4. To determine if organogenesis regeneration is possible for callus tissue developed from leaf somatic cells.

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CHAPTER 2. OSTRYA SEED SCARIFICATION

Abstract

Ostrya virginiana (Mill.) K. Koch (American Hophornbeam) is a member of Betulaceae and is primarily found in the eastern United States, usually as an understory tree. It grows in USDA hardiness zones 3-9. American Hophornbeam is commercially propagated only through seed, with no clonal propagation methods reported within the species. Currently, American Hophornbeam seed requires 60 days of warm stratification (daily temperatures of 30°C for eight h and 20°C for 16 h) followed by 120-140 days of cold stratification (4-5°C). The objective of this study was to determine if seed dormancy could be overcome by scarification, gibberellic acid (GA_3), and Potassium Nitrate (KNO_3) treatments. Seed were collected from accession lines located at the NDSU Horticulture Research Farm during late fall of 2012. Seed was scarified with a 5 h soak of concentrated (97%) sulfuric acid (H_2SO_4), or tip cut. After scarification, seed was treated with gibberellic acid (GA_3) at 250, 500, 750 mg/L or 0.5% potassium nitrate (KNO_3) (imbibition priming agent) for 24, 48, or 72 h. Treated seed was wrapped in a moist paper towel and placed inside a Ziploc® bag for 14 days at 20°C in dark conditions. Seed was considered germinated if the radicle emerging from the seed coat was ≥5mm. Results showed that sulfuric acid for scarification was a better treatment than tip cut for scarification. The presoaking of GA_3 and KNO_3 did not show a significant difference, as well as time of exposure to the hormone and imbibition priming agent with respect to seed germination. It was concluded that although sulfuric acid scarification eliminated the precise stratification requirement for seed germination, germination rates are not high enough to recommend replacing current stratification methods.

Introduction

Ostrya virginiana (Mill.) K. Koch (American Hophornbeam) is a member of the Betulaceae family and is primarily found in the eastern United States, usually as an understory tree within USDA hardiness zones 3-9 (Dirr, 2009). American Hophornbeam is commercially propagated only through seed as a result of the lack of a commercially viable clonal propagation method. The propagation process for American Hophornbeam is quite intensive with respect to stratification temperature requirements. American Hophornbeam seed requires a warm stratification period of 60 days with daily temperatures of 30°C for eight h and 20°C for 16 h, followed by a period of cold stratification of 120-140 days at 4-5°C for germination (Pitel et al., 1984). Improvement upon the current seed propagation methods can make seedling production more efficient for grafting of desirable clonal selections.

There are other ways to overcome heavy seed dormancy, for instance scarification. There are different methods of scarification such as chemical scarification, which is usually done with sulfuric acid or mechanical scarification. These methods both physically create abrasions on the seed to weaken the protective seed coatings including the pericarp and seed coat (testa). In American Hophornbeam, the pericarp adheres tightly to the seed coat (Marquis, 1991). Scarification induces imbibition and germination by allowing water to reach the permeable cells below the seed coat (Hartmann et al., 2011). Hormones play an important role in the germination of a seed. When germination requirements are met, the embryo releases gibberellic acid (GA_3), a naturally occurring plant hormone, into the aleurone layer of the seed where an enzyme begins converting starch into energy for the seed (Hartmann et al., 2011). Pitel et al. (1984) reported that 3 month stratification at 4° C, followed by scarification with concentrated (97%) sulfuric acid (H_2SO_4), and GA_3 at 500 mg/L for 24 h could overcome seed dormancy in American

Hophornbeam. Imbibition can also be controlled by the use of priming agents such as potassium nitrate (KNO_3) (Shim et al., 2008). The effect of imbibition priming agents on dormancy has been attributed to metabolic repair and a trigger for early metabolic events to begin while the seed is still in the lag phase of germination (Hartmann et al., 2011). The purpose of this study was to determine if different methods of scarification in combination with GA_3 or KNO_3 (imbibition priming agent) could be used as a substitute for the precise stratification requirement to overcome seed dormancy. This study is important in determining if the stratification requirement can be eliminated and germination rates can be improved. Reducing the stratification time will save money by using less energy and space to germinate the seed and removing the need for precision timing and temperature control required by the stratification process.

Materials and Methods

Source of Seed for Stratification

For the first run, American Hophornbeam seed were collected over a one-month period of November 2012 from the North Dakota State University (NDSU) Horticulture Research Farm near Abzaraka, ND. The membranous involucre was removed and all collected seed were prescreened for quality by submerging in tap water for 30 minutes. The seed that sunk were considered viable and used in the experiment; all other seed were discarded. Seed collected from Abzaraka, ND was depleted and therefore an alternate source was needed. Seed for the second run, material was received from Baileys nurseries, Inc. in Newport, MN. From this second source, two more runs of the study were conducted.

Scarification Treatments

Seed were scarified using concentrated (97%) sulfuric acid (H_2SO_4) (Fisher Scientific, Product no. A468-500) for 5 h. A mechanical scarification was also performed by cutting horizontally across the endosperm of the seed using scissors (Fig. 1). Intact seed were used as controls for all treatments.

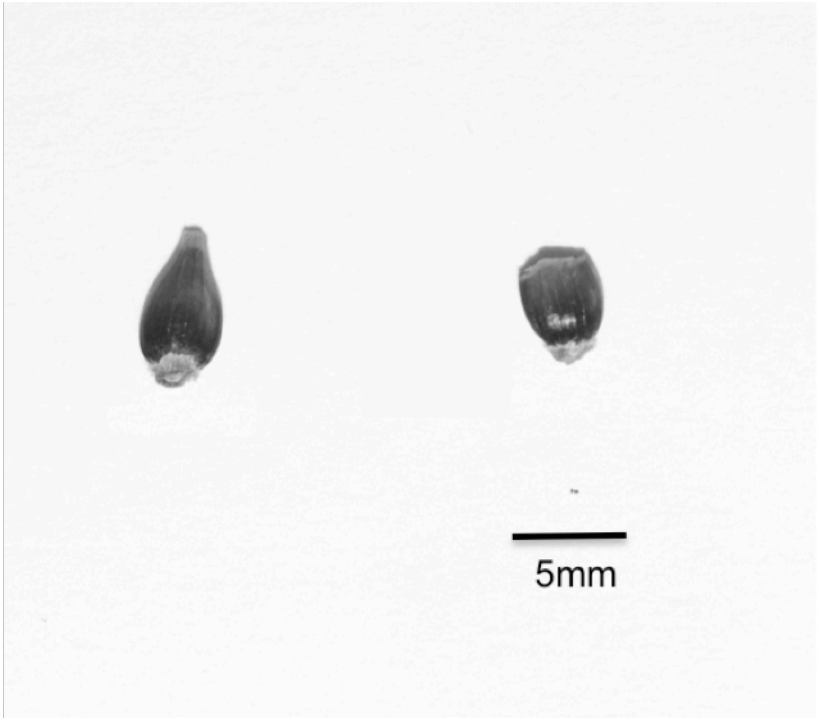

Figure 2. Intact American Hophornbeam seed (left), tip cut scarification (right).

Germination Hormone and Priming Agent Treatments

Different concentrations of gibberellic acid (GA_3) (Phytotechnology Laboratories, Product no. G500) were used (0, 250, 500, and 750 mg/L). Potassium nitrate (Phytotechnology Laboratories, Product no. P100) was used at a concentration of 0.5% as an imbibition priming agent to begin imbibition. Five seeds were used per replication. Seed were placed in flat top micro centrifuge tubes (Fisher Scientific Product no. 05-408-129) containing 0.5% KNO_3 or GA_3

at 0, 250, 500, 750 mg/L for a period of 24, 48, or 72 h with two replicates per treatment. Seed was wrapped with moist (distilled deionized water) paper towels and placed inside a Ziploc® bag for 14 days at room temperature (25°C) in the dark.

Data Collection and Analysis of Scarification Experiment

Data were collected after 14 days. Seeds were evaluated and considered germinated if radicle emerged was ≥5mm. Germination rates were determined from the percentage of seeds in each treatment that germinated after 2 weeks of moist conditions (germinated seed/total number of samples per treatment). All experiments were arranged as a completely random design (CRD). Five replications per treatment were used in each run. All data were analyzed using the General Linear Model (GLM) of SAS 9.3 (SAS Institute Inc., 2013).

Results and Discussion

Scarification Treatments

For the first run, there was a significant difference between germination rates of seed scarified with sulfuric acid as compared to tip cut scarification (Appendix Table 1, Table 1, Fig. 2). Germination rates were significantly higher with sulfuric acid in all combinations of GA_3 and KNO_3 (Table 1). Similar results were seen with a related species, *Carpinus orientalis* Mill. (Takos et al., 2001). They reported that soaking the seed in concentrated (97%) sulfuric acid for 4 h achieved the highest germination rate compared to all other methods of stratification and scarification. Sulfuric acid seems more practical for scarification as compared to the use of tip cutting for commercial germination. It has been reported that the best germination rate is achieved by complete removal of the pericarp through H_2SO_4 soaking (Pitel et al., 1984). This can be a cumbersome task and not practical for commercial propagation.

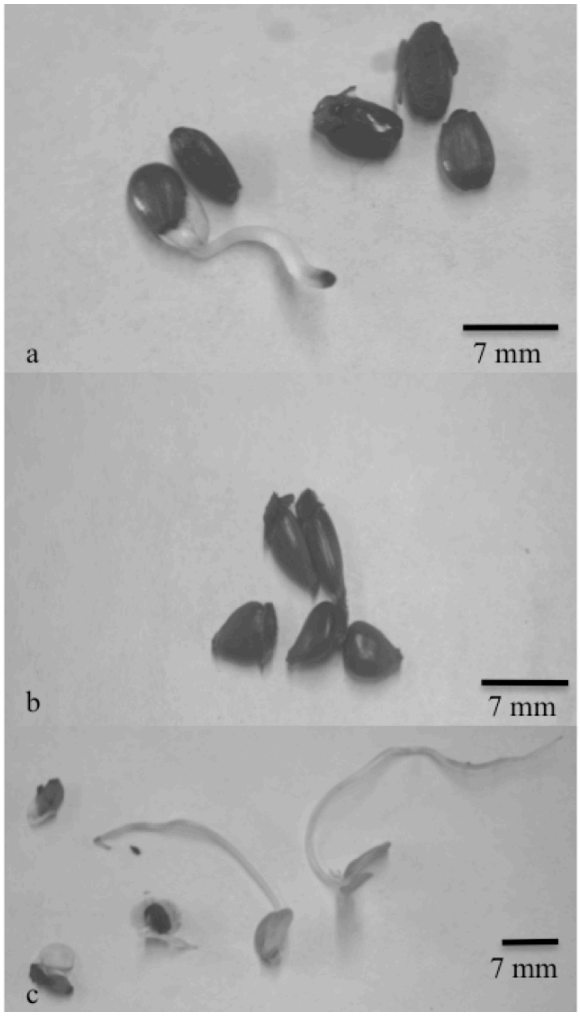

Figure 3. *Ostrya virginiana* (Mill.) K. Koch. seed under different scarification methods; (a) tip cut, 750 mg/L Gibberellic acid-3 (GA_3) for 72 h (b) intact, 0 mg/L GA_3 for 24 h (c) sulfuric acid, 500 mg/L GA_3 for 24 h.

Gibberellic Acid, Potassium Nitrate, and Exposure Time Treatments

For the first run, there was no significant difference between the different levels of GA_3 (0, 250, 500, 750 mg/L) (Appendix Table 1). When analyzed in combination with different exposure times (24, 48, 72 h) no significant differences were observed (Appendix Table 1, Table

1, Fig. 3). There was a significant difference between seed exposed to GA_3 and KNO_3 as compared to the control however there was no significant difference between the different GA_3 hormone levels or KNO_3 (Appendix Table 1, Table 2). There were similar results reported by Pitel et al. (1984) when using GA_3 that showed American Hophornbeam had a physiological dormancy that could be overcome by exposing the seed to GA_3 following a removal of the pericarp. Seed was placed into growth chambers for up to 60 days at a temperature of 20°C with supplemental incandescent lighting. Pericarp was completely removed and exposed to GA_3 then not stratified (control), stratified for 30 days, or stratified for 60 days. Germination rates were 80, 78, and 81%respectively showing no significant difference.

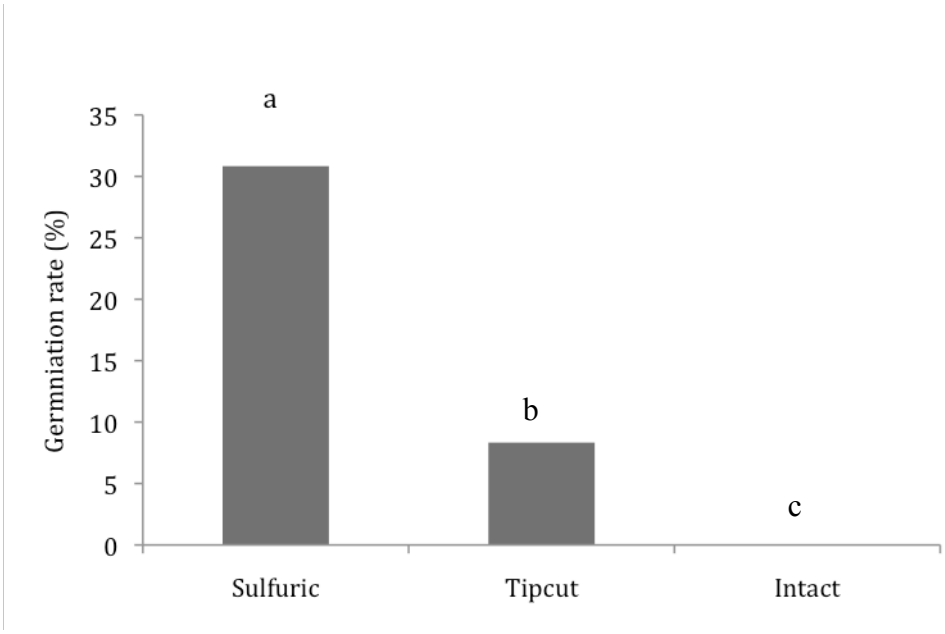

Figure 4. Effect of scarification treatments on germination rates of American Hophornbeam. Mean bar followed by the same letter is not significantly different ($\alpha<0.01$).

Table 1. Germination rates of American Hophornbeam seed after exposure to gibberellic acid (GA_3) or potassium nitrate (KNO_3).

Treatment	Mean Germination Rate (%)[a]
GA_3 (mg/L)	
0	4a
250	20b
500	16b
750	13b
KNO_3	12b

[a]Means averaged over runs

The difference in lighting and pericarp removal may have contributed to the reduction of the precise stratification requirements generally needed by American Hophornbeam. Potassium nitrate at the different exposure times did not have a significant effect in germination as compared to all other treatments (Appendix Table 1). Potassium nitrate has been shown to help imbibition in other species such as *Paspalum vaginatum* O. Swartz and *Prunus avium* L. by increasing the osmotic potential (Shim et al., 2008). There was a significant difference between all treatments and the control; however, there was no significant difference between treatments containing GA_3 or KNO_3 (Appendix Table 1, Table 2). These means include all levels of scarification and all exposure times within each hormone and imbibition priming agent treatments.

For the second and third run, none of the biological repetitions germinated (Appendix Table 1). The reason this could have happened was because of differences between the sources of seed. Each would have more similarities within the population sampled and would have greater differences between other sampled populations, therefore seed from a source in

Minnesota can behave completely different to treatments than seed from North Dakota. Data from all runs could not be combined due to unequal variances and error terms.

Conclusion

For the first run, the data suggest that weakening of the seed coverings (pericarp and testa) using concentrated (97%) sulfuric acid for 5 h as a scarification method produced the highest germination rates tested in this study. Gibberellic acid and KNO_3 as an imbibition priming agent had a significant effect on seed germination in this run, however, when compared with each other did not have a significant difference. Observations indicate that the impermeability of the seed coat and pericarp to water and gases can be overcome by scarification of the seed and exposure to either GA_3 or KNO_3. These results could not be confirmed through replication, however they were not a true replication as the source of seed was not the same. Further research using multiple seed sources would help make inferences as to whether these treatments can be used to surpass current stratification requirements. Even with the results of run one, stratification methods currently used for American Hophornbeam have higher germination rates than the data reported, although scarification allows for production of seedlings with less precision and energy.

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CHAPTER 3. ASEXUAL PROPAGATION

Abstract

American Hophornbeam (*Ostrya virginiana* (Mill.) K. Koch) is an underutilized ornamental landscape tree. This could be a result of commercial clonal propagation limitations and its slower growth habit. American Hophornbeam has desirable ornamental features including exfoliating bark and unique fruit clusters that resemble fruit of hops (*Humulus lupulus*). Also, the recent decline in ash (*Fraxinus spp.*), as a result of emerald ash borer, has increased American Hophornbeam's importance as it is a relatively pest free species. Currently, American Hophornbeam is seed propagated, with no clonal propagation reported within the species. The objective of this study was to develop a method of asexual (clonal) propagation for American Hophornbeam. Studies were developed to propagate American Hophornbeam asexually through softwood shoot cuttings, micropropagation, and organogenesis. A shoot experiment was developed to evaluate talc based indole-3-butyric acid (IBA) at 0, 0.3, and 0.8% or α-naphthalene-acetic acid (NAA) alcohol dip on rooting of semihardwood cuttings. Results indicated that no treatment tested produced significant rooting. A micropropagation experiment was also developed that evaluated different nutrient basal salt formulations (MS, LP, DKW, and WPM) with a cytokinin plant growth regulator (PGR) 2 μM 6-benzylaminopurine (BA). There was no significant difference between different nutrient salt formulations when shoot number and shoot length were measured. DKW was used as the basal salt formulation for stage II multiplication cultures with 7g/L agar and 3% sucrose. DKW was used as a nutrient salt formulation because microshoots from establishment experiment (Stage I) cultures had less visible nutrient deficiencies (chlorotic leaves) than all other nutrient salt formulations. Factorial combinations of BA (0, 4, 8 μM) and indole-3-butyric acid (IBA) concentrations (0 and 0.1 μM)

were compared to determine which plant growth regulator combination(s) would stimulate the proliferation of the most viable axillary or adventitious shoots. Results indicated that none of the treatments with BA (4 and 8 μM) produced significantly higher adventitious shoots than the control, but not significantly different from each other. Treatments with 4 μM BA regardless of IBA concentration or 8 μM BA in combination with 0.1 μM IBA produced significantly more axillary shoots than other treatments. All treatments with BA had significantly higher propagation numbers than the control. A subsequent factorial experiment was developed to evaluate thidiazuron (TDZ) (0.1 and 1 μM) in combination with IBA (0 and 0.1 μM). Lower concentrations of TDZ (0.1 μM) produced significantly higher adventitious shoot number, regardless of the presence of IBA than other treatments. Lower concentrations of TDZ (0.1 μM) produced longer adventitious shoots than other treatments as well as higher propagation numbers. In vitro rooting was evaluated utilizing WPM nutrient salt formulation and various IBA concentrations (0, 0.5, 1, and 2 μM). WPM was used because better rooting potential was observed than all other tested nutrient salts. Results indicated that IBA (0.5, 1, and 2 μM) produced significantly more roots than the control. Concentrations of 0.5 and 1 μM IBA produced significantly longer roots than all other evaluated treatments. An organogenesis experiment was evaluated to determine an adventitious shoot regeneration protocol from leaf callus. DKW was used as a nutrient salt formulation with 7g/L of agar and 3% sucrose. DKW was selected as a nutrient salt formulation for all subsequent micropropagation experiments because there were less visible nutrient deficiencies than all other nutrient salt formulations. A 4 x 2 factorial experiment was conducted comparing TDZ (0.1, 1, 5, and 10μM) or 10μM BA with 1-naphthalene-acetic acid (NAA) (0.1 and 0.5μM) for callus production from leaf tissues. Results indicated that there were no significance differences between any of the treatments. Callus was

normalized in medium containing DKW nutrient salts, 7g/L of agar, 3% sucrose, with 1 μM TDZ and 0.5 μM NAA to reduce residual effects from previous PGR exposure in preparation for microshoot regeneration evaluation. Subsequently, DKW was used as a nutrient salt formulation with 7g/L of agar and 3% sucrose. A 3 x 2 x 2 factorial experiment was designed with BA (0, 10, 20 μM), TDZ (0 and 0.1 μM), and NAA (0 and 1 μM) to evaluate the best PGR combination for microshoot regeneration. Results indicated that 10 μM BA had significantly higher adventitious shoot numbers than all other evaluated treatments. These methods of clonal propagation are useful tools for any future cultivars of the species developed and have opened up an opportunity for improvement of the species.

Introduction

American Hophornbeam (*Ostrya virginiana* (Mill.) K.Koch), member of the family Betulaceae, is native primarily to the eastern United States (Dirr, 2009). It grows in USDA hardiness zones 3 - 9. American Hophornbeam has desirable ornamental features including exfoliating bark and unique fruit clusters that resemble fruit of hops (*Humulus lupulus* L.). American Hophornbeam is an underutilized ornamental landscape tree; with limited improvement of the species partially as a result of a lack of a clonal propagation protocol and its slower growth rate as compared to other commercially produced trees such as maple (*Acer* spp.) and ash (*Fraxinus* spp.). Even though American Hophornbeam has a slower growth rate than other widely used species, a recent decline in ash (as a result of Emerald Ash Borer, *Agrilus planipennis* Fairmaire) and the potential decline in maple (as a result of Asian Longhorn Beetle, *Anoplophora glabripennis* Motschulsky) has increased American Hophornbeam's importance as it is a relatively pest free species (Dirr, 2009). Currently, American Hophornbeam is seed propagated, with no clonal propagation reported within the species. There is only one known

cultivar of American Hophornbeam, 'Camdale' - Sunbeam® American Hophornbeam (released from the North Dakota State University Woody Plant Improvement Program in 2011), which is currently not commercially available in part as a result of the lack of clonal propagation methods. Lacking clonal propagation has limited superior selections from being made and utilized (Pitel et al., 1984).

There are many methods of clonal propagation. The most commonly used method of asexual propagation is through stem cuttings. The type of stem cutting that is collected can have a major impact on rootability for a species. Stem cuttings can be classified depending on the physiological status of the stem cutting being utilized. Hardwood cuttings are those made of matured, dormant, firm wood. Semi-hardwood cuttings are cuttings made in late fall with partially matured wood. Softwood stem cuttings are cuttings prepared from the soft, succulent, new spring growth of deciduous or evergreen species (Hartmann et al., 2011). Stem cuttings are rooted using exogenous plant growth regulators (PGRs) to induce rooting from the cutting base. There are many studies in *Betula* spp. and *Alnus* spp., that have reported the use of both endogenous and exogenous plant growth regulators (PGRs) to root softwood cuttings (Yaguang et al., 2001; Pellett and Alpert, 1985; Ondruska and Schmidt, 1984; Schrader and Graves 2000; Henselova, 2002). A study was designed using semi-hardwood material and exogenous PGRs to root American Hophornbeam.

Another method of asexual propagation is using tissue culture through micropropagation. Micropropagation is the method of plant multiplication *in vitro*. Propagation of *Ostrya* spp. using micropropagation has not been reported. In the family; however, members of *Betula* spp. (Cheng et al., 2000; Perez and Postigo, 1989) and *Alnus* spp. (Barghchi, 1988; Perinet and Tremblay, 1987) have both been successfully propagated through micropropagation. One of the objectives

of this research was to develop a micropropagation protocol that can be utilized to commercially mass produce clonal varieties of superior selections of American Hophornbeam. In the establishment stage of micropropagation different nutrient basal salt formulations were considered. Murashige and Skoog (MS) (Murashige and Skoog, 1962), (WPM) Woody Plant Medium (WPM) (Lloyd and McCown, 1980), Long and Preece (LP) (Long et al., 1995), and Driver and Kuniyuki Walnut Medium (DKW) (Driver and Kuniyuke, 1984) were evaluated. MS and DKW are considered to be high nutrient basal salt formulations; WPM is considered a low nutrient basal salt formulation with LP as an average between high and low nutrient basal salt formulations (West and Preece, 2004). Nutrient basal salt formulation can have a significant effect on explant responses to supplemental PGRs in the growing medium (Preece, 1995).

There are other tissue culture methods used to propagate clonally such as organogenesis. Organogenesis is the regeneration of adventitious organs or primordia from undifferentiated cell mass (callus). There are issues that can arise from material propagated through organogenesis such as somaclonal variation. Somaclonal variation is genetic variation that is induced in plants produced in culture with a higher occurrence in adventitious production (Hartmann et al., 2011). In American Hophornbeam, there are no reported studies that have regenerated microshoots through somatic cells. Within the family, there are many species in *Betula* spp. and *Alnus* spp. (Cheng et al., 2000; Lin et al., 2010), which have successfully regenerated adventitious microshoots from callus. An experiment was developed to regenerate American Hophornbeam from leaf tissue derived callus.

Materials and Methods

Stem Cutting Propagation

Semihardwood cuttings were collected from the North Dakota State University (NDSU) Horticulture Research Farm near Absaraka, ND in August 2013. A modified method of propagation to that previously used for propagation of Seaside Alder (*Alnus maritime*, Betulaceae) was used (Schrader and Graves, 2000). Scharder and Graves (2000) utilized softwood cuttings but did not compare indole-3-butyric acid (IBA) to naphthalene-acetic acid (NAA); even though, all other procedures were repeated. Shoot tip cuttings were transported to the NDSU Lord and Burnham Greenhouse (NDSU Campus; Fargo, ND) for processing. Each cutting was cut into 15±1cm long segments. Leaves were removed from the basal half of each of the stems. Leaves on upper part of stem were cut in half to reduce transpiration. The basal 3±1cm of the stem was sprayed with distilled water and dipped in talc based indole-3-butyric acid (IBA) at 0.1 (Hormodin 1, OHP Inc. Prod. No. 983111), 0.3 (Hormodin 2, OHP Inc. Prod. No. 983221), and 0.8% (Hormodin 3, OHP Inc. Prod. No. 983380). These treatments were compared to three α-naphthalene-acetic acid (NAA) (Sigma Aldrich, Prod. No. N-0640) alcohol dips, talc (CVS® Prod. No.191684), and a control treatment (no talc or PGR). NAA was diluted in a solution of 50% ethanol (EtOH) by volume. Cuttings were dipped into 0, 0.25, and 0.5% NAA EtOH solution for 10 - 15 seconds. The treated cuttings were placed into a 1:1 perlite (PVP Industries Inc., Prod. No. 09217) to vermiculite (PVP industries Inc., Prod. No. 04202) mix by volume. Treated cuttings were placed in an intermittent mist propagation chamber with bottom heat (18±1°C) and an intermittent mist for ten seconds every ten minutes. After 6 weeks, treated cuttings were evaluated for number of roots and root length. A root was determined to be ≥ 5mm.

Micropropagation

Explant material was collected from the NDSU Horticulture Research Farm near Absaraka, ND in May 2012 to be placed in culture. In preliminary experiments, an issue encountered was the aseptic establishment of American Hophornbeam. Preliminary experiments indicated that the best disinfestation protocol tested was a pre-wash with 70% EtOH and three distilled water rinses. Excised shoots were then surface disinfested using a 0.825% sodium hypochlorite solution (bleach) with 1 ml/L of Tween® 20 (polyoxyethlenesorbitan monolaurate) (Phytotechnology Laboratories Prod. No. P-720) for 20 minutes and three sterile distilled deionized water rinses under aseptic conditions. Shoots were cut into segments (5±2mm) with each containing at least one node per segment. Explants were then placed into 25 x 150mm borosilicate glass culture tubes. An experiment using different nutrient basal salt formulations were used for the evaluation of aseptic explant establishment; Murashige and Skoog (MS) (Murashige and Skoog, 1962), Woody Plant Medium (WPM) (Lloyd and McCown, 1980), Long and Preece (LP) (Long et al., 1995), and Driver and Kuniyuki Walnut Medium (DKW) (Driver and Kuniyuke, 1984). MS and DKW are considered to be high nutrient basal salt formulations; WPM is considered a low basal salt formulation with LP as an average between high and low nutrient basal salt formulations (West and Preece, 2004). All nutrient salt formulations were supplemented with 2 μM 6-benzylaminopurine (BA), 7g/L agar (Phytotechnology Laboratories Prod. No. A111) and 3% sucrose. The pH was adjusted to 5.8 prior to autoclaving 1N potassium hydroxide (KOH). Explants were incubated approximately 30cm beneath cool white fluorescent lamps that provide a photon flux of approximately 40 μmol m^{-2} s^{-1} for a 16-h photoperiod at 25 ± 3° C. Data were taken six weeks after placement on various media treatments and evaluated on microshoot number and microshoot length. A microshoot was considered a shoot if it was

≥5mm. All shoots were subsequently transferred onto DKW basal medium with 2 μM BA, 7g/L agar and 3% sucrose for four weeks to normalize shoots before placing on Stage II (proliferation) media treatments to reduce residual effects of previous treatments. DKW was used as a nutrient salt formulation because microshoots from establishment experiment (Stage I) cultures had less visible nutrient deficiencies (chlorotic leaves) than all other nutrient salt formulations.

A broad spectrum PGR experiment was developed for stage II multiplication cultures using explant material previously in culture on media for normalization of microshoots (DKW basal medium with 2 μM BA, 7g/L agar and 3 % sucrose). Microshoots were divided into nodal segments (5±2 mm) containing at least one node and placed on a 3 x 2 factorial combination of BA (0, 4, 8 μM) and IBA (0, 0.1 μM) using DKW nutrient salts with 7g/L agar and 3 % sucrose. The pH was adjusted to 5.8 prior to autoclaving using 1N potassium hydroxide (KOH). Microshoots were incubated approximately 30cm beneath cool white fluorescent lamps that provide a photon flux of approximately 40 μmol m^{-2} s^{-1} for a 16-h photoperiod at 25±3°C. Data were taken on axillary shoot number, adventitious shoot number, and propagation number after six weeks in culture. A microshoot (axillary or adventitious) was considered a microshoot if it was ≥ 5 mm. Propagation number is based on the number of viable nodal segment propagules that were produced per treatment.

A second broad spectrum PGR experiment was developed for stage II multiplication cultures using explant material previously in culture on media for normalization of microshoots (DKW basal medium with 2 μM BA, 7g/L agar and 3 % sucrose). Microshoots were divided into nodal segments (5±2mm) containing at least one node and placed into a 2 x 2 factorial comparison between TDZ (0.1, 1 μM) and IBA (0, 0.1 μM) for microshoot proliferation evaluation. DKW nutrient basal salt medium was used with 7g/L agar and 3% sucrose. The pH

was adjusted to 5.8 prior to autoclaving using 1N KOH. Explants were incubated approximately 30cm beneath cool white fluorescent lamps that provide a photon flux of approximately 40 μmol m^{-2} s^{-1} for a 16 h photoperiod at 25 ± 3° C. Data was taken on axillary shoot number, adventitious shoot number, and propagule number after six weeks in culture. A microshoot (axillary or adventitious) was considered a microshoot if it was ≥ 5 mm. Propagation number is based on the number of viable nodal segment propagules that are produced per treatment.

For stage III rooting cultures, microshoots developed from in vitro multiplication cultures were placed on DKW basal medium with 2 μM BA 7g/L agar and 3 % sucrose for 4 weeks. Microshoots were placed on this medium to normalize shoots before placing on Stage III (rooting) media treatments to reduce variable PGR exposure and residual effects of previous treatments. Microshoots with at least one node (1.5 ± 0.5cm) were placed on DKW nutrient basal salt formulation medium with 7g/L agar and 3 % sucrose. The pH was adjusted to 5.8 prior to autoclaving using 1N KOH. Media was supplemented with IBA at 0, 0.5, 1, and 2 μM to evaluate microshoot rooting. Microshoots were incubated approximately 30cm beneath cool white fluorescent lamps that provide a photon flux of approximately 40 μmol m^{-2} s^{-1} for a 16 h photoperiod at 25 ± 3° C. Root number and root length were measured after 6 weeks. A microshoot was considered rooting if root length was ≥ 5 mm.

Organogenesis

An experiment to determine whether somatic cells can be used for adventitious microshoot regeneration was performed using leaf tissues as explant material. Leaf tissue was used from aseptic leaves of microshoots previously on DKW basal media with 2 μM BA 7g/L of agar and 3% sucrose. Leaves were cut into 4 mm^2 segments, which included sections of the midrib without the petiole. A 5 x 2 factorial experiment comparing cytokinins: TDZ (0.1, 1.0,

5.0, and 10.0 μM) and BA (10.0 μM) and auxins: 1-naphthaleneacetic acid (NAA) (0.1, and 0.5 μM) was evaluated for callus induction. WPM nutrient basal salt formulation was used with 7g/L agar and 3% sucrose. WPM produced higher callus formation at the base of explants in stage I (establishment) (results not shown) and thus was used to generate leaf callus. All tubes were wrapped with Parafilm® (Pechiney Plastic Packaging, Prod. No. PM-996) to prevent desiccation, and placed in dark conditions at 22±2°C for 8 weeks. Leaf callus was weighed, then placed on WPM with 1 μM TDZ + 0.1 μM IBA, 7g/L agar and 3 % sucrose to normalize the callus for 5 weeks. This was done because this treatment had the healthiest (green) callus. The pH was adjusted to 5.8 prior to autoclaving using 1N KOH.

An adventitious shoot regeneration experiment was conducted using DKW with 7g/L agar and 3% sucrose. Callus previously placed on WPM with 1 μM TDZ + 0.1 μM IBA, 7g/L agar and 3% sucrose to normalize the callus for 5 weeks. A 3 x 2 x 2 factorial with BA (0, 10, 20 μM), TDZ (0 and 1 μM), and NAA (0 and 1 μM) was evaluated. The pH was adjusted to 5.8 prior to autoclaving using 1N KOH. Callus were incubated approximately 30cm beneath cool white fluorescent lamps that provide a photon flux of approximately 40 μmol m^{-2} s^{-1} for a 16-h photoperiod at 25±3°C. Data was taken on adventitious shoot number and length after 6 weeks in culture. A microshoot was determined to be microshoot if ≥ 5mm in length.

Data Collection and Analysis

All experiments were arranged as completely random designs (CRD) and conducted twice unless otherwise stated. 5 replications were used in each treatment of every experiment. All data was analyzed using the General Linear Model (GLM) of SAS 9.3 (SAS Institute Inc., 2013). All mean comparisons were done using the student's T test of SAS 9.3 (SAS Institute Inc., 2013).

Results and Discussion

Stem Cutting Propagation

There was no significant difference between treatments tested (Appendix Table 2). The only treatments which produced roots were talc based 0.1% IBA and 0.5% NAA. In *Alnus glutinosa* subsp. *barbata* (C.A. Mey.) Yalt., 0.4% of IBA was used to induce rooting of cuttings collected in June and July (Ayan et al., 2006). American Hophornbeam cuttings in this experiment were collected in August, which could explain the decreased rootability of the cuttings as compared to *Alnus glutinosa* subsp. *barbata*. Some woody species have better rooting with semihardwood cuttings as compared to softwood cuttings (Hartmann et al., 2011). Semihardwood cuttings have been reported to provide a good rooting percentage for certain genotypes of hybrid hazelnuts (*Corylus ameriana* x *C. avellana*), a member of the Betulaceae (Ercisli and Read, 2001). Holloway and Peterburs (2009) reported that softwood cuttings of Mountain Alder (*Alnus incana* (L.) Moench ssp. *tenuifolia* (Nutt.) Breitung), another member of *Betulaceae*, collected June 20 (wild stands near Fairbanks, Alaska) treated with 0.3% IBA and placed in 1:1 perlite: vermiculite medium had significantly higher rooting (>80%) than semihardwood material collected in August (< 10%).

Another issue that could explain decreased rootability of American Hophornbeam is the lack of juvenile cutting materials. Collected cutting material for this experiment was from mature reproductive specimens. Mature cutting material can be difficult to propagate in some species such as Willow (*Salix* spp.), Sycamore (*Platanus* spp.), or Forsythia (*Forsythia* spp.) (Hartmann et al., 2011). Shoot forcing of epicormic buds to produce juvenile shoots has become a viable option in other species such as Silver Maple (*Acer saccharinum* L.) (Mansouri and Preece, 2009) and European White Birch (*Betula pendula* Roth.) (Cameron and Sani, 1994). Further research

on American Hophornbeam needs to be conducted with seasonal cutting collection times (physiological cutting status), PGR concentrations and shoot forcing for epicormic juvenile shoot production.

Micropropagation

For the first stage of micropropagation (Stage I - Establishment), data indicated that nutrient basal salt formulations: DKW, MS, WPM, and LP had no significant effect on microshoots number or length (Appendix Table 3, Table 3). Nas and Read (2003) reported that in the clonal production of Hazelnut hybrids (*Corylus americana* Marsh. x *Corylous avellana* L.) nutrient salt formulation were an important factor in production of micropropagated Hazelnuts. Nas and Read (2003) used NM, NMB5, WPM, NN, DKW, and MS with 5 g/L gelrite, 3% sucrose, and 22.2 μM BA + 0.049 μM IBA. Results indicated that NRM (low basal salt formulation with high vitamin levels) had higher multiplication rates and larger microshoots than all other treatments evaluated.

Table 2. American Hophornbeam explants placed into different salt formulations for 6 weeks.

	Mean[a,b]	
Salt formulation	Microshoot Number	Microshoot Length (cm)
LP	2.7a	1.3a
MS	4.0a	2.0a
WPM	4.5a	1.6a
DKW	8.0a	2.2a

[a] Means followed by the same letter are not significantly different ($\alpha<0.05$)

[b] Means were averaged over runs.

It was reported that WPM and MS produced increased amounts of callus but shoots that were more chlorotic than DKW, which was comparable with American Hophornbeam. DKW was selected as the nutrient basal salt formulation for all subsequent micropropagation

experiments because there were less visible nutrient deficiencies than all other nutrient basal salt formulations examined. It was also observed that WPM produced a large amount of callus at the base of the stems. For this reason, WPM was used as nutrient media for all subsequent organogenesis experiments.

In the second stage of micropropagation (Stage II - Multiplication) different experiments were conducted to determine which cytokinin and auxin concentration(s) produced the most microshoots, longest microshoots, and highest propagule number. It was observed while recording data that there were adventitious shoots being generated from callus and axillary shoots produced from nodes on the stem; and that overall combined shoot number was not significantly different among treatments. Data was subsequently taken on number of adventitious shoots, number of axillary shoots, and propagation number. The first proliferation study tested BA in factorial combination with IBA. Results indicated that there was a significant difference between each run of the experiment and within treatments when comparing adventitious and axillary shoot formation (Appendix Table 4, Table 4). There was also a significant difference between runs (Appendix Table 4, Table 4) and individual treatments when comparing number of axillary shoots (Appendix Table 4, Table 5). There were significantly more axillary shoots and adventitious shoots produced in run 1 than run 2 and significantly more propagules produced on average from run 1 than 2. Nodal segments used for the second run were smaller, overall, than nodal segments used in the first run. This could have been a contributing factor to the difference in runs. When data analysis was conducted on run x treatment, it was observed that there was no significant interaction (Appendix Table 4), which suggests that the variables behaved the same way within each run.

Table 3. Differences between runs of a factorial comparison of BA (0, 4, and 8 μM) and IBA (0 and 0.1 μM).

	Means[a]		
	Adventitious Shoots	Axillary Shoots	Propagation Number
Run 1	2.15a	1.05a	3.90a
Run 2	0.95b	0.35b	2.40b

[a] Means followed by the same letter are not significantly different ($\alpha<0.05$)

Table 4. Influence of 6-benzylaminopurine (BA) and indole-3-butyric acid (IBA) on American Hophornbeam shoots placed on DKW media after 6 weeks *in vitro*.

		Means[a,b]		
BA (μM)	IBA (μM)	Adventitious Shoots	Axillary Shoots	Propagation Number
8	0.1	1.62ab	1.38a	4.00a
8	0	2.87a	0.25b	4.63a
4	0.1	1.75a	1.13a	3.25a
4	0	1.50ab	0.75ab	3.75a
0	0	0.00b	1.00a	1.00b

[a] Means were averaged over runs

[b] Means followed by the same letter are not significantly different ($\alpha<0.05$)

There was also a significant difference between all treatments and the control when propagation numbers were compared. Although there is a significant difference between treatments when analyzing all variables, treatments behaved the same when compared within runs with no significant with run x treatment interaction (Appendix Table 4). All treatments of cytokinins (4 and 8 μM BA) regardless of presence of IBA produced significantly more adventitious shoots than the control (0 BA and 0 μM IBA) (Fig. 4). This same trend was reported for *Betula papyrifera* and *Betula pubescens* x *B. papyrifera* when culturing on WPM medium supplemented with 8.9 μM BA (Brand and Lineberger, 1992). Microshoots were primarily produced through direct adventitious regeneration in both species. With American Hophornbeam, treatments that produced increased numbers of adventitious shoots did not produce as many axillary shoots (Table 5). The highest axillary shoot numbers were produced by

treatments with 8 μM BA and 0.1 μM IBA, 4 μM BA and 0.1 μM IBA, 4 μM BA and 0 μM IBA, and the control (0 μM BA and 0 μM IBA) which was the original microshoot placed into culture (Appendix Table 4, Table 5) There was no significance difference between any of the treatments when looking at propagation number except when compared to the control (Appendix Table 4, Table 5, Fig. 5). Even though there is no significant difference between any of the treatments containing PGRs when looking at propagation number, treatments with higher axillary shoots should be utilized over media that produces increased levels of adventitious shoots if somaclonal variation is present. The potential for somaclonal variation in adventitious shoots is higher than with axillary shoots. Axillary shoots did not have deformation of the leaves and were easier to divide into nodal segments than adventitious shoots. Jokinen and Tormala (1991) reported that somaclonal variation has been minimal when European White Birch (*Betula pendula* Roth.) has been propagated through axillary proliferation as compared to adventitious proliferation.

The subsequent experiment evaluated another cytokinin (TDZ) in factorial combination with IBA. Results indicated that there was a significant difference between treatments that included higher concentrations of TDZ compared to the lower concentrations regardless of concentration of IBA (Appendix Table 5, Table 6, Fig. 6). Shoots placed on 0.1 μM TDZ and 0.1 μM IBA had the highest shoot number produced, but were not significantly different from shoots in 0.1 μMTDZ and 0 μM IBA (Table 6). Dai et al. (2006) reported that Paper Birch (*Betula papyrifera*)performed better in WPM media with 4-8 μM for shoot proliferation and Asian White Birch (*Betula platyphylla*) performed better on WPM medium supplemented with 20 μM BA.

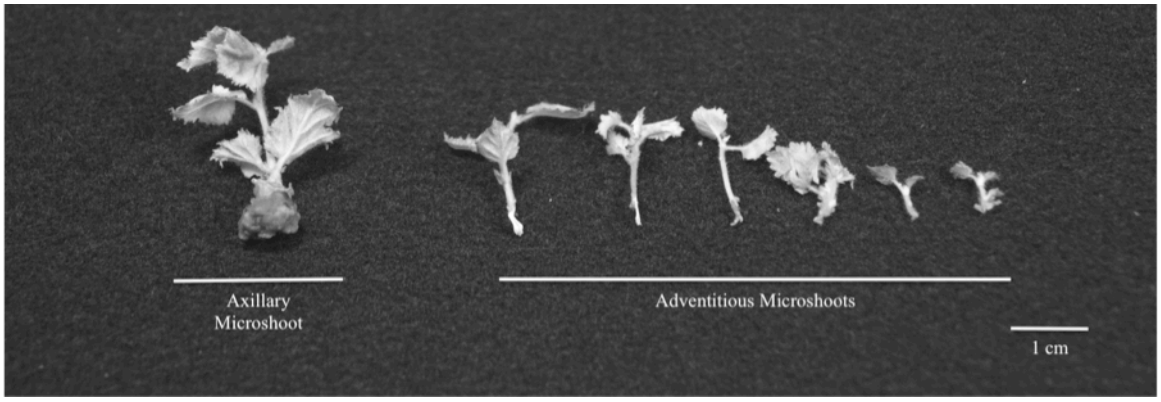

Figure 5. American Hophornbeam adventitious microshoots produced on DKW medium supplemented with 8 µM BA and 0 µM IBA for 6 weeks.

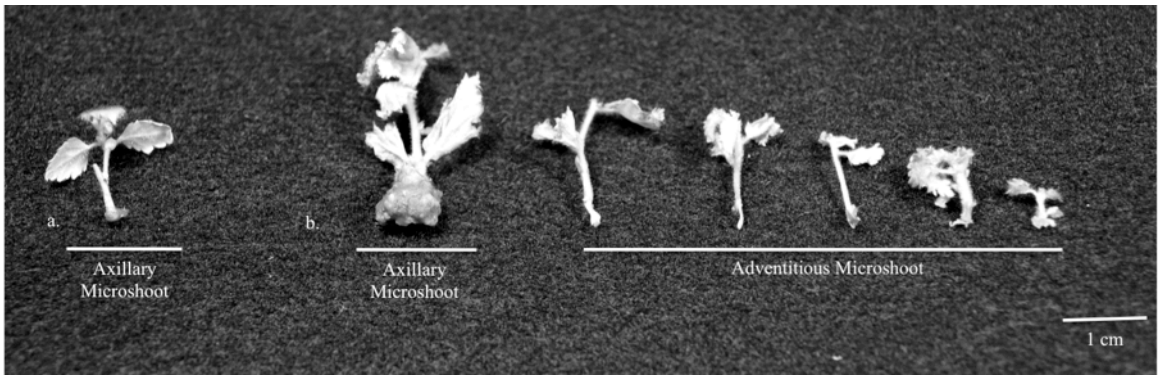

Figure 6. American Hophornbeam microshoots on DKW nutrient salt media with (a) control (0 µM BA and 0 µM IBA) (b) 8 µM BA and 0 µM IBA for 6 weeks.

Like American Hophornbeam, when exposed to TDZ, shoots with thick stems were produced. When cultured on TDZ medium, American Hophornbeam produced only adventitious microshoots and with no Hophornbeam, when exposed to TDZ, shoots with thick stems were produced. When cultured on TDZ medium, American Hophornbeam produced only adventitious microshoots and with no axillary proliferation (Fig. 6). Higher concentrations of TDZ produced shorter microshoots, which also had deformed leaves (Table 6, Fig. 6). West and Preece (2004) reported that elevated concentrations of TDZ were herbicidal to Hibiscus (*Hibiscus moscheutos* (L.)) explants and microshoots. Although the number of microshoots was fairly similar between

adventitious microshoots in media with BA and TDZ, the propagation numbers were higher in BA than TDZ experiments (Data not analyzed). TDZ treatments generated all adventitious microshoots as compared to BA treatments that produced both adventitious and axillary microshoots.

Table 5. Effects of TDZ and IBA on shoot number, shoot length, and propagation number after 6 weeks in culture.

Treatment (μM)		Means[a,b]		
TDZ	IBA	Shoot Number	Average Shoot Length (cm)	Average Propagation Number
1	0.1	0.9b	0.78bc	0.70bc
1	0	0.5b	0.387c	0.30c
0.1	0.1	2.1a	1.21ab	1.12ab
0.1	0	1.3ab	1.47a	1.6a

[a] Means were averaged over runs
[b] Mean followed by the same letter is not significantly different (α = 0.05)

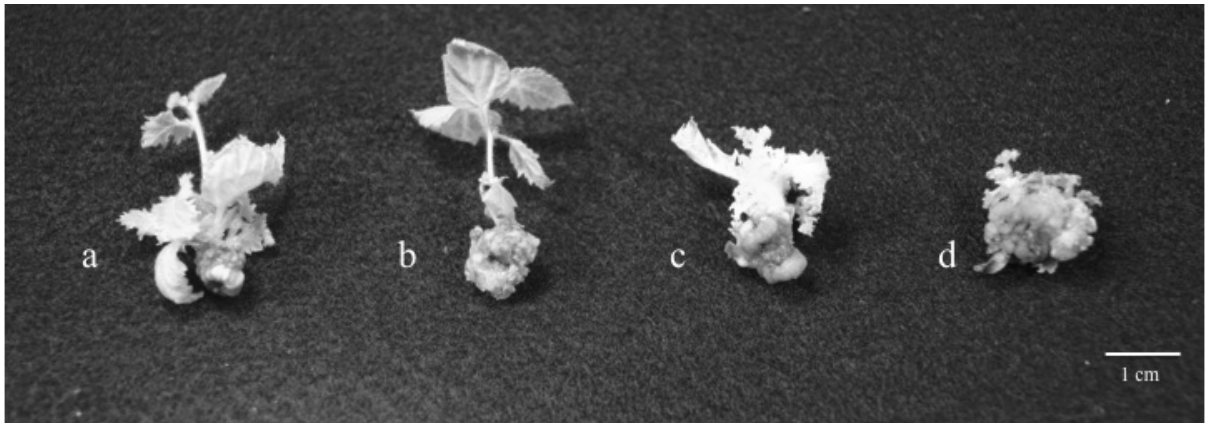

Figure 7. Adventitious microshoots of American Hophornbeam on DKW nutrient salt formulation and (a) 0.1 TDZ, 0.1 IBA (b) 0.1 TDZ 0 IBA (c) 1 TDZ, 0 IBA (d) 1 TDZ, 0.1 IBA.

In the rooting experiment, there was a significant difference between root number in the runs (Appendix Table 6, Table 5), however when an analysis was done comparing the interaction between run and treatment, there was no statistical significance (Appendix Table 6). It can be

concluded that the treatments behaved the same within runs. The difference in runs could be caused by a difference in size of the microshoots placed within each run. Smaller microshoots were used for run 1 compared to microshoots used in run 2. There was significance between treatments for average root length (Appendix Table 6, Table 7).

Table 6. Differences between runs of a comparison of IBA (0, 0.5, 1, and 2 μM) induced root development in DKW nutrient salt formulation after six weeks in culture.

	Means[a]	
	Root Number	Root Length
Run 1	1.39b	1.92a
Run 2	2.26a	2.07a

[a] Means followed by the same letter are not significantly different ($\alpha < 0.05$)

There was no significant difference between any of the concentrations of IBA (0.5, 1, and 2 μM) in number of roots developed; however, 2 μM IBA and the control had significantly shorter roots than 1 μM IBA. Jansson and Welander (1990) reported that *Betula albosinensis*, *Betula costata*, *Betula ermanii* cv. Mount Apoi, and *Betula jacquemontii* rooted *in vitro* using WPM nutrient basal salts and 0.5 μM IBA. They also reported that longer shoots placed into culture produced significantly more and longer roots. In American Hophornbeam, the difference in runs in terms of root number could have been influenced by microshoot size. The best evaluated treatments to use for *in vitro* rooting of American Hophornbeam were 0.5, 1, and 2 μM IBA (Table 8, Fig. 7). Higher concentrations (>2 μM) of IBA were herbicidal to microshoots with decreased size and chlorotic leaves (data not shown).

Table 7. The effect of IBA concentrations on rooting microshoots of American Hophornbeam.

	Mean[a,b]	
IBA (µM)	Root number	Root length (cm)
0	0.4b	0.42c
0.5	1.75a	2.35ab
1.0	2.4a	3.04a
2.0	2.5a	1.89b

[a] Means were averaged over runs
[b] Mean followed by the same letter is not significantly different ($\alpha < 0.05$)

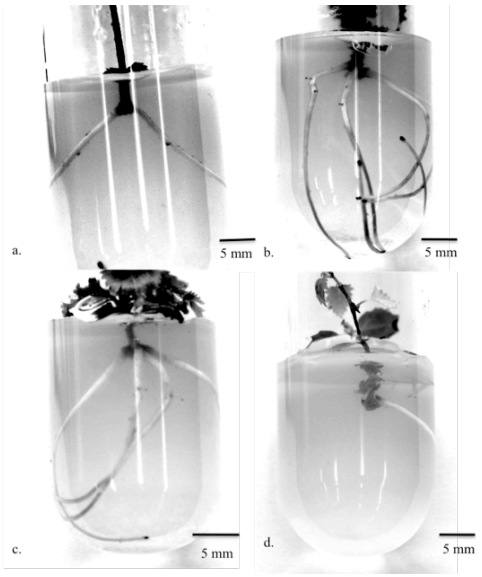

Figure 8. *In vitro* rooting of American Hophornbeam on DKW nutrient salts with (a) 0.5 µM IBA (b) 1 µM IBA (c) 2 µM IBA (d) 0 µM IBA after 6 weeks in culture.

Organogenesis

As previously stated, WPM nutrient salt media was observed to produce more callus at the base of explants in the establishment stage of micropropagation; therefore, it was chosen for all subsequent organogenesis experiments. When producing callus from leaf tissues, data

indicated that there was a significant difference between runs however there was no significant difference between any of the callus weights from any evaluated treatment (Appendix Table 7). There was no significant run x treatment interaction. Simola (1985) reported in *Betula pendula* f. *purpurea (*Andre*)* C. K. Schneid*.* , the best-tested callus induction was achieved using high nutrient basal salt formulation medium with concentration of cytokinins (2.3 or 4.6 μM, Kinetin) and high concentrations of auxin (9.0 or 22.6 μM, 2,4-D). In American Hophornbeam, high concentrations of cytokinins (5 μM TDZ, 10 μM TDZ, 10 μM BA) had no significant difference on callus production as compared to lower concentrations of cytokinins (0.1 and 1 μM TDZ) regardless of IBA concentration.

For callus regeneration, Cheng et al. (2000) reported that it is possible to regenerate microshoots from leaf callus tissues within Betulaceae. *Betula platyphylla* 'Fargo' (Dakota Pinnacle® Asian White Birch) was placed on media containing WPM nutrient basal salts and either BA (0,10, 20, 30 μM) or TDZ (0, 1, 2, 3). Results indicated that media containing 10, 20, and 30 μM of BA had 87.5, 100, and 100 % of explants forming adventitious microshoots respectively. In American Hophornbeam, 10 μM BA had significantly higher adventitious shoots develop than any other treatment (Appendix Table 8, Fig. 8, Table 9). Adventitious shoots were developed by other treatments but were not significantly different than treatments that did not produce any shoots. Organogenesis can be a powerful tool to proliferate microshoots with less explant material; however, there is the increased potential for somaclonal variation between the original clonal selection and the adventitious microshoots produced (Kaeppler, 2000).

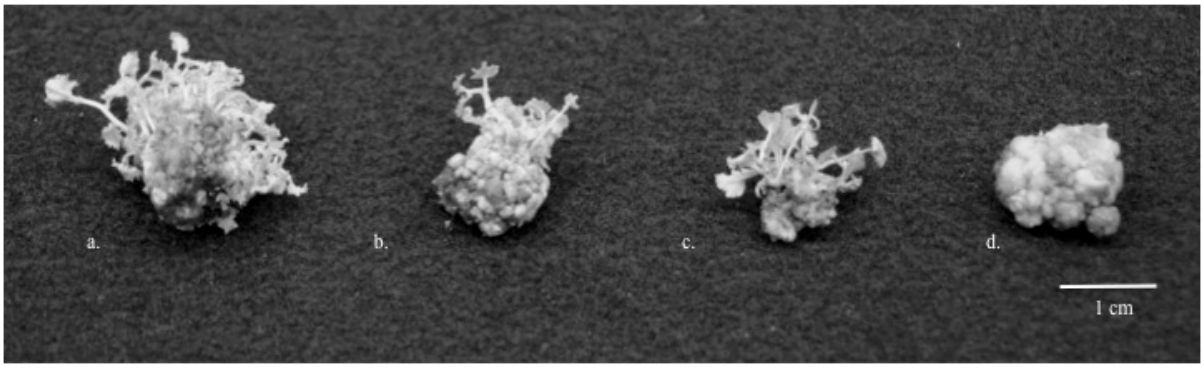

Figure 9. American Hophornbeam microshoots regenerated from leaf callus placed in WPM nutrient salt media with (a) 10 µM BA (b) 10 µM BA and 0.1 µM NAA (c) 20 µM BA (d) 10 µM BA and 1 µM TDZ for 6 weeks.

Table 8. Comparison of the effects of Benzylaminopurine (BA), Thidiazuron (TDZ), and 1-Naphthaleneacetic acid (NAA) on American Hophornbeam leaf callus shoot regeneration.

BA (µM)	TDZ (µM)	NAA (µM)	Shoot Number[a,b]
0	0	1	0.7b
0	1	1	0.0b
0	0	0	0.0b
0	1	0	0.4b
10	0	1	0.9b
10	1	1	0.0b
10	0	0	3.80a
10	1	0	0.4b
20	0	1	0.5b
20	1	1	0.0b
20	0	0	0.8b
20	1	0	0.0b

[a] Means were averaged over runs
[b] Means followed by the same letter are not significantly different ($\alpha<0.05$)

Conclusion

Although clonal propagation is generally more expensive than sexual propagation, the higher expense is justified if improvements of the species have been done and clonal propagation

is needed to maintain genetic traits. Stem cuttings can be an inexpensive method of clonal propagation for many woody species. Results indicated that semi-hardwood cuttings of American Hophornbeam did not significantly root with any of the exogenous applications of plant growth regulators. Stem cuttings for this experiment were collected from reproductive trees and could have been a significant contributing factor to the decreased rootability of the cuttings. Further research needs to be conducted to compare softwood, semihardwood, and hardwood cuttings for rootability as well as different PGR types and variable PGR concentrations for root formation. Rejuvenation of mature material needs to be explored as well to produce juvenile shoots (from epicormic buds) through methods such as shoot forcing.

Data from the micropropagation experiments suggest that American Hophornbeam can be propagated asexually through micropropagation. When using micropropagation as a method of clonal propagation, there are two types of microshoots that American Hophornbeam (*Ostrya virginiana* (Mill.) K. Koch) produces, axillary and adventitious. Axillary shoots are better to use with respect to the decreased potential for somaclonal variation than adventitious shoots but have lower production numbers. The best axillary shoots were produced by 4 and 8 μM BA with 0.1 μM IBA; however, the average number of shoots produced for these two treatments may not be high enough for the standards of the commercial industry. *In vitro* rooting was achieved with all concentrations of IBA (0.5, 1, and 2 μM) evaluated and showed no significant difference between concentrations. Further research needs to be conducted on *ex vitro* rooting of microshoots to determine an acceptable PGR concentration for rooting.

Organogenesis was also proven to be a viable method of asexual propagation for American Hophornbeam. Data from the organogenesis study suggests that microshoots can be regenerated from callus developed from leaf tissue. There was a significant difference between

10 μM BA and all other treatments when looking at callus regeneration. If somaclonal variation can be verified to be absent from American Hophornbeam propagated in this way then adventitious shoot regeneration could be successfully utilized (10 μM BA). The tissue culture protocols suggested in this study allow for clonal propagation of American Hophornbeam. Cultivars such as 'Camdale' - Sunbeam® American Hophornbeam can now be clonally propagated using the tissue culture protocols from this study.

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CHAPTER 4. GENERAL CONCLUSIONS

The recent decline in tree species such as ash (*Fraxinus* spp.) by Emerald Ash Borer ((*Agrilus planipennis* Fairmaire) and maples (*Acer* spp.) by Asian Longhorn Beetle (*Anoplophora glabripennis* Motschulsky) has elevated American Hophornbeam's importance as a street and landscape tree in part because of its low pest problems. Current reported methods of propagation have been limited to sexual propagation. The long and precise stratification requirements for American Hophornbeam were focused on in order to improve them. Scarification was evaluated as a treatment as well as GA_3 and KNO_3 to replace the dormancy requirements. It was found that scarification of American Hophornbeam seed could break seed dormancy without stratification but is not commercially viable as a result of low germination rates. A subsequent experiment was conducted to attempt to clonally propagate semihardwood shoots using talc-based IBA and NAA. Results indicated that semihardwood cuttings had limited rootability and further evaluation should be undertaken. A micropropagation evaluation of American Hophornbeam was conducted to determine whether tissue culture of shoots was a viable method of propagation. It was found that microshoots produced both axillary and adventitious microshoots. Microshoots from these experiments were subsequently successfully rooted on media containing IBA (0.5, 1, and 2 μM). Regeneration of microshoots from leaf callus was also tested. It was determined that microshoots could be regenerated using leaf callus of American Hophornbeam. Currently there is only one commercial cultivar of American Hophornbeam - Sunbeam® American Hophornbeam (*Ostrya virginiana* 'Camdale') released from the NDSU Woody Plant Improvement Program in 2011. It is not commercially available partially as a result of a lack of clonal propagation capabilities. The clonal methods developed from this study have potentially opened the door to future improvements of the species.

APPENDIX A. AMERICAN HOPHORNBEAM SEED SCARIFICATION EXPERIMENT

Variable: Run 1 Germination Rate

Source of Variation	df	MS	F
Rep	1	0.001	0.6746
Scarification (S)	2	0.750	<.0001
Treatment (T)	4	0.059	0.0475
S x T	8	0.027	0.3288
T x Time	10	0.040	0.0927
S x T x Time	20	0.031	0.2025
Error	44	0.023	

Variable: Run 2 and 3 Germination Rate

Run	1	0	0
Rep	4	0	0
Scarification (S)	2	0	0
Treatment (T)	4	0	0
S x T	8	0	0
T x Time	10	0	0
S x T x Time	20	0	0
Error	400	0	

APPENDIX B. RUN 1 EFFECT OF SCARIFICATION, EXPOSURE TO HORMONE OR IMBIBITION PRIMING AGENT, AND EXPOSURE TIME ON AMERICAN HOPHORNBEAM GERMINATION RATES

Scarification	GA3	KN03 (%)	Exposure Time	Germination[1,2,3] Rate (%)
Sulfuric acid	0		24	30
	250		24	40
	500		24	70
	750		24	50
		0.5	24	23
	0		48	0
	250		48	30
	500		48	0
	750		48	30
		0.5	48	30
	0		72	10
	250		72	40
	500		72	50
	750		72	20
		0.5	72	40
Tip cut	0		24	0
	250		24	13
	500		24	20
	750		24	0
		0.5	24	13
	0		48	0
	250		48	10
	500		48	0
	750		48	10
		0.5	48	0
	0		72	0
	250		72	50
	500		72	0
	750		72	10
	0	0.5	72	0

APPENDIX C. SHOOT CUTTING EXPERIMENT

Variable: Average Root Number				
Source of Variation	df	MS	F	Pr > F
Run	1	0.113	1.91	0.1711
Rep	4	0.113	1.91	0.1182
Treatment (T)	7	0.055	0.94	0.4806
Error	67	0.058		
Total	79			
Variable: Average Root Length				
Source of Variation	df	MS	F	F
Run	1	0.569	2.08	0.1540
Rep	4	0.569	2.08	0.0933
Treatment (T)	7	0.255	0.88	0.4882
Error	67	0.274		
Total	79			

APPENDIX D. MICROPROPAGATION ESTABLISHMENT

Variable: Average Shoot Number

Source of Variation	df	MS	F	Pr > F
Run	1	26.04	2.14	0.1595
Rep	2	3.792	0.29	0.7528
Treatment (T)	3	31.04	2.36	0.1072
Error	17	13.129		
Total	23			

Variable: Average Shoot Length

Source of Variation	df	MS	F	Pr > F
Run	1	0.38	0.50	0.4898
Rep	2	0.17	0.22	0.8036
Treatment (T)	3	0.82	1.09	0.3805
Error	17	0.75		
Total	23			

APPENDIX E. MICROPROPAGATION BAXIBA ADVENTITIOUS MICROSHOOTS VS AXILLARY SHOOTS

Variable: Adventitious shoot Number

Source of Variation	df	MS	F	Pr > F
Run	1	14.4	5.94	0.0217
Rep	3	6.17	2.54	0.0773
Treatment (T)	4	8.41	3.47	0.0207
Run x Treatment	4	2.46	1.02	0.4171
Error	27	2.43		
Total	39			

Variable: Axillary shoot number

Source of Variation	df	MS	F	Pr > F
Run	1	4.9	11.21	0.0024
Rep	3	0.40	0.92	0.4467
Treatment (T)	4	2.66	6.09	0.0013
Run Treatment	4	0.96	2.20	0.0955
Error	27	0.437		
Total	39			

Variable: Propagation Number

Source of Variation	df	MS	F	Pr > F
Run	1	21.03	11.45	0.0022
Rep	3	5.23	2.85	0.0583
Treatment (T)	4	26.38	14.36	<.0001
Run x Treatment	4	5.15	2.37	0.0750
Error	27	1.836		
Total	39			

APPENDIX F. MICROPROPAGATION TDZXIBA

Variable: Average Shoot Number

Source of Variation	df	MS	F	Pr > F
Run	1	0.400	0.29	0.5927
Rep	4	3.038	2.22	0.092
Treatment (T)	3	4.667	3.42	0.0309
Run x Treatment	3	1.867	1.37	0.2733
Error	28	1.366		
Total	39			

Variable: Average Shoot Length

Source of Variation	df	MS	F	Pr > F
Run	1	0.1904	0.40	0.5345
Rep	4	0.4907	1.02	0.4144
Treatment (T)	3	2.2936	4.76	0.0083
Run x Treatment	3	0.093	0.19	0.9002
Error	28	0.4815		
Total	39			

Variable: Average Propagule Number

Source of Variation	df	MS	F	Pr > F
Run	1	1.3801	2.58	0.1198
Rep	4	0.4892	0.91	0.4701
Treatment (T)	3	3.1198	5.82	0.0032
Run x Treatment	3	0.2081	0.39	0.7623
Error	28	0.5359		
Total	39			

APPENDIX G. MICROPROPAGATION ROOTING

Variable: Average Root Number

Source of Variation	df	MS	F	Pr > F
Run	1	8.70	6.79	0.0130
Rep	5	1.01	0.73	0.6048
Treatment (T)	3	9.45	6.88	0.0010
Run x Treatment	3	2.40	1.75	0.1763
Error	33	1.37		
Total	45			

Variable: Average Root Length

Source of Variation	df	MS	F	Pr > F
Run	1	0.25	0.17	0.6819
Rep	5	1.30	0.90	0.4954
Treatment (T)	3	13.11	8.98	0.0002
Run x Treatment	3	1.83	1.25	0.3059
Error	33	1.46		
Total	45			

APPENDIX H. LEAF CALLUS INDUCTION

Variable: Callus Weight

Source of Variation	df	MS	F	Pr > F
Run	1	213105.12	50.43	<0.0001
Rep	2	2771.1598	0.66	0.5237
Treatment (T)	9	3035.1517	0.72	0.6895
Error	47	4225.3545		
Total	59			

APPENDIX I. LEAF CALLUS REGENERATION

Variable: Shoot Number

Source of Variation	df	MS	F	Pr > F
Run	1	7.01	1.57	0.2139
Rep	4	4.31	0.96	0.4312
Treatment (T)	11	11.2	2.50	0.0084
Error	92	4.47		
Total	119			