Propagation Studies of Sugar Maple (*Acer saccharum* Marsh.)

BY

TERESA ALEXANDRA SUTANTO

A Thesis

Submitted to the Faculty of Graduate Studies in
Partial Fulfillment of the Requirements for the
Degree of

MASTER OF SCIENCE

Department of Plant Science

University of Manitoba

Winnipeg, Manitoba

ACKNOLEDGEMENTS

I would like to acknowledge the help of many people during my graduate study:

Dr. Claudio Stasolla for his encouragement, guidance and support throughout the program.

Dr. Philip Ronald and Dr. Muhammad Tahir, for serving on my advisory committee and Dr. Ronald for his time and guidance, particularly on the cutting experiment.

Dr. Rob Gulden and Dr. Gary Crow for the generous time helping me on the statistical analysis of the data.

Bert Luit for the assistance in the lab and the companion during the trips to Morden and Portage La Prairie for sample collections.

Jeffries Nurseries Ltd. for supplying the plant materials for the project.

Rob and Sue MacLeod of Glenlea Greenhouses and Dave Klassen of Shannon Oaks Tree Farm for providing their time and for contributing space in their greenhouses for the cutting experiment.

Kevin Baron for helping with seed collection, Mohamed Elhiti for teaching the techniques for cell fixation and staining, and every lab members for making the lab enjoyable!

Lorne Adam for the valuable insights and help on the digital image analysis using the computer software.

Ian Brown for the setting the propagation box and helping with selecting rooting medium for the cutting experiment.

Rhanissa Hirawan for drawing the illustration materials used in the thesis.

Fellow graduate students for their friendship and support.

Committee members of Agri-Food Research and Development Initiative (ARDI) for the financial support, as well as Province of Manitoba for awarding Manitoba Graduate Scholarship.

And lastly, my parents, Hartono Sutanto and Fonny Hadi Gunawan, and my brother, Vito, for their continued support and love. Thanks to you all!

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ABA, abscisic acid
ACC, 1-aminocyclopropane-1-carboxylic acid
APS, American Phytopathological Society
ANOVA, analysis of variance
AOSA, Association of Official Seed Analysts
BS, brassinosteroids
CFIA, Canadian Food Inspection Agency
CFS-AFC, Canadian Forest Service – Atlantic Forestry Centre
CKX, cytokinin oxidase/dehydrogenase
BAP, 6-benzylaminopurine
EAB, emerald ash borer
2,4-D , 2,4-dichlorophenoxyacetic acid
DKW, Driver and Kuniyuki Walnut
GDD, growing degree day
IBA, indole-3-butyric acid,
ISTA, International Seed Testing Association
LS, Linsmaier-Skoog
MS, Murashige-Skoog
NAA, 1-naphthaleneacetic acid
NTSC, National Tree Seed Centre
2iP, 2-isopentenyl adenine
IAA, indole-3-acetic acid
GA_3, gibberellic acid
PAA, phenylacetic acid
PPM™, Plant preservative Mixture
SAS®, Statistical Analysis Software
STS, silverthiosulfate
TDZ, thidiazuron
WPM, woody plant medium
Z or ZEA, zeatin

ABSTRACT

Sutanto, Teresa Alexandra. M.Sc., The University of Manitoba, April, 2010. Propagation Studies on Sugar Maple (*Acer saccharum* Marsh.) Major Professor; Claudio Stasolla.

Sugar Maple (*Acer saccharum* Marsh.) is a very important tree species and is known not only for its sap in the production of maple syrup, but also for its superior hardwood quality and popular ornamental properties. In the effort to improve the diversity of the hardwood tree species in Manitoba, an effective propagation method for sugar maple is needed. The study tested several propagation techniques namely in vitro shoot organogenesis, induced embryo conversion and rooting of greenwood cuttings. Shoot multiplication was achieved using bud and embryo explants, however the rate of shoot production was very low implying that the culture conditions required some optimizations. Dormant isolated embryos were induced to germinate and convert into whole plants in vitro, eliminating the need for long stratification period. The study found the highest embryo conversion frequencies by the addition of cytokinin 6-benzylaminopurine (BAP) at 0.5-1.5 mg/L or thidiazuron (TDZ) at 0.01 mg/L into the culture medium. Greenwood cuttings of several hardy cultivars of *A. saccharum*, including 'Jefcan', 'Bailsta' and 'Green Mountain' were compared for rooting capacity. In 2008, cutting type, rooting hormone and collection time were found to significantly influence rooting. In the following year, the study was expanded to compare different rooting conditions, using peat-perlite mix in fog system, sand beds under intermittent misting, and commercial peat plugs under automated misting system. Rooting was improved by selecting for medial-type cuttings and by promoting cutting survival through the the use of peat-based rooting medium and the maintenance of cool temperatures during the rooting period. The application of auxin did not increase rooting frequency of 'Jefcan' cuttings, but considerably improved the quality of roots produced, which may affect cutting survival upon transplantation.

1.0 INTRODUCTION

Because of Manitoba's climate, the nursery industry in the province is dependent on a limited number of tree species with sufficient cold hardiness to survive the harsh winter conditions. In order to expand the selections, growers are continuously on the search for new tree species. Additionally, nursery growers in North America have been forced to accelerate the search due to the emerging threat by invasive pests, particularly the Emerald Ash Borer (EAB). Known as one of the worst insect pests, EAB is believed to be native to eastern Asia. The pest kills a tree by feeding under the bark causing disruption of water and nutrient flow throughout the tree (McCullough and Usborne 2008). Since almost all ash species are susceptible to the pest, millions of ash trees have been destroyed in south western Ontario, Michigan, and the nearby states (Ronald 2006). The loss of ash resources in the Michigan area was projected to exceed $1.7 billion, based on the value of timber as commodity (Poland and McCullough 2006). Moreover, the infestation has been noted to slowly move towards the prairies. In Manitoba, ash species comprise about 60 to 70% of the shade trees produced and sold locally (Ronald 2006); hence, the economical and environmental impact of this pest would be catastrophic, both in urban spaces as well as in the forested areas (Poland and McCullough 2006). For this reason, Manitoba needs new hardwood tree species.

Besides being one of the Canadian Food Inspection Agency (CFIA)'s recommended alternatives to ash, sugar maple (*Acer saccharum*) also features an oval to rounded crown and attractive fall colors in shades of red, orange and yellow. For these reasons, sugar maple has become a well-liked ornamental tree. In addition, sugar maple tolerates Manitoba's alkaline soils and can be used successfully throughout the Southern Canadian prairies. The biggest barrier to sugar maple establishing itself as a predominant tree species in the Canadian prairies is the lack of an effective propagation system (Ronald, pers. comm.). Presently all sugar maple sold in Manitoba are produced from seedlings or by bud-

grafting (Figure 1.1). Seedlings of sugar maple are variable in the fall color and are considered less desirable than cloned cultivars. Bud-grafting enables cloning of sugar maple cultivars, but requires the use of seedling rootstock. These seedling rootstocks are often of questionable cold hardiness since many originate from the Eastern United States where annual seed production is more consistent (Ronald, pers. comm.).

The nursery industry would enthusiastically welcome a procedure that would enable cold-hardy selections of sugar maple to be produced on their own roots. The protocol may involve tissue culture micropropagation or the more traditional rooting of softwood cuttings under mist. The desired result would be a well-rooted sugar maple liner; a small plant produced for further growing in a production nursery, with satisfactory crown and root hardiness to thrive in the prairie region. The development of an efficient propagation system for sugar maple would allow Manitoba nurseries to generate a large number of trees over a short period of time. With this ability Manitoba's competitiveness in the national tree industry would be greatly enhanced.

Moreover, an efficient propagation system would have the potential to expand the current maple syrup industry in Manitoba. At the present time Manitoba maple (*Acer negundo*) is the primary source of maple syrup in the prairie. The syrup production from each tap of a Manitoba maple is approximately one-quarter to one-half of that of a sugar maple (Kendrick 2004). Thus, a large scale production of cold hardy sugar maple cultivars would have tremendous benefits for this industry.

The objective of the proposed research project is to develop an effective propagation system for sugar maple. The research used several approaches including; hormone-mediated embryo conversion, in-vitro shoot organogenesis, and rooting of softwood cuttings.

Figure 1.1 A dormant bud of sugar maple (*Acer saccharum*; circled) grafted onto a seedling rootstock. This propagation method known as bud grafting is implemented by nurseries for the clonal production of sugar maple (picture courtesy of Philip Ronald)

2.0 LITERATURE REVIEW

2.1 Introduction to sugar maple (*Acer saccharum* Marsh.)

Sugar maple belongs to the family Aceraceae. Members of this family are deciduous trees that are widely spread across North America, Europe, Asia and northern Africa (Olson and Gabriel, 1974). As an important tree species, sugar maple is known for its sap as well as for the quality of its wood. The sap produced by the tree is used for the production of maple syrup. Although saps from other maple species can be used for syrup production, sap from the sugar maple is still considered of superior quality. Additionally, sugar maple trees yield strong, hard wood with dense grain and light color often used for furniture and flooring. In the horticultural industry, sugar maple is admired for its oval to rounded crown shapes and attractive fall color, which ranges from bright yellow to orange to intense red-orange (Figure 2.1); hence popular as a street and garden tree (Horsley et al., 2002). The tree is commonly found in hardwood forests of eastern United States and Canada, including Maine, New York, Vermont, Nova Scotia, New Brunswick and Ontario (USDA ARS, National Genetic Resources Program).

The maple syrup industry is unique to North America, with majority of the production in eastern United States and Canada. Currently Canada is the world's largest producer of maple food products, with annual production valued at 211 million dollars in 2008 (Statistics Canada 2008). The province of Quebec accounts for approximately 90% of Canada's maple syrup production (Environment Canada 2006). Maple syrup production in Manitoba is relatively small and local; however this may change as more climate-adapted trees are planted across the province as an effort to replace the diseased tree species as well as diversify the province's urban forests.

The sugar maple cultivars used in this study were mainly 'Jefcan' and 'Bailsta'; while a small study was done using 'Green Mountain' in 2009. The primary cultivar used in the study, 'Jefcan', is a winter-hardy selection of sugar maple introduced by Jeffries Nurseries Ltd. of Portage La Prairie,

Manitoba. It has yellow-orange fall foliage (Figure 2.1) and prefers rich, well-drained soil in a sheltered location. Another superior selection of sugar maple, 'Bailsta', displays glossy, deep green foliage that changes into orange in fall. The cultivar 'Green Mountain' is the most widely planted sugar maple cultivar in the United States. The leaves, which are thick and leathery, turn orange-red in color in the fall, as shown in Figure 2.1.

Figure 2.1 Two cultivars of sugar maple (*Acer saccharum* Marsh.); 'Green Mountain' (left) and 'Jefcan' (right) showing different fall leaf colors (Pictures courtesy of Philip Ronald).

2.2 Plant propagation: an overview

The practice of plant propagation originated thousands of years ago when human civilization started (Harlan 1992). Hunting and gathering were followed by domestication, which involved plant breeding or selection, plant propagation, and plant production. Over time, agriculture has developed from primitive techniques into more advanced and organized systems, and today our knowledge has improved significantly allowing us to study the intricate details of a plant's nature, and manipulate its desirable traits for the purpose of improving the quality of human life.

Angiosperms or flowering plants are the most widespread group of land plants. Their lifecycle includes two biological generations known as sporophyte, or the vegetative generation, and gametophyte, which represents the reproductive stage. Shoot and root growth corresponds to sporophytic stage, in which the cells divide mitotically or asexually. This can be observed in the shoot and root meristems, as well as the secondary meristem or the cambial meristem. The gametophytic phase is found inside a flower bud, which encloses several anthers and a pistil. The anthers and the pistil contain pollen grains and ovules, respectively. These are the key components in pollination. In angiosperms, pollination is followed by double fertilization which involves two separate fusions, one giving rise to an endosperm and the other forming a zygote (Hartmann et al. 1997).

Plant propagation makes use of different plant propagules for the multiplication and production of plants. A propagule is defined as any part of a plant that can be used to produce a new plant or a population of plants (Hartmann et al. 1997). Examples of propagules include seeds, cuttings, buds, scions as well as specialized structures like bulbs and tubers. Among these, seed is the only one that is produced via sexual fusion of gametes; the rest are propagules for vegetative or asexual propagation.

2.2.1 Sexual propagation

Most plant species reproduce through seed production. A seed is a matured ovule containing an embryo, which is typically the product of fertilization (Fahn 1982). In rare cases, embryos may also be produced asexually from the unfertilized reproductive cells within the ovule. Asexually-produced seeds generally are indistinguishable from those created via fertilization (Bewley and Black 1994). This event is known as apomixis, which produces an offspring that is a clone of the parent and is referred to as an apomict (Hartmann et al. 1997). The basic components of a seed include an embryo and two covering layers; the endosperm and seed coat or testa (Finch-Savage and Leubner-Metzger 2006). In angiosperms, seeds may allocate most of their storage materials to the cotyledons or the endosperm depending on the species (Hartmann et al. 1997).

A fertilized ovule develops into a seed through three physiological stages of development, namely histodifferentiation, cell expansion, and maturation drying (Bewley and Black 1994). During the first stage, seed size increases rapidly due to active cell division. Moreover, the seed fresh and dry weights also increase steadily. At the end of this stage, the embryo reaches the cotyledonary phase. Angiosperms and gymnosperms have different characteristics in their developmental process (Hartmann et al. 1997). Differences also exist between monocotyledonous and dicotyledonous angiosperms. Since sugar maple is a dicotyledonous angiosperm, only embryogenesis of this category will be covered in this review.

Embryogenesis in dicots consists of typical stages including proembryo, globular, heart, torpedo and cotyledon stages (Steeves and Sussex 1989). The proembryo stage follows after the egg is fertilized by the sperm nuclei producing a single-celled zygote, which then divides at an angle to produce a smaller apical cell and a larger basal cell. Lineage wise, the basal cell will generate the hypophysis that eventually forms the radicle; whereas the smaller apical cell will develop to form all other cells of the

embryo (Hartmann et al. 1997). The basal cell also gives rise to the suspensor, which comprises a column of single or multiple cells. The suspensor physically pushes the embryo into the embryo sac in order to expose it to the nutrients. Additionally, the suspensor's filamentous cells also aid in absorbing nutrients, which are then transferred to the developing embryo. The suspensor continues to deliver nutrients as well as growth regulators for the embryo until the ability of the embryo to seek nourishment from the endosperm is refined (Schwartz et al. 1994). Besides, in certain species, the bottom cell of the suspensor may expand and become implanted into the surrounding tissue of the ovule to serve as an anchor (Hartmann et al. 1997). Normally the suspensor begins to senesce around the end of heart stage, suggesting that its role as an embryonic organ has been completed (Schwartz et al. 1994; Hartmann et al. 1997).

After several longitudinal and transverse divisions, a globular embryo is formed. At this stage the structure displays the initiation of the three primary tissue systems; dermal, vascular and fundamental. The outer layer of cells will develop into the epidermis, while the inner cells will differentiate into the cambial meristem and the cortex (Steeves and Sussex 1989). The embryo continues to divide and grow in size, and eventually localized growth starts to become noticeable on the two sides of the shoot apical meristem, forming the two lateral cotyledon primordia (Hartmann et al. 1997). This delineates the heart stage of the embryo development. Later, the procambium extends into the cotyledons as the growth continues. The initiation of cotyledons and axis is usually followed by the differentiation of the root meristem, which takes place at the opposite ends of the axis. This results in the formation of a torpedo embryo characterized by root and shoot meristem at its opposite poles (Goldberg et al. 1994). Since the embryo is contained in a closed space of an ovule, sometimes the cotyledons will bend back as the embryo matures, as with the case of sugar maple embryos.

After embryogenesis is completed, the seed progresses to the cell expansion stage, which involves the accumulation of storage reserves such as carbohydrates, lipids and proteins (Bewley and Black 1994). The organs in which these materials are stored vary among species but usually are either the cotyledons or the endosperm. These food reserves will supply energy to the germinating seedling before it establishes its photosynthetic capacity (Brown et al. 1982; Bewley and Black 1994). The storage materials are produced by the maternal tissues and transferred to the seeds through a vascular strand connecting the seed and the mother plant. This connecting structure, referred to as vascular trace, although is not a direct connection to the embryo; it allows transport of molecules to the seed coat. From the seed coat the molecules are diffused through the cells to reach the embryo (Bewley and Black 1994). Moreover, during the diffusion process, the seed also scans for viruses and large molecules. In addition to food reserves, the DNA and RNA of the seed also increase during cell expansion (Hartmann et al. 1997).

A seed is physiologically mature when it reaches the maximum dry weight. At this time, the seed will readily germinate when removed from the seed coat or fruit (Miles et al. 1988). The last stage in seed development involves rapid water loss. The seed maturation process is marked by the synthesis of a new set of proteins, most of which are categorized as late embryogenesis abundant (LEA) proteins (Shih 2008). These proteins are highly stable and hydrophilic, which contribute to their function as protectants. Since they bind to water, they help stabilize the membranes and other cell parts as the cell dries (Dure 1993; Shih 2008). Early in germination, the embryo often has not regained its capacity to synthesize mRNA; therefore, during the maturation process, seeds also store specific conserved mRNAs, which are readily translated into proteins if needed (Dure 1993).

At the end, the seed is either quiescent or dormant. A seed is said to be quiescent if it germinates when exposed to the appropriate conditions; therefore, quiescence can be described as the

retardation or delay of growth and metabolism processes due to environmental factors nullifying these processes (Bewley and Black 1994). On the other hand, dormancy is the inability to germinate, within a particular time period, under any environmental conditions that are otherwise favourable for germination if the seed is non-dormant (Baskin and Baskin 2004). Moreover, dormancy can be defined as the mechanism that increases survival through unfavourable situations (Finch-Savage and Leubner-Metzger 2006). Dormancy that develops when a seed is still attached to the plant is considered primary dormancy; whereas secondary dormancy is induced by adverse environmental conditions after the seed has been detached from the parent plant (Taiz and Zeiger 2002). Furthermore, after-ripening, or the transition period required to remove primary dormancy, involves complex interactions between internal and external factors (Hartmann et al. 1997). Depending on species and the level of dormancy, the seeds may require exposure to specific environmental conditions for a particular length of time in order to germinate (Foley, 2005). On the other hand, dormancy caused by the presence of a hard seed coat which restricts radicle growth or prevents water and/or oxygen from reaching the embryo is not a true dormancy (Hartmann et al. 1997). True dormancy is a physiological state of a seed and in order to overcome the condition, the seed requires treatments with low temperatures and/or specific types of light over a period of time. In some cases, physical scarification is also necessary when the dormant condition is caused by both exogenous and endogenous factors.

Similar to other events that occur in a plant's life, the seed development process relies on the actions of several hormones. Plant hormones serve in the communication and control of different growth and developmental processes occurring in different parts of a plant. In seed development, the roles of hormones include regulating embryogenesis, directing storage reserve accumulation, as well as managing the growth and development of fruit tissue that houses the seeds (Hartmann et al. 1997). Plant hormones auxins and gibberellins play crucial roles in the stimulation of fruit growth. Evidences have shown that the application of gibberellic acid allows the continuation of development in fruits with

aborted or unfertilized seeds (Bukovac 1963). Another group of plant hormones, cytokinins, are essential for cell division and differentiation; thus, the hormone level is usually highest during cell division and differentiation phase of embryogenesis. In addition, polar auxin transport is suggested to be involved in the establishment of the embryo axis during embryogenesis (Taiz and Zeiger 2002). Auxin level is known to increase during seed development. Furthermore, the free form of auxin is more pronounced during the early developmental stages, and it is later replaced by the inactive conjugated form as the seed matures (Hartmann et al. 1997). The roles of ABA in seed development and maturation include stimulating the production of crucial storage proteins, assisting in the accumulation of food reserves, and preventing precocious germination (Taiz and Zeiger 2002). ABA level is highest during the cell expansion phase, and it remains relatively high through the seed maturation stage (King 1982). The role of hormones during plant growth and development is discussed in more detail in the subsequent sections.

A young plant that is grown from a germinated seed is referred to as a seedling (Brown 2008). The process of transitioning from an embryo into a plant is known as embryo conversion (Forest Genetic Council of British Columbia). Since seeds are produced from the fertilization of an egg by a pollen, half of their DNA is paternal and the other half is maternal (Hartmann et al. 1997). Plant breeders use this variability as an opportunity to select for more desirable traits, such as higher yield, special qualities and appearance. The variety produced from the process is adapted for cultivation and referred to as cultivated variety or cultivar (Brickell et al. 2004). Plant breeding, as a method of plant improvement, has been expanded to include new sectors such as genetic engineering and biotechnology (Hartmann et al. 1997). Several techniques from many disciplines have contributed to this advancement; including the discoveries of molecular biology of genes, transfer systems of genetic information, technology to clone genes as well as part of chromosome, and ability to manipulate and transform plant parts and whole plants.

The first step in seed propagation is to stimulate germination. In order for germination to occur, a seed must fulfill certain conditions. First, the embryo within the seed must be viable or alive. Moreover, the seed needs to be exposed to suitable environmental conditions, which include temperature, moisture, oxygen and sometimes the presence of light. In addition, germination is also directly regulated by the seed's dormancy state (Bewley and Black 1978). In contrast, some seeds may exhibit vivipary or precocious germination, a phenomenon where seeds germinate without completing the last stage of maturation drying, and are still attached to the mother plant (Bewley and Black 1978, Hartmann et al. 1997).

Germination can be divided into three phases; imbibition phase, lag phase and radicle emergence (Hartmann et al. 1997). Upon completion of seed development, seeds generally contain less than 15% moisture. In order to germinate, the seeds must regain water that has been lost during the drying via imbibition. Seeds imbibe water through their water-permeable seed coat. This imbibition phase will occur as long as the seeds are dry, regardless if the embryos are dead or alive. The imbibition phase is marked by rapid water uptake. The second phase usually involves very little water uptake, if any, and it is known as the lag phase. During this phase, the seed is physiologically active, as shown by the following activities: mitochondria maturation and activation, protein synthesis, reserve mobilization and metabolism, and enzyme production (Bewley and Black 1994). These processes initiate and accommodate the next stage, radicle emergence, through the production of enzymes that loosen the cell wall and affect the water potential via protein synthesis needed for radicle extension (Simon 1984). Although radicle emergence is the first visible sign of germination, its occurrence is not due to cell division but rather to cell elongation (Haber and Luippold 1960). Furthermore, soon after the radicle penetrates the seed coat, cell division commences, initially at the radicle tip and then at the shoot apex (Rogan and Simon 1975). Therefore, cell division is restricted to the two growing points located at opposite ends of the embryo axis; the shoot and root meristems.

Seed propagation has undeniably played a very important role in nature's maintenance of various ecosystems. This is supported by the fact that approximately 250,000 angiosperm species and 700 gymnosperm species have been perpetuated on the basis of seed propagation (Dirr and Heuser 1987). Moreover, the majority of food crops and bedding plants are grown from seed (Brown 2008). Propagating plants using seed is an inexpensive way to produce a large quantity of plants in a short time. However, seed propagation cannot be used to produce clones of a particular plant because seeds from the same plant usually possess different genotypes (Hartmann et al. 1997). Clonal propagation is generally performed on a plant that has been selected for its desirable traits, for example plant height, flower colour and fruit characteristics. Retention of genetic traits can be accomplished via asexual or vegetative propagation.

2.2.2 Asexual or vegetative propagation

Many plants have the ability to asexually propagate themselves; while some others require human intervention to achieve asexual propagation (Brown 2008). Propagation of plants via established vegetative structures is made possible by the plant's ability to dedifferentiate to form a new meristem. This new meristem is called an adventitious meristem and its formation is initiated by mitotic division (Dirr and Heuser 1987). Parts of a plant that are commonly used for vegetative propagation include stem, root, leaf and bud. Some examples of vegetative propagation include cutting, layering, grafting or budding, and propagation via separation and divisions of specialized structures such as bulbs and corms (Dirr and Heuser 1987; Brown 2008).

Since vegetative propagation is asexual, the resulting plant has an identical genotype as the source or the parent; therefore, they can be referred to as clones (Dirr and Heuser 1987; Hartmann et al. 1997; Acquaah 2005; Brown 2008). However, there may still be some variation among the clones due to

environmental effects, and occasionally, spontaneous mutations (Dirr and Heuser 1987; Acquaah 2005). Vegetative propagation offers some advantages such as homogeny of population, preservation of preferred genotypes, shortened time to maturity and, in the case of grafting, unification of several genotypes into a single plant (Hartmann et al. 1997). The hastened time to maturity is particularly beneficial for breeding trees or woody perennials (Acquaah 2005). With regards to sugar maple propagation, cutting, grafting or budding, and tissue culture propagation are the most relevant. Therefore, only these techniques are discussed in this review.

2.2.2.1 Cutting propagation

A cutting is defined as a vegetative material collected from any of the three primary plant organs; leaf, stem or root (Hartmann et al. 1997). Under the appropriate conditions, the cutting can be induced to regenerate a new plant (Acquaah 2005). In the horticultural industry, cutting propagation is popular among propagators because it maintains the integrity of the stock plant and is more cost-effective as compared to grafting. In addition, cutting propagation also produces own-rooted plants; thus eliminating graft incompatibility (refer to the next section on grafting for further details; Dirr and Heuser 1987; Hartmann et al. 1997). In terms of cost, rooting success of 50-60% is comparable to 90% success with grafting (Dirr and Heuser 1987). A disadvantage to cutting propagation is that the production cycle takes another year before the plants reach a particular size and are ready to be sold.

A leaf cutting may or may not include an axillary bud that is attached on a short stem. The use of leaf cuttings to propagate woody species is less common because of low success rates. When working with woody species, stem cutting is the most commonly used cutting type (Dirr and Heuser 1987). Generally, stem cuttings can be divided into softwood, semi hardwood and hardwood cuttings. Softwood cuttings are usually collected in spring and early summer; whereas semi-hardwood cuttings are obtained later in the summer season. These types of cuttings are susceptible to wilting due to the

presence of leaves; therefore, it is important to keep cuttings in shade and maintain moisture during the root induction period. Generally, a cutting is about 5 to 15 centimetres in length, and has several nodes. Examples of plants that are propagated using softwood cuttings are rose, plum, and lilac; while those propagated with semi hardwood cuttings are azalea, magnolia and rhododendron (Dirr and Heuser 1987; Acquaah 2005). Hardwood cuttings are collected after the trees have lost their leaves in the winter or early spring before the growth break. This type of cuttings is generally longer, approximately 12 to 25 centimetres (Dirr and Heuser 1987; Acquaah 2005). The trees are usually in a dormant state, and although hardwood cuttings of some species are almost impossible to root, hardwood cuttings of grapes, figs and willow have been noted to root successfully (Acquaah 2005).

Another source of cuttings is the root. Lilac, apple and horseradish are frequently propagated by root cuttings. This type of cutting is typically collected either in late winter or early spring, when the plant is dormant (Hartmann 1997). As a general rule, cuttings collected from parts of a plant closer to the main trunk are more likely to regenerate, whether to form roots or shoots. The size of the cutting varies depending on the diameter of the root, for instance a root with 0.5 cm diameter is usually cut into 8-cm pieces. Root cuttings can be stuck vertically or horizontally, while maintaining polarity. After shoots are formed, they can be cut and treated as stem cuttings or the whole root-shoot transplanted (Dirr and Heuser 1987; Hartmann 1997).Additionally, sometimes root cuttings need to be treated with fungicide before planting, by dipping the cuttings or wetting the rooting medium with the fungicide solution. Certain rooting hormones have incorporated fungicide in their formulation to simplify the process (Dirr and Heuser 1987).

Some species, such as sugar maple (*Acer saccharum*) and Norway maple (*A. platanoides*), are considered difficult-to-root species (Dirr and Heuser 1987). Plants that belong to this category usually have a narrow window to achieve high rooting percentage. This is shown in softwood cutting

experiments on flowering crabapple, *Malus* spp (Burd and Dirr 1977; Chapman and Hoover 1981). The observation was that the cuttings collected in the month of May in Illinois (Burd and Dirr 1977) and those collected in July from Michigan area (Chapman and Hoover 1981) produced high rooting percentages. Nevertheless, cuttings collected shortly after resulted in a drastic drop in rooting percentages. This demonstrated that these species root well within a specific narrow window. In the cases with softwood and semi hardwood cuttings, some propagators recommend reducing total leaf area in order to prevent excessive transpiration; however, evidence for this practice is still ambiguous. Multiple-noded cuttings of *Acer rubrum* 'Red Sunset' have been shown to produce more profuse root system compared to single-noded, smaller cuttings, implying that maintaining a relatively adequate leaf area may result in larger root system (Dirr and Heuser 1987).

The success of cutting propagation depends on many variables incorporated into the process; these include nutritional status of the stock plant, hormone level, juvenility of the stock plant, and environmental factors (Acquaah 2005). Since the nutritional status of the stock plant influences the rootability of the cuttings, it is important to select for healthy and pathogen-free plants to collect cuttings from. Terminal shoot cuttings that contain an actively growing tip generally have lower carbohydrate levels than more mature cuttings, and may root the same or differently (Dirr and Heuser 1987; Hartmann et al. 1997). Adequately maintained stock plants, properly fertilized and sprayed, usually provide good quality cuttings.

Moreover, juvenility of the stock plant is also a factor that is known to influence rooting (Rosier et al. 2005). Cuttings from certain species may root well regardless of the age of the stock plant; these species are considered easy-to-root. When working with difficult-to-root species, such as sugar maple, juvenility of the source plant should be carefully taken into account. How juvenility influences the rooting capacity of the cuttings is still unknown; however, as a plant ages, it is suggested to accumulate

compounds that may be inhibitory to the rooting process (Dirr and Heuser 1987). A study by Paton et al. (1970) found inhibitory compounds in adult tissue of *Eucalyptus* plant. Additionally, as plants age, other studies have observed reduced contents of some phenolic compounds, which supposedly function as a cofactor in the rooting response of auxin. Cuttings can be treated in order to rejuvenate them, for example, by spraying cytokinins or gibberellins (Dirr and Heuser 1987). The practice has been shown to improve rooting on various species but is not commonly used commercially. Something to bear in mind is that juvenility of a plant, as well as the effect on rooting response vary among species. In addition, the timing of cutting collection is a vital part of the process. Some species only have a small window during the year when their cuttings can be rooted successfully (Tousignant et al. 2003), and cuttings of other species can be collected almost year-round and root just as well regardless of timing. Several factors are represented by timing of collection, and they are mainly related to how the environmental conditions affect the plant's physiological state (Dirr and Heuser 1987).

Apart from the physiological state of the cuttings, environmental conditions during rooting also play an important role. To promote and/or accelerate rooting, several factors can be manipulated; these factors include rooting hormone, bottom heat and above-ground temperature (Alegre et al. 1998). Other important factors include rooting medium and mist system. The type of rooting medium to use depends on the species, cutting type, season and the overall propagation system. Several functions are to be served by the rooting medium; these include fixing the cutting in place for the duration of root formation, supplying moisture to the cutting, allowing air penetration and gas exchange at the base of cutting, as well as limiting light infiltration in order to create dark conditions at the cutting base (Hartmann et al. 1997). Common rooting medium is composed of perlite and peat. Some propagators often use sand on its own because of its wide-spread availability and low cost (Dirr and Heuser 1987). A large selection of premixed propagation media is available in the market; this can help shorten production steps as well as save time and labor (Hartmann et al. 1997).

A key factor in successful rooting is the maintenance of humidity and temperature of the cuttings, which has been resolved by the development of mist system. Intermittent mist regulates the volume of water delivered to the cuttings and reduces the risk of excess moisture in the media. Misting can be regulated by an electronic timer which is adjusted manually, or can be automatically adjusted based on the cutting conditions as detected by a sensor. For example, a controller may utilize a flat plate to simulate an actual leaf. The plate allows the controller to estimate whether the leaf is dry and needs misting (Dirr and Heuser 1987). The time cuttings take to root varies among species; generally ranging from a week to a few months. Misting can be reduced or stopped once cuttings have rooted. Generally, over the winter season rooted cuttings are stored at a temperature slightly below freezing, approximately -2°C, and in the spring these cuttings are transplanted (Dirr and Heuser 1987; Hartmann 1997).

2.2.2.2 Grafting of budding propagation

Grafting is a vegetative propagation method that involves the fusion of two plants or plant parts so that they will continue to grow as a composite plant (Hartmann et al. 1997; Acquaah 2005). The process is considered successful when the graft has healed completely and the vascular tissues of the graft components have connected (Acquaah 2005). Grafting is composed of at least two parts to produce the new plant; a scion and a rootstock. In some cases, a third part may be incorporated and is termed an interstock because it is placed between the scion and the understock. A scion consists of a short stem having two or more dormant buds, which will grow into the upper portion of the new plant. Budding is used to describe the process where the scion only has one bud attached to a segment of bark. The other part of grafting, the understock, is the bottom part of the graft, which is also known as the rootstock (Dirr and Heuser 1987; Hartmann 1997). This feature may become an advantage when the scion cultivar needs a more adapted root system to survive and grow well, for reasons such as plant size and disease tolerance (Dirr and Heuser 1987). In some cases, another component can be introduced in

between the two existing components. An interstock is a piece of stem slotted in between the scion and the understock, and is used generally to control disease and avoid problems that may exist when a particular scion is grafted onto a certain rootstock. Other reasons to include interstock are for production of special tree structures and to benefit from its growth-controlling characteristic. Examples include the use of an upright-growing interstock to add height in order to produce "tree" roses and the insertion of dwarfing 'Malling 9' apple as interstock to reduce tree growth and promote flower and fruit production (Hartmann et al. 1997).

As one of the oldest method of plant propagation, grafting is a popular and valuable method of propagation. Generally grafting is used when the species is difficult or impossible to propagate with other vegetative propagation techniques. The process is considered labour intensive and costly; yet it is still a popular and valuable method of propagation. Besides its use as a propagation method, grafting can be used for specialized repair of damaged trees (Dirr and Heuser 1987; Hartmann 1997). Furthermore, grafting can be difficult since not all species are compatible as a grafting pair. The complexity is increased as reciprocal grafts may behave differently, for example, a graft of *Prunus persica* (peach) as scion on *P. cerasifera* × *P. munsoniana* 'Mariana' (plum) may not be successful even though 'Mariana' plum is compatible as scion on peach (Dirr and Heuser 1987). In general, plants that are closer taxonomically have a greater chance of showing graft compatibility (Dirr and Heuser 1987; Hartmann et al. 1997; Acquaah 2005). Additionally, the grafted plant may display growth patterns that are not detected when the parts are grown separately due to a unique interaction that takes place when they are grafted. Some of these novel growth patterns may be of high importance to the horticultural value of the plant; while others, which can be harmful to the plant and sometimes fatal, result in graft incompatibility (Hartmann et al. 1997).

Graft incompatibility is suggested to be caused by undesirable physiological interaction between the grafting partners or anatomical aberrations of vascular tissue in the transition zone (Hartmann et al. 1997). Incompatibility may also be related to genetic differences or infections by viruses and mycoplasma (Dirr and Heuser 1987; Hartmann 1997). Thus, it is important to use a pathogen-free understock and scion. Moreover, several factors including aeration, temperature, humidity, and the inherent capacity to produce callus that will unite the scion to the understock, may influence the success of a graft. Warm temperature is known to facilitate callus formation and optimal temperature ranges between 10 to 30°C. Additionally, temperature sensitivity varies among species; apple is known to have a wider range of callus-promoting temperature compared to walnut. Most importantly, moisture around the graft junction must be continuously maintained during the healing period; this is often achieved by using grafting wax or simple plastic covering (Dirr and Heuser 1987). External symptoms indicating incompatibility may include failure to form proper union, early defoliation, premature death within one or two years, overgrowths at, above or below the graft union, significant differences in the rate of growth and vigour between scion and rootstock, or disintegration of the components at graft union (Hartmann et al. 1997).

2.2.2.3 Tissue culture propagation

Plant tissue culture holds a lot of potential in that many areas of plant improvement and biological studies can benefit from it. Based on the concept of totipotency of single cells, tissue culture utilizes specific chemical and physical conditions for the culturing of cells, tissue or organs and regeneration of whole organisms (Schleiden 1938). These defined culture conditions include pH range, temperature, light period, as well as the addition of various compounds with specific roles in plant growth regulation. The practice of tissue culture as a propagation method has greatly benefitted some species. In the case of orchid, normal propagation is very slow, whereas shoot tip cultures of orchids can

produce a large number of protocorms, which can be harvested and subsequently divided and recultured to produce new plants in a shorter time period (Dirr and Heuser 1987).

One way to regenerate plantlets through tissue culture is via organogenesis (Brown and Thorpe 1986). It usually begins with the formation of shoot primordia, followed by the growth of leafy vegetative shoots. These shoots are then induced to develop roots in order to produce plantlets ready for transfer into soil. Three different stages of organogenesis include competence, canalization and morphogenesis (Sugiyama 1999). In the first stage, the cultured cells develop competence to react with the hormones supplied in the culture media. During canalization, dedifferentiation of cells is observed, and depending on the hormonal balance, for example the auxin to cytokinin ratio, cells may initiate shoot or root development. In the final stage, morphogenesis may proceed independently of exogenously supplemented hormones.

Cytokinin is a group of plant growth regulators that are required for normal plant shoot development (Werner et al. 2001). It was discovered when scientists sought for factors involved in stimulation of cell division or cytokinesis (Hartmann et al. 1997). In 1940s and 1950s, Skoog and his coworkers at the University of Wisconsin tested a variety of substances, and detected the stimulation of shoot growth activity when they added autoclaved herring sperm DNA into the culture medium. Further investigation and characterization led to the discovery of first cytokinin, which was named kinetin. Kinetin is a synthetic analog cytokinin and is a derivative of aminopurine. The most abundant form of natural cytokinin is zeatin, initially found in extracts of the immature endosperm of corn (*Zea mays*; Taiz and Zeiger 2002). Although roots can be cultured continuously on a simple nutrient medium, the addition of cytokinin is crucial for stimulating and maintaining growth of isolated stem tissues in culture. In addition, the ratio of auxin to cytokinin delineates the differentiation plot of cultured callus tissue, where a high auxin to cytokinin ratio promotes root formation and a low auxin to cytokinin ratio

stimulates the development of shoots (Taiz and Zeiger 2002). The biological roles exercised by cytokinins during growth and development are discussed in the next section.

Many maple species have been successfully propagated in vitro, either by vegetative cuttings or shoot proliferation. These include silver maple (*A. saccharinum*; Preece et al. 1991a, 1991b), red maple (*A. rubrum*; Wann and Gates 1993), Norway maple (A. platanoides; Durkovic 1996), sycamore maple (*A. pseudoplatanus*; Wilhelm 1999), Japanese maple (*A. palmatum*; Fernandez-Lorenzo et al. 2000) and kawakami maple (*A. caudatifolium* Hayata; Durkovic 2003). Although not all studies compared responses between juvenile and mature explants, generally positive results were achieved when juvenile explants were used. In kawakami maple (*A. caudatifolium*), mature explants did not successfully regenerate shoots and failed to propagate (Durkovic 2003). However in the case of red maple, explants from mature trees produced positive results during in vitro regeneration (Wann and Gates 1993).

Propagation via tissue culture can also be achieved through somatic embryogenesis, which is defined as the differentiation process of somatic or non-gametic cells to form embryos, which are bipolar structures with both shoot and root meristems (Smith 1992; Hartmann et al 1997). In general, these embryos appear and behave in the same way as their zygotic counterpart, in that they can mature and germinate (Smith 1992). Their development also includes stages similar to that of zygotic embryos, except that somatic embryos generally have smaller cotyledons and they exist on their own, without endosperm and seed coat (Gray and Purohit 1991). In a study on cell suspension systems by Steward et al. (1958), it was observed that a treatment of carrot cell cultures with coconut milk resulted in the development of embryo-like structures. The induction of somatic embryogenesis usually involves treating callus cells with a short auxin pulse, followed by hormone-free medium to allow for embryo development (Smith 1992). Somatic embryogenesis produces clones of the original plant, thus creating the potential for high rates of clonal propagation. A study conducted on *Theobroma cacao* plants to

assess the field performance of plants produced via somatic embryogenesis compared to those propagated by other traditional methods found that somatic-embryo-derived cacao plants showed normal phenotypes and comparable growth parameters to those propagated by other means (Maximova et al. 2008).

2.3 Role of plant growth regulators in propagation

Within a plant, cells and organs communicate in very intricate ways, which generally involve various compounds and ions. One group of molecules involved in this system is represented by plant hormones. Plant hormones are defined as low-molecular weight molecules that occur naturally within plants at very low concentrations and are responsible for regulating the plant's growth and development activities (Raven et al. 1999). These molecules have specific chemical structures and can be grouped into six major categories; auxins, cytokinins, gibberellins, ethylene, abscisic acid, and brassinosteroids (Arteca 2006). Besides these, some natural and synthetic chemicals when applied exogenously to plants can induce hormonal effects. The term plant growth regulator (PGR) is used as a collective term for these substances and the naturally-occurring plant hormones (Hartmann et al. 1997).

As for many other aspects of plant growth and development, seed dormancy and germination are closely controlled by hormones. Gibberellin is highly correlated to seed germination since its activities include activation of reserve mobilization and enzyme actions. Gibberellin levels are generally maintained relatively high during the seed development and decline as the seed matures. Furthermore, external application of gibberellin has been shown to promote dormancy release. In contrast, abscisic acid (ABA) acts the opposite way, by imposing inhibitory effects on germination. Additionally, ABA prevents precocious germination in non-dormant seeds (Hilhorst and Karssen 1992). The application of ABA can also offset the effect of gibberellins; however the effect is temporary and seeds will germinate upon removal of ABA. The role of cytokinin in dormancy and germination is not as pronounced, but

cytokinin is known to counteract some germination-inhibiting effects, including the effect of ABA (Hartmann et al. 1997). Similarly, the gaseous hormone ethylene is recognized to alleviate dormancy caused by hormone reactions and environmental conditions (KeÇpczyński and KeÇpczyńska 1997). In addition, ethylene is a natural germination-stimulant in certain species, such as snowberry (*Syphoricarpos*), honeysuckle (*Lonicera*), and corn (*Zea*) (Hartmann et al. 1997). Table 2.1 summarizes the effects of each hormone in various events during plant growth and development, and also the application of hormones in plant propagation.

2.3.1 Auxins

Auxin, the first plant hormone to be discovered, primarily functions in the regulation of stem and coleoptiles elongation (Taiz and Zeiger 2002). Auxin is synthesized principally in the apical buds and is transported in a polar manner moving from the apical to the basal end also known as basipetal transport, thus creating a gradient from the shoot to the root tip. Various growth and development processes are regulated by this polar gradient, including stem elongation, leaf senescence, wound healing, and apical dominance (Taiz and Zeiger 2002). Main functions of auxin are control of cell division and expansion, stem extension, fruit development, branch growth initiation and leaf abscission (Acquaah 2005). The growth-promoting mechanisms of auxin is suggested to involve the stimulation of proton transport across cell wall resulting in increased extensibility of the wall, or the induction of transcription of mRNAs which are essential for continuous growth (Hartmann et al. 1997). In roots, auxin is known to stimulate cell elongation at low concentrations, but has inhibitory effects at high concentrations. Indole-3-acetic acid (IAA) is the naturally-occurring auxin that is found in all plants; while indole-3-butyric acid (IBA) has been found to exist in some, such as mustard and corn (Taiz and Zeiger 2002). Synthetic auxins, including 1-naphthaleneacetic acid (NAA), and 2,4-dichlorophenoxyacetic acid (2,4-D), have the same functions as naturally-occurring ones; nevertheless, they persist longer and are not catabolised as rapidly when applied to living tissue (Hartmann et al. 1997).

In propagation, auxin is commonly used to induce adventitious root formation in cuttings, particularly when working with the difficult-to-root woody species (Acquaah 2005). In addition, auxin is also commonly used to control morphogenesis in tissue culture propagation. As a rooting stimulant, IBA is the most preferred form of auxin because of its persistence, optimal auxin activity and immobility within the plant. These characteristics allow IBA to persist and stay around the application site and stimulate rooting, rather than creating unwanted effects in other parts of the plant. Beside IBA, NAA is also commonly used as a rooting hormone. NAA toxicity is higher than that of IBA; hence it is important to maintain a proper concentration to avoid damage to the plant. Since IAA is readily degraded, it is not as effective in promoting root formation in cuttings. In cutting propagation, several methods of hormone application are quick dip, prolonged dip, and powder or gel dip (Brown 2008). StimRoot®#2 (Plant Products Co. Ltd., Bramalea, Ontario, Canada) and Dyna Gro® Root Gel (Dyna-Gro Nutrient Solutions, Richmond, California, U.S.) are both commercial rooting hormones available in the market. StimRoot®#2 is in the form of talc or powder, whereas Dyna Gro® Root Gel is in gelatinous form. The auxin in StimRoot®#2 is in form IBA at 0.4% and in Dyna Gro® Root Gel is 0.5% of IBA and 0.25% of NAA (Quist [cited 2009]).

2.3.2 Cytokinins

As the name implies, cytokinin is a group of compounds that enhance cell division or cytokinesis (Hartmann et al. 1997). Cytokinin is present in high concentrations in actively-dividing tissues, such as the embryonic and meristematic tissues (Taiz and Zeiger 2002). Moreover, cytokinin is synthesized in the developing embryos, young tissues including leaf and fruit, as well as in the roots. Physiologically, the primary role of cytokinin is associated to the stimulation of cell division. In addition, cytokinin plays a role in the morphogenesis of shoots and roots, and the maturation of chloroplasts. Together with auxin, cytokinin also regulates the plant cell cycle. In tissue culture the use of cytokinin helps in the preservation of the meristematic state of cells, resulting in continuous production of callus (Acquaah

2005). The ratio of cytokinin to auxin is a crucial component of tissue culture which determines the developmental fate of callus. Shoot fate is encouraged under high cytokinin to auxin ratio, whereas root fate is favoured upon low cytokinin to auxin ratio (Taiz and Zeiger 2002). Cytokinins can be divided into naturally-occurring and synthetic cytokinins. Isopentenyl adenine (2iP) and zeatin (Z) are examples of naturally-occurring cytokinins; whereas kinetin, benzylamino purine (BAP), and thidiazuron (TDZ) are some of the commonly used synthetic cytokinins (Hartmann et al. 1997; Acquaah 2005).

2.3.3 Gibberellins

Gibberellins are mainly known for their ability to promote stem elongation. They have been found in various forms; but merely several are known to display physiological activities (Hartmann et al. 1997). Commercially, gibberellic acid (GA_3) and a mixture of GA_4 and GA_7 are available; although GA_3 is more commonly used. Gibberellin biosynthesis occurs in shoot apex, roots, embryos and cotyledons of developing seeds (Taiz and Zeiger 2002; Acquaah 2005). The main effect of gibberellins on plant growth is the promotion of shoot elongation via cell division and growth. In addition, gibberellins also play a role in seed dormancy and germination, as well as flower and fruit development. Gibberellins have also been observed to stimulate growth in specific genetically dwarf plants (Cooper 1958). Other roles of GA involve the regulation of seed enzyme synthesis and the stimulation of flowering in long-day plants and biennials (Hartmann et al. 1997). Carrot (*Daucus carota*) and cabbage (*Brassica oleracea* var. *Capitata*) are plants that require exposure to long days or cool temperatures for flowering. With the application of gibberellic acid, these environmental treatments are no longer needed (Acquaah 2005). Other commercial applications of gibberellins include the yield increase of sugarcane, the size improvement of seedless grapes, and the acceleration of barley malting process (Taiz and Zeiger 2002). Gibberellins have been used to treat dormant seeds to promote germination (Chen and Chang 1971). Furthermore, the use of gibberellic acid is common in plant tissue culture practices especially for the promotion of callus growth and shoot multiplication. A study by Brian and his colleagues (1960) found that root formation of

pea and bean cuttings was consistently inhibited upon treatment with gibberellic acid. The authors suggested it was due to a direct local effect inhibiting the early cell divisions that are responsible for conferring the meristematic fate to the mature shoot tissue.

2.3.4 Abscisic Acid

Based on its effect on plant growth, abscisic acid (ABA) is considered to be a growth retardant (Acquaah 2005). A product of carotenoid degradation known as mevalonic acid is known to be the precursor of ABA (Hartmann et al. 1997). The synthesis takes place in the chloroplasts and the end-product, ABA, is transported throughout the plant via xylem and phloem (Taiz and Zeiger 2002). In addition, ABA is responsible for regulating water and ion uptake in the roots as well as controlling stomatal movement (Hartmann et al. 1997; Acquaah 2005). The growth-inhibiting properties of ABA are mainly exercised on cell division and elongation (Hartmann et al. 1997). Although ABA is clearly involved in leaf senescence, it only stimulates leaf abscission in a few species (Taiz and Zeiger 2002). Furthermore, ABA levels have been shown to increase in response to water stress (Hartmann et al. 1997). ABA serves as a stimulant for seed and bud dormancy. Moreover, ABA-imposed seed dormancy can be reverted by applying gibberellins, because the two hormones have an antagonistic relationship. ABA applications in horticulture industry mainly involve the inhibition of cell division and elongation, for example in the production of dwarf plants such as poinsettia, petunia, and geraniums (Acquaah 2005).

2.3.5 Ethylene

The gaseous plant hormone, ethylene, is famous for its fruit-ripening activity (Taiz and Zeiger 2002). With amino acid methionine as a precursor, biosynthesis of ethylene produces an intermediate compound known as 1-aminocyclopropane-1-carboxylic acid (ACC), which has the ability to directly produce ethylene effects. Ethylene synthesis is induced by light, carbon dioxide, cytokinins, auxins, and exogenous carbohydrates (George et al. 2007). Furthermore, wounding and stress also increase

ethylene levels. The effects of ethylene on plant growth include the promotion of flowering, the induction of lateral buds, as well as the stimulation of senescence and abscission of leaves and fruits (Abeles 1992). Horticulturally, ethylene can be applied to facilitate uniform ripening (Acquaah 2005). Furthermore, in cucumber and pumpkin, commercial ethylene spray is used to boost the number of female flowers, thus increasing the fruit production. In plant propagation, ethylene may be used to induce adventitious root formation, aid in dormancy release and promote seed germination in certain species (Hartmann et al. 1997).

2.3.6 Brassinosteroids

The brassinosteroids (BS) are the newest class of plant hormones. Although there are more than 30 compounds listed in this class, brassinolide is the frequently used naturally-occurring form of brassinosteroids (Arteca 2006). BS were first identified around 1970s (Khripach et al. 2000) and since then many studies have shown the involvement of these hormones in various physiological processes. BS are required for normal growth and development, as shown by dwarf and stunted characteristics of mutants with defects in BS biosynthetic pathways and those that were insensitive to BS, respectively (Arteca 2006). Their roles in plant growth and development include cell expansion, etiolation, reproductive development and vascular differentiation (Clouse and Sasse 1998). BS is also known to play a role in stress and disease resistance (Hazra and Pore 1998). Furthermore, Cutler (1991) reported a potential economic value of BS as yield-promoting compounds although the field tests showed inconsistent results. More recently, large-scale field trials in China, Japan and Union of Soviet Socialist Republic (USSR) confirmed the practical value of yield-promoting property of BS (Khripach et al. 2000). Increase in yield had been observed in many crops including wheat, corn, watermelon and cucumber (Arteca 2006). Moreover, the application of BS has been shown to include promotion of germination and reduced fruit abortion and fruit drop. Swamy and Seeta Ram Rao (2006) found that the application of synthetic BS improved root formation and growth of geranium stem cuttings.

Table 2.1 Summary of the roles of plant hormones during growth and development, as well as in plant propagation (Dirr and Heuser 1987; Hartmann et al. 1997; Acquaah 2005).

Hormone	General roles	Roles in propagation
Auxin	Apical dominance Stem elongation Leaf senescence Wound healing Stimulate root growth	Stimulate rooting in cutting prop Morphogenesis in micropropagation
Cytokinin	Promote cell division Maintain meristematic state Shoot and root morphogenesis Chloroplast maturation	Morphogenesis in micropropagation Initiate shoot growth from cuttings overcome dormancy imposed by abscisic acid or heat
Gibberellin	Stimulate shoot elongation Promote germination in seeds Flower and fruit development	Control seed germination Relieve some types of dormancy Micropropagation
Abscisic acid	Inhibit cell division and elongation Control water and ion uptake in roots Regulate stomata movement Induce leaf senescence Promote seed and bud dormancy	Micropropagation via somatic embryogenesis Inhibit seed germination
Ethylene	Fruit ripening Induce flowering and lateral bud Stimulate leaf and fruit senescence and abscission	Aid in dormancy release in certain species Increase rooting of cuttings
Brassinosteroids	Cell expansion Vascular differentiation Reproductive development Improve stress and disease resistance	Promote seed germination Increase root formation and growth in cuttings

3.0 MATERIALS AND METHODS

3.1 Embryo Conversion

3.1.1 Plant materials

Seeds for embryo conversion study were ordered from the National Tree Seed Centre (NTSC). NTSC is located at the Canadian Forest Service – Atlantic Forestry Centre (CFS-AFC) in Fredericton, New Brunswick. Its purpose is to obtain, store, and provide seed of known origin and quality for use in scientific research and ex-situ conservation of Canadian tree and shrub species. In addition, NTSC is also actively involved in gene conservation of Canadian-native tree and shrub species, to preserve woody plant biodiversity in Canada. Upon collection, seeds were tested and subsequently dried prior to storage. All seeds were stored in hermetically sealed jars at 20°C. NTSC also performs germination tests regularly to monitor seed viability. The testing procedure was standardized according to rules of the International Seed Testing Association (ISTA) or Association of Official Seed Analysts (AOSA). Using four replicates of 50 seeds from each lot, this test began by soaking the seeds in cool tap water for a period of 72 hours. Afterward, the seeds were transferred onto a moistened germination medium in a container that was kept at 1-5°C for 12 weeks; this period is referred to as the stratification period. Subsequently, the seeds were placed in a germinator or programmable growth chamber that was set to 20°C with 8 hours of light and 16 hours of darkness, while maintaining humidity level of 85%. At day 21, germination was assessed and recorded.

For the embryo conversion study, three seed lots were used in each treatment; 20061304, 20061307 and 20061309 (Figure 3.1). Seeds from lot 20061309 were larger and lighter in colour than the other two seed lots. Table 3.1 also shows that seed lot 20061309 had the highest thousand seed weight (TSW) among the three lots. Germination test performed in 2008 revealed germination rates ranging from 75 to 95% for the three seed lots; with seed lot 20061304 having the highest germination

rate (95%), followed by 20061307 at 85% and 20061309 at 75%. As indicated in the seed lot number, these seeds were collected in the fall of 2006 from Perth-Andover, NB. Locations of these trees are specified in Table 3.1.

3.1.2 Culturing procedures

First the dry seeds were cracked open and the seed coats were discarded. Then the embryos were wrapped with cheesecloth and secured with a rubber band to facilitate the transfer of seeds between steps during sterilization. The sterilizing solution was made using commercial bleach, Ultra Javex® bleach by Clorox, which had a concentrated formula containing 6.98% sodium hypochlorite. The sterilizing mixture was consisted of 17.2% bleach and was prepared with autoclaved distilled water. This solution contained an equal amount of sodium hypochlorite, which is the active sterilizing ingredient in bleach, as a 20% solution made with regular, non-concentrated Javex® bleach. In addition, approximately 4 drops of Tween®20, a wetting agent, were added into the sterilizing solution. A magnetic stirrer was used to ensure an even distribution of sterilizing ingredient in the solution. The cheesecloth-wrapped seed bundle was submerged in the solution for 20 minutes, and then rinsed three times using autoclaved distilled water. The seeds were put into a sterile Petri dish and stored overnight, between 18-24 hours, at 4°C as a cold treatment. The day following the sterilization, the sterile rehydrated embryos were ready to be dissected and plated onto media. Sterile water was used to keep the seed coat moist and to facilitate dissection. Intact, naked embryos were then transferred onto semi-solid media in magenta jars. Each jar contained between five to eight embryos and each magenta jar represented one replicate.

The media for all the treatments used the same basic medium recipe, which contained 4.33 g/L Sigma-Aldrich® Murashige-Skoog basal salt mixtures and was supplemented with 1.25 ml/L LV vitamin stock, 5 ml/L LV iron stock, 10 g/L sucrose, 50 mg/L of each glycine, glutamine, serine, and 100 mg/L of

myo-inositol (Appendix I). Phytagel was added at 3.5 g/L to produce semi-solid media and the medium was adjusted to pH 5.8 before autoclaving. The various treatments involved different plant growth regulators at varying levels of concentrations. Initially, the experiment compared the effect of auxin, cytokinin and gibberellin as represented by indole-3-butyric acid, 6-benzylaminopurine and gibberellic acid (GA_3) respectively. Furthermore, additional treatments were performed using other compounds with cytokinin activity, including zeatin and thidiazuron. Several concentrations were used for each compound, with ranges depending on its relative efficiency. In most cases, these hormones were used within the range of 0.5 to 1.5 milligrams per litre of media. Some exceptions included thidiazuron with a range of 0.001 to 0.005 mg/L, and gibberellic acid (GA_3) between 0.1 to 1.0 mg/L (Table 3.2). All isolated embryo cultures were incubated at 16-hour photoperiod at 23 °C for six weeks.

3.1.3 Data collection and analysis

Contamination was carefully monitored and jars with contamination were immediately removed and the non-contaminated embryos were transferred onto fresh media. However, in rare cases where contamination developed later, usually after three weeks, they might have not been removed from the contaminated media since transfer onto new media might have suspended growth and/or damaged vulnerable organs. At the end of the six-week period, the growth of these embryos was assessed. The response of the embryos to the treatment was measured in terms of their conversion success, which was defined as the completion of conversion of an embryo into a whole plant. Conversion of an embryo into a plant with both shoot and root systems was a complete conversion, whereas those that developed only a shoot or a root system were considered partially converted. Depending on the hormone and the seed lot, each treatment had between 60 to 90 embryos which were divided into 10 to 15 jars. The conversion rate of the embryos into whole plantlets was assessed proportionally to the total number of embryos in each jar, meaning the total number of converted embryos divided by the total number of embryos in the particular jar. Moreover, contaminated embryos were not included in the

calculation. Subsequently, the data were subjected to analysis of variance (ANOVA) using the Mixed procedure of Statistical Analysis System® version 9.1 (SAS Institute Inc., USA). Square root transformation and grouping by hormone were done to improve the homogeneity and normality of variances. Results from the analyses are reported in percentages.

Figure 3.1 Dry sugar maple (*Acer saccharum*) seeds from National Tree Seed Centre; three seed lots: 20061304, 20061307 and 20061309 from Perth-Andover, North Brunswick

Table 3.1 Location of source trees and thousand seed weight (TSW) of each seed lot used in embryo conversion study of sugar maple (*Acer saccharum*)

Seed lot	Latitude	Longitude	Elevation (m)	TSW (g)
20061304	46.85	-67.64	278	87.36
20061307	46.85	-67.65	241	98.87
20061309	46.85	-67.64	304	103.48

Table 3.2 Various concentrations of plant growth regulators (milligrams L^{-1}) used to stimulate sugar maple (*Acer saccharum*) embryo conversion from isolated mature embryos cultured on basal medium (Appendix I)

Plant growh regulator		Concentration (mg/L)		
Auxin	Indole-3-butyric acid (IBA)	0.5	1	1.5
Cytokinins	6-benzylaminopurine (BAP)	0.5	1	1.5
	Zeatin (ZEA)	0.5	1.5	-
	Thidiazuron (TDZ)	0.001	0.005	-
Gibberellin	Gibberellic acid (GA)	0.1	0.5	1.5

3.2 Shoot organogenesis

3.2.1 Plant materials

Dormant bud collection began in early spring of 2008 before bud break. As the weather warmed up, the buds started to reactivate to generate new growth. The bud collection spanned a period of approximately eight weeks, from April to May. Sources included sugar maple trees from Jeffries Nurseries Ltd. (Portage La Prairie) as well as public and private trees in Winnipeg, Manitoba (Talon Bay, Wellington Crescent, and Byng Place). In addition, the study also utilized seeds as an explant source. A small pilot project was done in 2007 using fresh immature seeds collected from several sugar maple trees in the Winnipeg area. The project was not repeated due to unavailability of fresh seeds in the area the following year. The study mainly used mature dry seeds as the source and these were ordered from the National Tree Seed Centre (NTSC) of Fredericton, New Brunswick. The study used several seed lots that were collected from different locations in various years, between 2002 and 2006. Moreover, shoot tips and petiole segments were also tested for organogenic capacity. These explants were collected in June 2008 from the trees in Winnipeg.

3.2.2 Culturing procedures

3.2.2.1 Culturing procedure of bud explants

Upon collection, the buds while still attached to the shoot were rinsed under running tap water for about one hour to remove surface dirt. The bud scales were then removed to expose the shoot meristem. This needed to be done very carefully in order not to damage the delicate structure. The shoot meristem tip of the dormant buds were very small, they had several green spikes of leaf primordia measuring about 2-3 mm in length. In the case of reactivated buds, their scales were generally more loose and easier to remove; their shoot meristem also had started growing in size. Likewise,

rejuvenating buds were prepared by removing their bud scales. After their scales removed, they were kept on moistened filter paper to prevent drying out.

At first the naked shoot bud tip explants were sterilized in 10% bleach solution for four minutes. A Plant Preservative Mixture™ (PPM) wash was implemented after detecting high contamination rates; however, some of the bud scales were left on to provide some protection to the vulnerable shoot meristem. The final sterilization procedure for bud explants began with a 4% PPM™ wash for two hours, followed by three water rinses and transfer onto a medium (Appendix II) to preserve the moisture. The plate was sealed and kept at room temperature overnight. The next day, the explants were washed with 70% ethanol for 30 seconds then 20% bleach solution for 25 minutes and rinsed three times using autoclaved water. The bleach solution was made using regular, non-concentrated Javex® bleach. After the rinses, all the bud scales were removed completely, and the explants were re-sterilized with 10% bleach solution for 1 minute, then washed three times in sterile autoclaved water and plated onto the appropriate medium (Appendix II).

The organogenic process consisted of four different steps; shoot induction, shoot multiplication, shoot elongation and rooting. Each step was characterized by a medium containing a specific hormonal composition (see Appendix II). The pH of all the media was adjusted to 5.8 prior to autoclaving at 121 °C for 25 minutes. Initially, explants were plated onto induction media but later this step was skipped. Every four weeks, the explants were subcultured onto a fresh medium or transferred to the next stage depending on their response to the treatments. The bud explants were incubated in a tissue culture cabinet set at 16/8-hour (light/dark) photoperiod at 23°C. Furthermore, the cultures were regularly checked to prevent spread of contamination to the sterile explants.

3.2.2.2 Culturing procedure of embryo explants

Sugar maple seeds, both fresh and dried, were also used as a source of explants. The dry embryos were extracted from the seeds and then soaked in running tap water for 1 hour prior to sterilization. Next, the embryos were immersed in a 10% solution of regular Javex® bleach for 15 minutes, and then rinsed three times using sterilized distilled water. The sterile embryos were subsequently placed in a sealed petri dish, before being kept at 4 °C overnight. The following day, the embryos would have been fully rehydrated and ready for dissection. In the beginning, contamination was relatively high. The strength and duration of sterilization had to be increased several times in order to suppress contamination. To reduce the level of contamination, the time period was increased to 15 minutes, then to 20 minutes. In addition, the concentration of the bleach solution was increased from 0.6 to 0.9% sodium hypochlorite. The sterilization procedure that gave reasonable survival rate among the mature dry embryos involved treatment with 0.6% sodium hypochlorite for 20 minutes. The level of contamination with the adjusted concentration and duration was about 4%. Moreover, the fresh seeds collected from residential trees in Winnipeg were sterilized using a solution of 0.9% sodium hypochlorite for 15 minutes. Some of these embryos were small and still in a liquid endosperm. Prior to sterilization, a shallow incision was made on the seed coat to allow penetration of the sterilizing chemical.

Using sterile forceps and scalpel the embryos were dissected into segments of cotyledons and embryo axes (Figure 3.2). The cotyledon segment represented approximately the middle third of a whole cotyledon. As for the embryo axis, both ends were removed such that the segment did not include the apical meristem. After dissection, the segments were placed on the prepared media supplemented with hormones to induce organogenesis (Figure 3.3). Similar to the bud explants, the embryo explants also had four types of media; induction, maintenance, shooting and rooting. All four were made from a basal medium outlined in Appendix III. The induction media for embryo explants were supplemented with two types of cytokinin, benzylamino purine and thidiazuron, to induce

formation of adventitious shoots; whereas the maintenance media contained no growth regulator to preserve growth and elongation of the microshoots. Explants were kept in dark in an incubator set at 23°C and contamination was monitored closely. Explants were regularly subcultured at 4-week interval or transferred to the appropriate medium based on their responses.

3.2.2.3 Culturing procedure for shoot tips and petioles

Shoot tip and petiole segments were cut to about one to two centimetres in length. These explants were sterilized in 70% ethanol for 30 seconds then in 10% regular Javex® bleach solution for 15 minutes, followed by three rinses using sterile distilled water. Explants were placed on the agarized medium and slightly pushed in to ensure contact. The medium for shoot tips used a basal medium (refer to Appendix IV) supplemented with benzylamino purine or BAP at 2.5 µM; whereas the media for petiole segments had thidiazuron at 0.04, 0.1 or 0.2 µM. Explants were transferred to new plates every four-week period. All media were adjusted to pH 5.8 and autoclaved at 121 °C for 25 minutes. All cultures were assessed for shoot formation every four weeks.

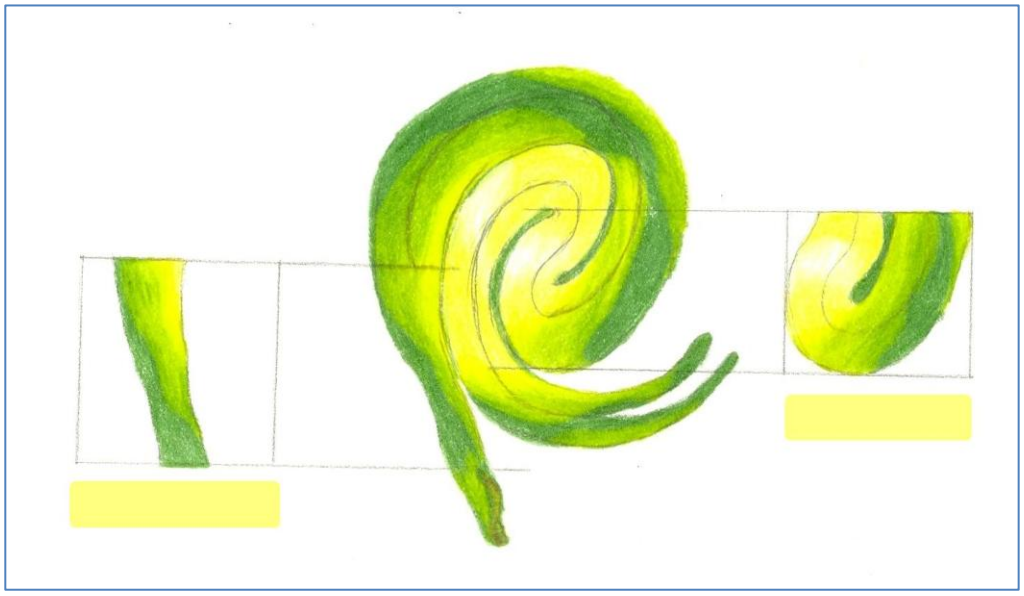


Figure 3.2 Illustration of the longitudinal section of a sugar maple (*Acer saccharum*) embryo from which explants for shoot organogenesis were harvested; embryo axis (left box) and a middle segment from the cotyledons (right box; drawing courtesy of Rhanissa Hirawan).

Figure 3.3 Plated *Acer saccharum* embryo explants on induction medium after two weeks in culture

3.3 Rooting of softwood cuttings

3.3.1 Experimental year 2008

3.3.1.1 Plant materials and preparations

The experiment in 2008 consisted of three collection dates: July 2, July 9 and July 14, with accumulated growing degree days (GDDs) of 496, 578 and 640, respectively. Cuttings used in the rooting study were from juvenile trees, ranging from 3-4 years in age. Juvenile trees are those that have not started producing seeds. The trees were located in a field nursery located south of Portage La Prairie, Manitoba (Young Farm of Jeffries Nurseries Ltd.). The experiment in 2008 was performed on two sugar maple cultivars, 'Jefcan' and 'Bailsta'. The 'Jefcan' trees were one-year older than the 'Bailsta' trees. The collected cuttings were divided into two groups based on the presence of the shoot apex; terminal and medial (Figure 3.4). Generally, cuttings of terminal type had two or more nodes with leaves attached, although these leaves were often younger and smaller in size. Medial cuttings were more likely to have mature leaves and usually only had one pair of leaves attached.

All cuttings were kept moist by dipping them in cool tap water before putting them in plastic boxes, in which they were transported and stored prior to planting. These cuttings were prepared immediately after collection. Cuttings were to have at least two nodes and the leaves on the bottom node were removed and discarded. Furthermore, cuttings were kept overnight in darkness at low temperatures, approximately 4°C. The following day, the cuttings were taken to the planting site in Glenlea (Glenlea Greenhouses). Next, the cuttings were treated with the appropriate rooting hormone and directly inserted into the rooting medium. The hormone treatments are listed in Table 3.3. Hormone solution or powder was applied to the bottom of each cutting (approximately 2 cm of the cutting base). Indole-3-butyric acid (IBA) solutions were prepared by dissolving the appropriate amount of IBA (Sigma-Aldrich® I5386) in 70% ethanol, whereas naphthaleneacetic acid (NAA; Sigma-Aldrich®

N0640) was dissolved in 1 M sodium hydroxide (NaOH). The solution with the combination of IBA and NAA also used 1 M NaOH as the solvent. For the treatments with hormone solutions, the base of the cutting was submerged for ten seconds. Several factors were analyzed in the experiment, including cultivar, cutting type, collection date, and hormone treatment. Overall, the experiment utilized two cultivars, two cutting types, three collection dates, and nine hormone treatments. For one planting date, each hormone treatment had approximately 10-30 cuttings per cutting type.

3.3.1.2 Rooting conditions and procedures

All cuttings were inserted into a sand bed inside a plastic-covered greenhouse at the Glenlea site (Figure 3.5). To prepare the sand bed, first, the ground was graded to have a slope for drainage, and then a five-inch layer of three-quarter limestone was added, which was followed with a layer of builder's sand or 5" sand. To enhance rooting, the greenhouse was equipped with an intermittent misting system which can be adjusted according to the weather and needs. Generally, on a sunny day, the misting would start at 8:30 a.m. for 5 seconds every 15 minutes and increased to misting for ten seconds every eight minutes by noon, and then slowly decreased. The duration of misting and the intervals vary with air temperature, cloud cover and the root development of the cuttings. The heat from the sunlight and the frequent misting often resulted in high humidity levels within the greenhouse. To help lower temperature as well as prevent moisture build-up, the greenhouse had an automated exhaust fan. It was controlled based on temperature, which was set to about 27°C.

The rooting period lasted for 12 weeks. As the weather cooled down towards fall season and cuttings started to root, the misting was reduced and eventually turned off. Also, the greenhouse was built using poly material which can be modified to aid the transition of these rooted cuttings into cooler conditions or winter hardening. The modification was done by rolling the plastic on the sides of the

greenhouse; thus allowed air movement and the cuttings were able to adapt slowly to the changing weather conditions.

3.3.1.3 Data collection and analysis

After 12 weeks, the cuttings were harvested and rooting was assessed. Rooted cuttings were brought back to the lab for further measurements, including length of the longest root, number of primary roots, fresh and dry weights of the whole roots. The primary roots were those that emerged directly from the bottom of the cutting, instead of a branch of another root. The roots were washed carefully by dipping the roots into a beaker of water and patted dry using a paper towel, then removed from the cutting to be weighed. Subsequently the roots were put into an envelope of aluminum foil and properly labelled. These foil envelopes were then put into an oven set to 32°C for 72 hours. Some roots took longer to dry depending on the amount and thickness of roots. Dried roots were then weighed and results were recorded. Data on rooting frequencies were analyzed using Proc Genmod of Statistical Analysis Software (SAS®) version 9.1 (SAS Institute Inc., USA) to assess the effects of each factor including cutting type, cultivar and rooting hormone application on root formation. Mean separation group was calculated using the Wald chi-square statistics. The primary root counts, root length and fresh and dry weights of the rooted cuttings were analyzed using Proc Mixed of SAS®. To meet the assumptions for the analysis of variance (ANOVA) model, the data was initially square root transformed. The non-transformed means are reported in the Result section in Chapter 6.

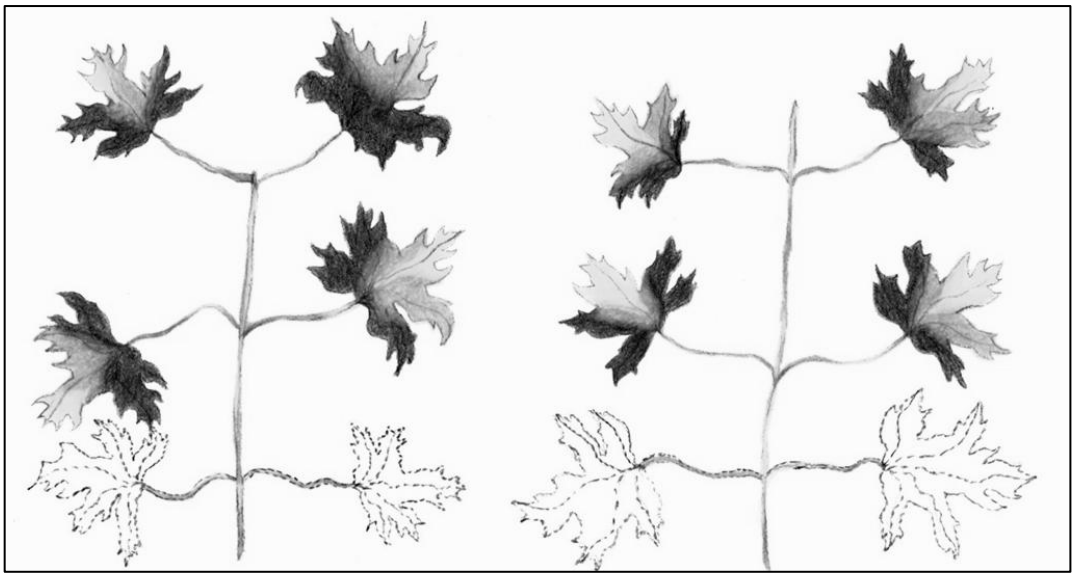


Figure 3.4 Illustration of the two types of cuttings that were used in the cutting propagation study of sugar maple (*Acer saccharum*) in July 2008; terminal (left) and medial (right) cutting types (drawing courtesy of Rhanissa Hirawan).

Table 3.3 Composition of hormone treatments used to stimulate rooting of cuttings of *Acer saccharum* collected in July 2008. Abbreviated names may be used to refer to the treatment in text.

Rooting hormone	Concentration (g/L)	Abbreviated
StimRoot®#2 (IBA at 4 g/L)		SR2
Indole-3-butyric acid (IBA)	5	IBA-5
	10	IBA-10
1-Naphthaleneacetic acid (NAA)	5	NAA-5
	10	NAA-10
IBA + NAA	5+5	IBA-5/NAA-5
	5+10	IBA-5/NAA-10
	10+5	IBA-10/NAA-5
	10+10	IBA-10/NAA-10

Figure 3.5 Plastic-covered greenhouse at Glenlea Greenhouse where the *Acer saccharum* cuttings (right far corner) were planted in sand beds for rooting (picture was taken at harvest, 12 weeks after planting)

3.3.2 Experimental year 2009

3.3.2.1 Cutting collection and preparation

Based on the results obtained from the 2008 cutting experiment, several parameters were adjusted and some new parameters were added to the 2009 experiment. Due to extreme winter damage observed on the 'Bailsta' trees (Figure 3.6), the experiment in 2009 did not include the cultivar 'Bailsta' for the majority of the study. Moreover, only one type of cutting, the medial type, was used in the experiment. The medial cuttings comprised the third and the fourth nodes, counted from the terminal or youngest node (Figure 3.7). The experiment in 2009 was composed of four collection dates. Collection started when the trees had grown a good amount of green shoots, approximately 40 cm in length. The collection dates were June 17 (collection 1), July 1 (2), July 7 (3) and July 13 (4).The collections followed the recommended range of accumulated heat units (growing degree days or GDDs) of Tousignant, et al. (2003) that suggested after accumulation of 270 GDDs above 5°C. The actual growing degree days for each collection were 264 (June 17), 474 (July 1), 546 (July 7) and 614 (July 13). This was the accumulated GDDs up to the end of day of before the collection date; for example, for collection 1 which was done on June 17, 2009, 264 is the GDDs reported at the end of June 16, 2009. Another parameter added in the 2009 experiment was the leaf factor. One of the leaves from the second-youngest node of each cutting was removed and stored in an airtight plastic bag for the leaf analysis (Figure 3.7). These leaves were scanned using a flatbed scanner at a resolution of 240 dpi to produce digital images, which were then used for leaf area and color analysis.

The main experiment consisted of a total of four hormone treatments and a control treatment (Table 3.4). Two commercial hormones were used in the study, StimRoot® #2 and Dyna Gro Root Gel®. StimRoot® #2 was used in the 2008 experiment, and consists of IBA at 4000 ppm, or equivalent to 4 g/L. The other commercial hormone, Dyna Gro Root Gel, as the name implies is in form of a gel. It contains

both IBA and NAA at 5 g/L and 2.5 g/L respectively. The two other hormone treatments were from the previous study in 2008; they were IBA at 5 g/L (IBA-5) and a mixture of IBA at 10 g/L and NAA at 5 g/L (IBA-10/NAA-5). The solutions were prepared as described in the previous section (2008 experiment). Moreover, distilled water was used as a control.

For the first, second and fourth collections, each treatment had two replicates of 20 cuttings. For the third collection, the cuttings were divided into two groups, and then planted at two different sites; Glenlea and Morden and each treatment had one replicate of 25 cuttings at Glenlea and 27-28 cuttings at Morden. The Morden site only involved four treatments; control, StimRoot® #2, IBA at 5 g/L and the combination of 10 g/L of IBA with 5 g/L of NAA. Additionally, a side study was conducted to examine the effect of acidic pH on rooting of cuttings. Another treatment using water with pH at 4.0 (pH was adjusted using pH buffer 4.0) was applied on two replicates of 20 cuttings from the first and second collections, and one replicate of 25 cuttings from the third collection. The pH level (4.0) was selected based on the pH of IBA solution at 5 g/L.

In addition to the 'Jefcan' field cuttings, the fourth collection date included cuttings from container trees. Each of these trees was grown in a number-10 nursery container or a pot. Container trees are grown under more-controlled conditions, especially during the winter season, when the trees were tipped and covered with flax straw and poly sheets in order to protect from harsh temperatures and to prevent winter damage to the root systems (Ronald, pers. Comm.). Other than the different environmental conditions during the winter season, these container trees were also of younger age (three-year old) compared to those in the field (five-year old). Similarly, these trees were provided by and maintained at the Jeffries Nurseries Ltd., Portage La Prairie. The experiment on container trees was conducted on 'Jefcan', 'Bailsta' and Green Mountain® cultivars, involving two treatments; control and IBA at 5 g/L. Each treatment was performed on two replicates of 15 cuttings.

All collected cuttings were kept moist by a quick dip in cool tap water. They were prepared by removing leaves from the bottom node. The cuttings were then stored in darkness overnight in either plastic boxes or black plastic bags at cool temperatures. The following morning the cuttings were transported to the planting site, treated with the appropriate rooting hormone or solution, and then immediately planted into the rooting medium. Adventitious roots normally would form around a node; thus, the cuttings were prepared to have most bottom node inserted into the hormone treatment and into the rooting medium.

Figure 3.6 Trees of sugar maple (*Acer saccharum*) cultivar 'Bailsta' in late spring of 2009, showing extensive winter damage

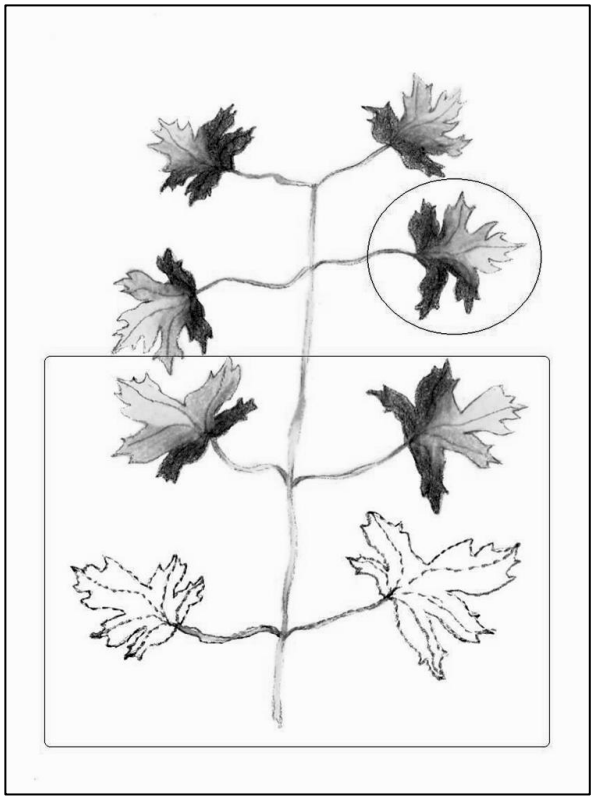


Figure 3.7 Illustration of a cutting for the experiment on rooting of softwood cutting of sugar maple (*Acer saccharum*) in 2009. The first and second youngest nodes were removed and leaves on the bottom node were discarded (dotted lines). One of the leaves on second-youngest node (circled) was collected for leaf analysis. The bottom half (box) was the final cutting to be planted (drawing courtesy of Rhanissa Hirawan)

Table 3.4 List of hormone treatments in *Acer saccharum* cutting experiment in 2009 (IBA: indole-3-butyric acid; NAA: naphthaleneacetic acid)

Composition	Treatment
Distilled water	Control
IBA 5 g/L	IBA-5
IBA 10 g/L+NAA 5 g/L	IBA-10/NAA-5
IBA 5 g/L+NAA 2.5 g/L++ (gel)	Dyna Gro Root Gel®
IBA 4 g/L++ (talc)	StimRoot®#2

3.3.2.2 Rooting conditions and procedures

Another new factor in 2009 experiment consisted of three different rooting conditions; peat-perlite pot mix in a misting box, sand beds in Glenlea greenhouse, and peat plugs at Shannon Oak Tree Farm. The misting box was kept in one of the greenhouses at the University of Manitoba. Cuttings from the first collection were stuck in a 1:1 mixture of perlite and Sunshine® Professional Peat Lite Mix #4 (Sun Gro Horticulture Canada Ltd., Vancouver, British Columbia) in 3x3 inches pot. These were then put in a misting box with a timer set at 10 seconds of fine mist for every minute. Due to limited space available in the misting box, each pot was planted with two cuttings, both receiving the same hormone treatment. Misting cycles can be adjusted to the weather conditions and the cutting needs.

For the second collection date, the cuttings were rooted in sand beds at the Glenlea site (Glenlea Greenhouses). The greenhouse had an automatic misting system, which can be manually set to have different mist frequencies depending on the time of the day (refer to previous section for further details). Cuttings from the third collection date were divided into two groups; one group was planted in sand bed at Glenlea and another was inserted into the rooting medium at the Morden site (Shannon Oak Tree Farm, North of Morden; Figure 3.8). The Morden site utilized a different medium for rooting, contained in ellepots® (A.M.A. Plastics Ltd., Figure 3.8). Furthermore, the mist system at Morden site included a screen balance, which predicts the leaf conditions and regulates the misting depending on the needs (Figure 3.9). All cuttings from the final or fourth collection were stuck in the sand bed at Glenlea.

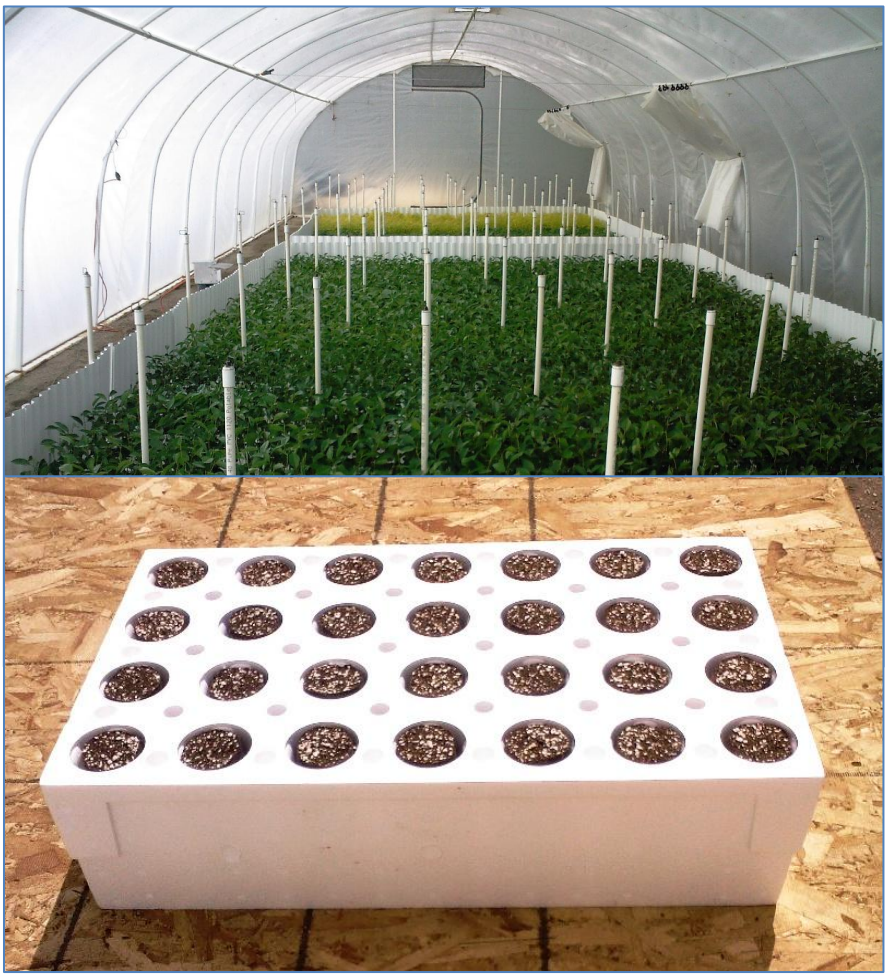


Figure 3.8 The inside of a plastic-covered greenhouse at the Morden site (Shannon Oaks Tree Farm), where the propagation trays were kept (top), and a sample of the plug tray with rooting media (bottom)

Figure 3.9 Screen balance timer used to estimate when mist is needed to maintain humidity and moisture in the rooting experiment with sugar maple (*Acer saccharum*) 'Jefcan' cuttings at the Morden site (Shannon Oak Tree Farm)

At the end of the rooting period the cuttings were assessed for root formation, and from those which rooted, the number of primary roots were counted and recorded. The measurement taken of the rooted cuttings from the 2009 experiment was non-destructive; thus the rooted cuttings were kept and transplanted the following spring. Rooting assessment on cuttings was performed at the end of week 12 of each planting. A gentle pull was used to test if a cutting had formed roots. Cuttings at Glenlea were removed from the ground using a potato fork. However, since it was considered early to harvest the cuttings, the rooted cuttings at Glenlea were immediately transplanted into pots following assessment. Rooted cuttings were always kept moist to ensure that the roots did not dry out before potting.

Over the winter, these rooted cuttings were stored in pots at -2°C. At the Morden site, assessment was done by taking out the plugs of soiless mix from the tray and lightly pulling the cutting. The number of primary roots was estimated by counting the number of roots that were visible around the outside of each plug at about three centimetres from the top surface of the plug. Although assessment was performed at week 12, the cuttings remained in the greenhouse until approximately week 19, and were transferred outside of the greenhouse to slowly harden for about two weeks, before being put into storage. To prepare the cuttings for storage, the rooted cuttings, removed from their tray, were lined up in groups of seven, then rolled up and wrapped with a plastic sheet. The rolls of cuttings with the plugs were put in a storage room that was maintained at -2°C. These cuttings will be taken out of storage and transplanted when the weather is appropriate for re-growth in the spring.

3.3.2.3 Data collection and analysis

Statistical analysis on the results was performed using basic statistical formulas as well as Statistical Analysis Software (SAS®) version 9.1 (SAS Institute Inc., USA). Proc Genmod of SAS® was used to test for significance of each factor including the effects of hormone treatments on root formation. Mean separation group was calculated using the Wald chi-square statistics. The primary root counts of

the rooted cuttings were also analyzed using the Mixed Procedure of SAS®. To meet the assumptions for the Analysis of Variance (ANOVA) model, the data on the number of primary roots were initially square root transformed. In chapter 6, the non-transformed means are reported.

The scanned leaf images were analyzed for leaf area and leaf color using the American Phytopathological Society (APS) Assess® software. Leaf images were selected and measurements of leaf area, average color saturation and intensity were requested. The leaf area collected from all field 'Jefcan' cuttings (excluding those from container trees) were pooled to test if leaf area had any effect on rooting of cuttings. The average leaf area values for rooted and non-rooted cuttings were analyzed using Proc Genmod of SAS®. Pearson correlation value was calculated by using the Proc Corr on SAS® to assess the relationship between leaf parameters and the number of primary roots. Data on color saturation and intensity were analyzed in the same way as leaf area for relationship with rooting frequency and the number of primary roots formed per rooted cutting.

4.0 CHAPTER 1: EMBRYO CONVERSION

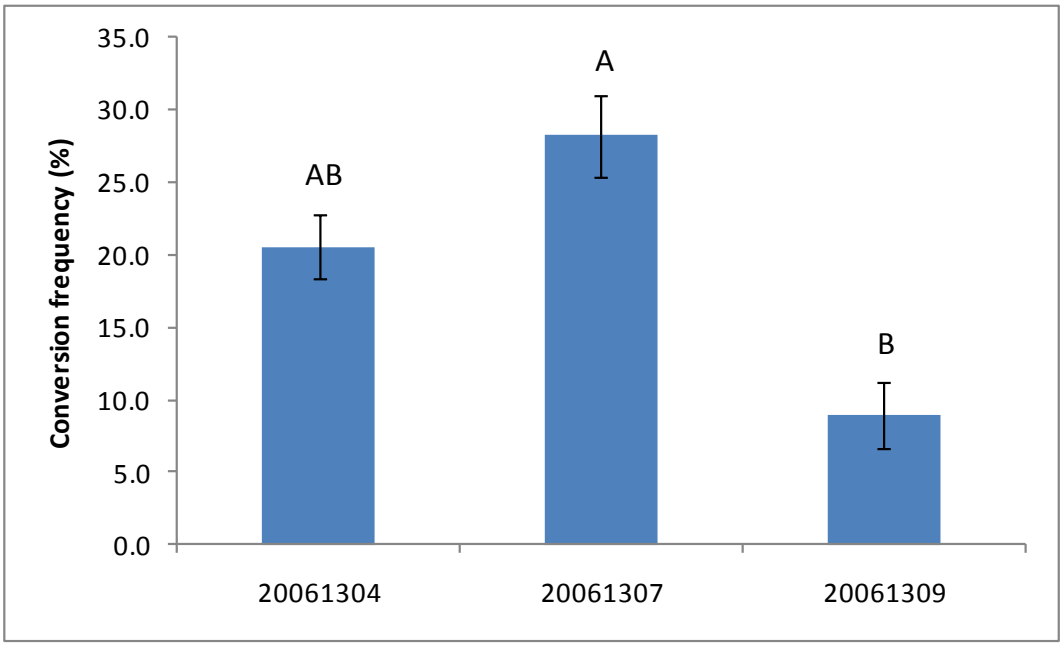
4.1 Results

The jars with the isolated embryos were kept in a tissue culture cabinet which was set at 16-hour photoperiod and 23°C for a period of six weeks. The embryos generally started to unroll and elongate after one week, and then slowly accumulated chlorophyll, which made them turn green. Some embryos also built up other pigments, as shown by their red or purple color. Embryos that did not unroll or turn green by the end of the second week usually died. The number of dead embryos varied among the seed lots and the hormone treatment applied. At the end of the fourth week usually most embryos had already fully expanded their cotyledons and some had begun to form bud-like shoot structures from their apical meristem. These structures continued to grow into true leaves. Furthermore, the radicle would swell and elongate. This part of the embryo remained white, throughout the culture period. At some point, a small extension emerged and developed into a root.

4.1.1 Effects by genotypic differences

The genotypic influence on embryo conversion among the three seed lots used in the study was confirmed to be significant ($p=0.0070$). Similar to their reported germination percentages, the three seed lots exhibited a gradient in their conversion scores. Seed lot 20061307 showed the highest average conversion over the general hormone treatments, followed with 20061304 and 20061309 (Figure 4.1). Although seed lot 20061304 had the highest germination (95%), its conversion frequency (20.6%) was not considerably different ($P>0.05$) from that of 20061309 (8.9%). In addition, the difference in conversion between seed lots 20061304 (20.6%) and 20061307 (28.2%) was reported to be statistically insignificant at $p\leq0.05$. Statistically, only embryos of seed lot 20061307 were more likely to convert into whole plantlets than those from seed lot 20061309. Overall, it was observed that the seed lot 20061309 performed more poorly in comparison to other seed lots in each hormone treatment.

Figure 4.1 Effects of genotype on *Acer saccharum* embryo conversion (%) as demonstrated by three seedlots 20061304, 20061307 and 20061309 as an average over several hormone treatments.



Values are the mean of 10 to 15 replicates of five to eight embryos in treatments of control, benzylamino-purine, indole-3-butyric acid, and gibberellic acid at various concentrations (refer to Appendix I for further details). Means with the same letters are not significantly different at $p \leq 0.05$.

Although the percentage of 20061309 embryos that formed both shoots and roots was rather low, many embryos from this seed lot produced shoots without roots (partial conversion). Partially-converted embryos were also observed in other seed lots, but the phenomenon was most commonly found among embryos of seed lot 20061309. These unrooted plantlets need to be induced specifically to form roots to achieve full conversion in order for them to survive ex vitro conditions.

4.1.2 Effects of plant growth regulators

4.1.2.1 General hormone comparison

The analysis of the results demonstrated that both seed lot and hormone treatments played important roles in the conversion of *A. saccharum* embryos into plantlets ($p < 0.0001$). Additionally, interaction between the two factors was also significant ($p = 0.0124$); thus results are reported separately for each seed lot to simultaneously present the interaction between the two factors (Figure 4.2). Healthy characteristics of fully converted embryos included an elongated hypocotyl, well-developed leaves with bright green coloration and optimal turgidity, generally with an average area of approximately 10 cm^2, and an elongated root system. Moreover, some embryos exhibited red and/or purple coloration, particularly on their expanded cotyledons and/or the hypocotyl and the area around the shoot apical meristem. Unhealthy characteristics of embryos included white-yellow, attenuated cotyledons, and were observed in the treatment with the lowest concentration of gibberellic acid (0.1 mg/L). Many of these embryos had begun to expand their cotyledons, but then stopped and started to lose the chlorophyll. There were also some embryos that died after their cotyledons had fully expanded. For some seed lots, none of the embryos in this treatment produced any shoot or root (0 [zero] % conversion frequency; Figure 4.2). Moreover, callus development was observed on the basal end of some embryos from seed lot 20061309 in the treatment with 6-benzylaminopurine (BAP) at 1.0 mg/L despite the absence of root growth.

Figure 4.2 summarizes the conversion percentages of the general hormone treatments. Overall, supplementation of hormones enhanced embryo conversion, except in the case of gibberellic acid (GA_3 or GA) at 0.1 mg/L (for seed lots 20061307 and 20061309) and 0.5 mg/L (for 20061309). These seed lot and hormone treatment combinations had zero conversion rates; hence were of inferior quality than their respective control treatments (16.8% among 20061307; 1.3% among 20061309). Furthermore, statistical analysis revealed that in all three seed lots, conversion percentages of the control embryos were not significantly different from those in the auxin indole-3-butyric acid (IBA) and gibberellin (GA) treatments, except for seed lot 20061304 embryos in treatment with GA at 0.5 mg/L. To summarize, only the treatments with cytokinin 6-benzylaminopurine (BAP) did significantly improve the embryo conversion consistently in all the three seed lots studied. Moreover, the positive effect of BAP treatments seemed to be concentration-independent since all three levels had means that were not significantly different from each other. Nonetheless, the middle and higher concentrations (1.0 and 1.5 mg/L) seemed to be more likely to stimulate embryo conversion than the lowest concentration (0.5 mg/L).

In the general hormone comparison, across all three seed lots, the application of cytokinin BAP produced the highest percentage of embryos producing both shoot and root (complete conversion). Although BA treatment produced the highest conversion, the lowest conversion was observed in different hormone treatments depending on the seed lot, indicating the interaction between the two factors. Among 20061304 embryos, conversion was slightly lower among the auxin IBA treatment as compared to gibberellin GA treatments, if the mean of 0.1 mg/L GA was not accounted. In seed lots 20061307 and 20061309, embryo conversion was comparatively the lowest in the gibberellic acid (GA) treatments.

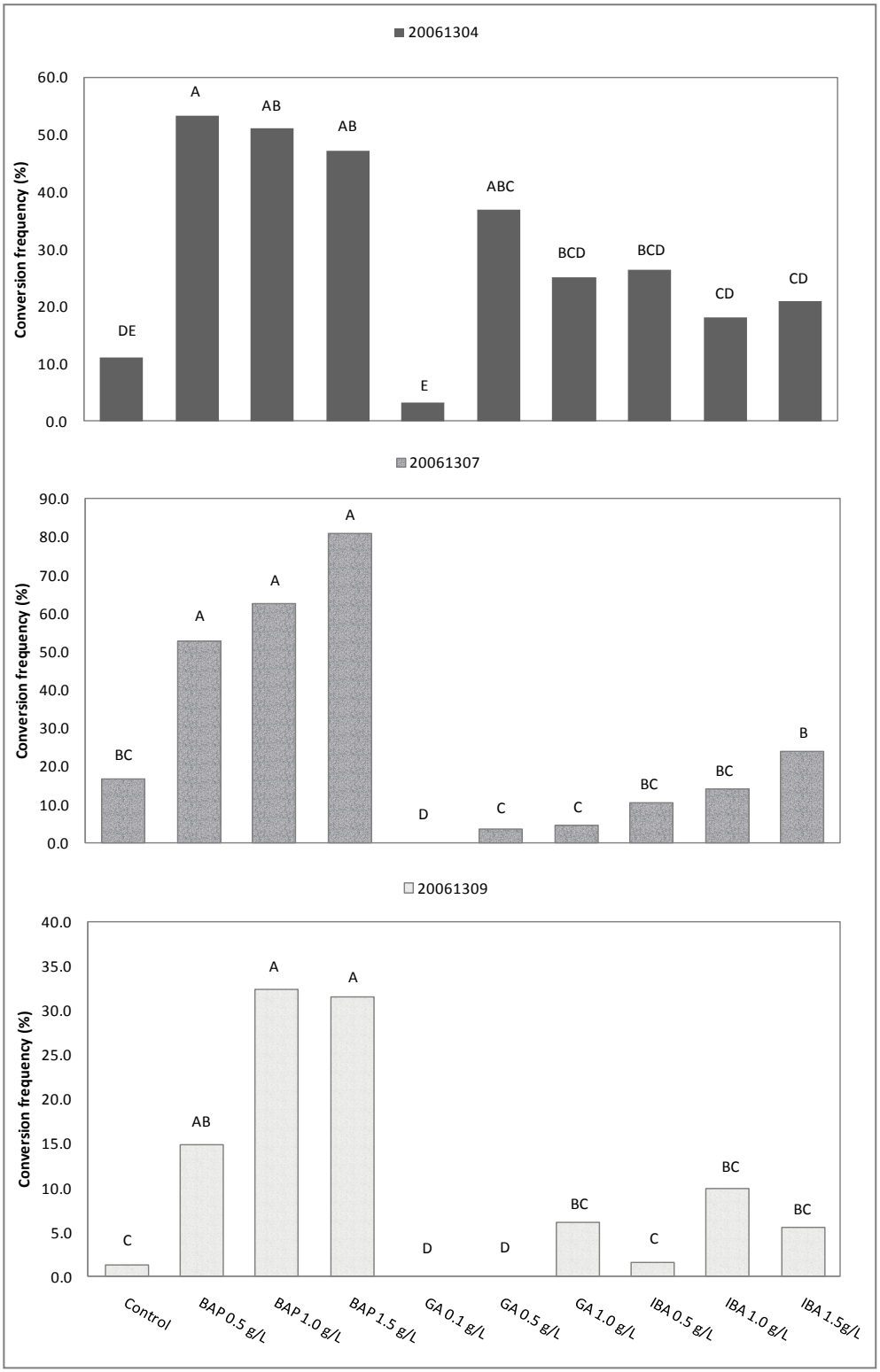


Figure 4.2 Percentages of embryos from three seed lots of *Acer saccharum* that converted into plantlets over a period of six weeks in culture media supplemented with various concentrations of hormones (Appendix I; BAP: 6-benzylaminopurine, IBA: indole-3-butyric acid, GA: gibberellic acid [GA_3]) Values are the mean of 10 to 15 replicates of five to eight embryos and means with the same letters are not significantly different at $p \leq 0.05$.

4.1.2.2 Cytokinin comparison

Taken as a whole, embryo conversion was noticeably higher among the cytokinin treatments than in the general hormone treatments (Figures 4.2 and 4.3). Within the cytokinin treatments, depending on the seed lot, the most substantial promotion was observed in the treatment with either 6-benzylaminopurine (BAP) or thidiazuron (TDZ, Figure 4.3). In all seed lots, conversion rates of these treatments were not significantly different from each other; however BA treatments were more likely to produce healthy-looking seedlings. Moreover, the application of certain hormones at specific concentrations had resulted in stunted appearance and reduced organ size. Based on the recorded observations, seedlings in thidiazuron treatments (both at 0.01 and 0.05 mg/L) were more likely to have abnormal phenotypes which included enlarged shoot apical meristems, thickened cotyledons, stunted hypocotyls as well as small pointy leaves. Treatment with 0.005 mg/L of thidiazuron produced the highest number of stunted seedlings with small shoots and the phenotypes were generally more severe. In addition, several embryos in the treatment with combination of BA and TDZ were noticed to have produced multiple shoots (Figure 4.4). Axillary shoot proliferation was observed around the shoot apex, producing a cluster of shoots that lacked elongation.

Among the hormone treatments, some embryos were detected to exhibit excessive purple coloration, most evidently in their cotyledons and hypocotyls. Some embryos accumulated more pigments than others. This observation was particularly common among embryos in BAP+TDZ treatment. In addition to promoting embryo conversion, hormone supplementation also influenced embryo survival rate. This was observed in the number of dead embryos in the hormone treatments being lower than in control. Among the three seed lots, the numbers of dead embryos from each seed lot corresponded with its germination and conversion rates. Overall, stimulation of embryo conversion process was achieved most efficiently by the application of cytokinin 6-benzylaminopurine at 0.5-1.5 mg/L and thidiazuron at 0.001 mg/L.

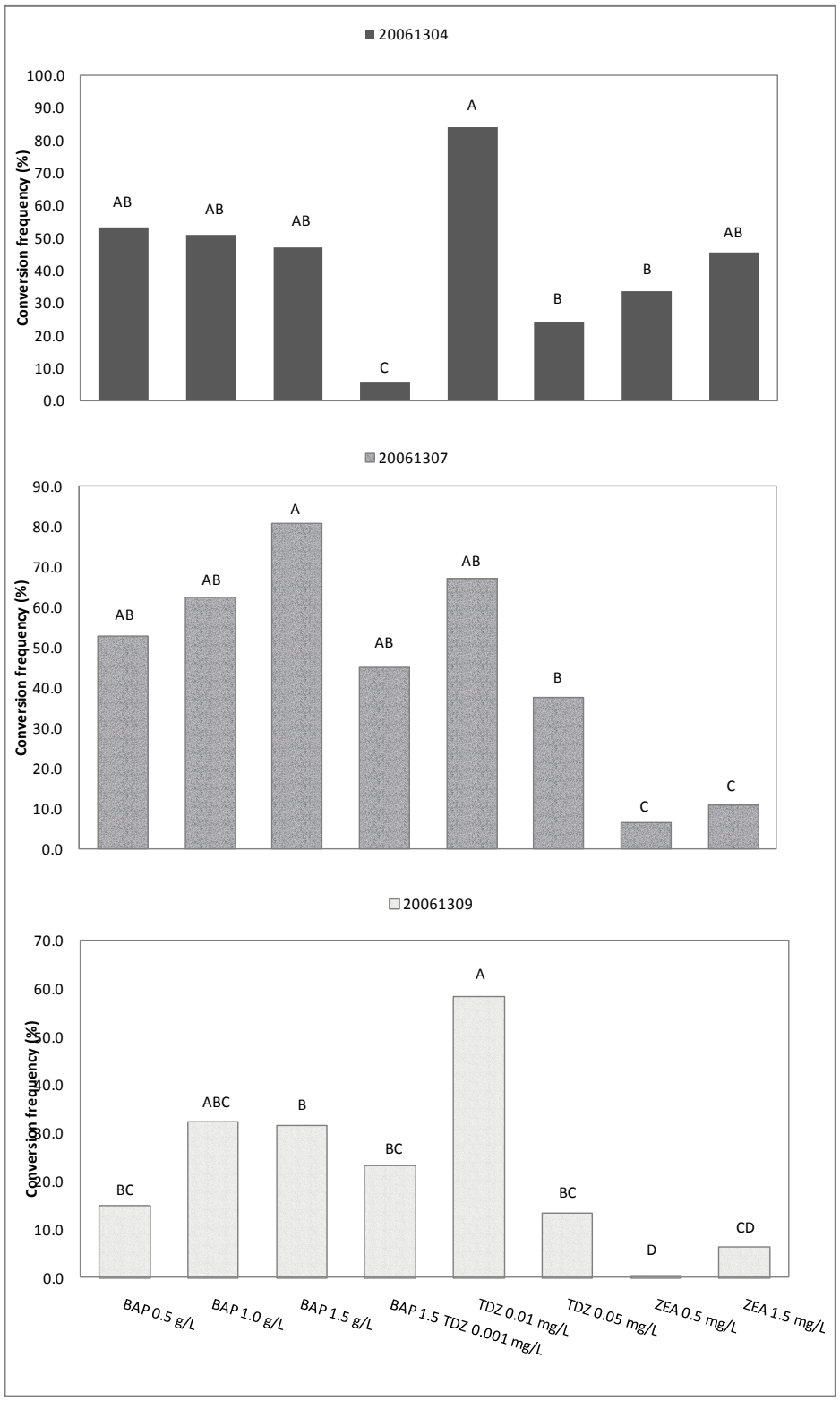


Figure 4.3 Percentages of embryos from three seed lots of *Acer saccharum* that converted into plantlets over a period of six weeks in culture media supplemented with various concentrations of cytokinins (Appendix I; BAP: 6-benzylaminopurine, TDZ: thidiazuron, ZEA: zeatin). Values are the mean of 10 to 15 replicates of five to eight embryos and means with the same letters are not significantly different at $p \leq 0.05$.

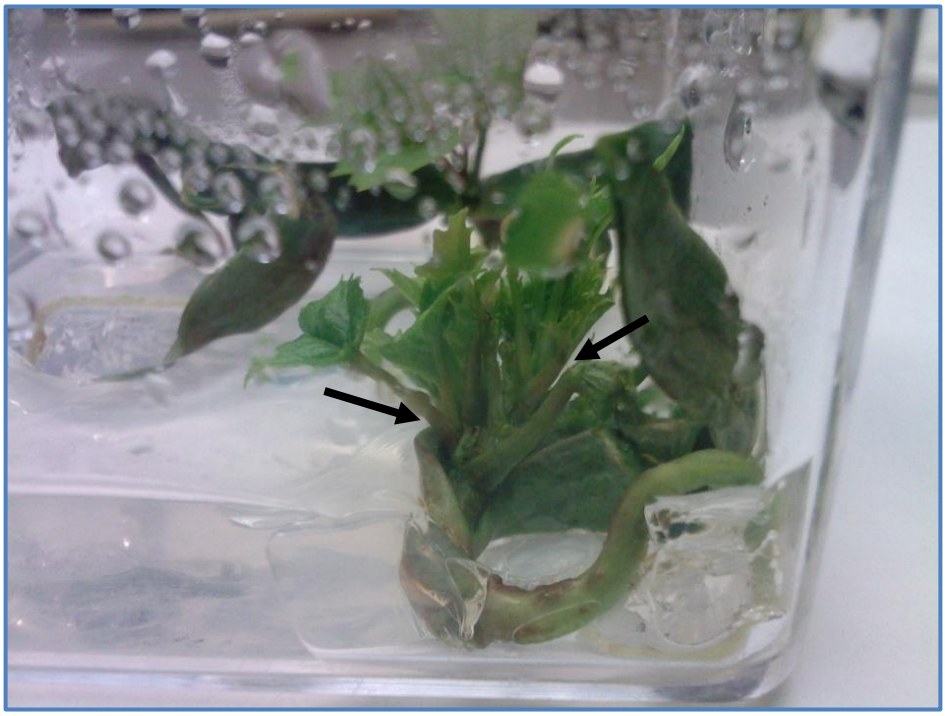


Figure 4.4 Multiple shoots emerging around the shoot apical meristem of a sugar maple (*Acer saccharum*) embryo treated with 1.5 mg/L benzylaminopurine and 0.001 mg/L thidiazuron following a six-week culture period.

4.2 Discussion

As expected the seed lot variation substantially influenced embryo conversion ($p = 0.0070$). The differences in conversion among the three seed lots mimicked the gradient in their germination percentages (Figure 4.1). The seed lots 20061304 and 20061307 behaved differently in terms of their conversion frequencies, when compared to their germination percentages. However the difference in conversion was statistically insignificant. In addition, seed lot 20061309 had the lowest germination and conversion among the three seed lots. Upon stimulation by some growth regulators, embryo conversion was increased; nevertheless, within each hormone treatment seed lot 20061309 always had the lowest conversion. The limited response of this seed lot might be because the seeds were in a deeper state of dormancy or had a different hormonal balance and required specific treatments and/or hormone concentrations. Another explanation for the low germination and conversion rates would be the physiological state of the embryo, including whether the embryo is still alive.

The general hormone comparison assessed the efficiencies of auxin, cytokinin and gibberellin in stimulating embryo conversion. The results demonstrated that cytokinin BAP at 0.5-1.5 mg/L was consistently the most effective in stimulating embryo conversion in all three seed lots (Figure 4.2). Cytokinins are well-known for their roles in various growth and developmental processes in plants, including control of seed germination (Khan 1971; Mok 1994). In addition, Stirk et al. (2005) showed that cytokinin metabolism was active through all phases of germination, starting from imbibition, radicle emergence and the early stages of seedling establishment. A study by Nikolic et al. (2006) reported that exogenous cytokinins ZEA, BA and TDZ at various concentrations within the range of 0.08-3.50 µM, approximately 18 µg/L-0.8 mg/L of BAP, accelerated and significantly improved germination of *Lotus corniculatus* L. seeds.

Moreover, studies on *Pinus radiata* (Lin and Leung 2002) and coconut (Pech y Aké et al. 2007) isolated embryo cultures showed that gibberellic acid increased germination and promoted embryo conversion into plants. In contrast, Stojičić et al. (2008) reported that conversion of *Pinus heldreichii* embryos did not require the addition of exogenous growth regulators. According to the same authors, GA_3 at 1.15 µM, approximately 0.4 mg/L, gave the most benefits on the development of isolated *Pinus heldreichii* embryos by accelerating and synchronizing the embryo germination and increasing seedling dry weight although the use of growth regulators was deemed unnecessary for the normal development of these embryos. Our study was not able to verify the stimulatory effect by gibberellic acid on *A. saccharum* embryos due to substantial variation in conversion responses among seed lots and GA_3 concentrations. In seed lot 20061304, GA at 0.5 mg/L produced a relatively high conversion rate of 36.8% (Figure 4.2), which was among the highest and was not significantly different from those of the cytokinin BAP treatments. Nonetheless the stimulatory effect was not observed in the other seed lots (Figure 4.2). In addition, the lowest concentration (0.1 mg/L) of GA_3 constantly inhibited embryo conversion in all seed lots, yielding conversion rates that were lower than the respective control. The results of this study demonstrated that the effect of gibberellins on the embryo conversion of *A. saccharum* varies with different genotypes and may be dependent on its concentration.

Lin and Leung (2002) found exogenous applications of auxins and cytokinins in cultures of isolated zygotic embryos of *Pinus radiata* often had inhibitory effects on germination as well as on seedling establishment, particularly during cotyledon and root growth. Another study with *Pinus heldreichii* demonstrated that the exogenous supply of BAP, kinetin, GA_3, IBA and naphthaleneacetic acid (NAA) did not affect the germination of isolated *P. heldreichii* embryos (Stojičić, et al. 2008). The same study also found that the growth of isolated embryos was reduced by the presence of the cytokinins kinetin and BAP, as well as the auxin IBA. Likewise, the current study observed that some *A.*

saccharum embryos were able to grow vigorous shoots without developing any roots, a trend that might be related to the effects of exogenous auxins and cytokinins.

When various types of cytokinins were tested, we observed that the average conversion frequency was higher, which confirmed that cytokinins, regardless of type, were more proficient in stimulating conversion (Figure 4.3). Similar to what was observed in the current study, some cytokinin treatments also have been shown to suppress seedling elongation of *L. corniculatus* although seedlings still continued to produce shoots and roots (Nicolic et al. 2006). Additionally, cytokinins have also been reported to increase anthocyanin synthesis in suspension cultures of carrot (Ozeki and Komamine 1986), in red clover seedlings (Dedio and Clark 1971) and in *Arabidopsis thaliana* plants (Deikman and Hammer 1995). Some *A. saccharum* embryos in this study were observed to develop an excessive amount of anthocyanin, resulting in purple tissues. In addition to cytokinin-related induction, anthocyanins production may also be stimulated by light and wounds (Deikman and Hammer 1995). In addition, physical aberrations such as stubby hypocotyls and crinkled cotyledons were observed among *P. heldreichii* embryos treated with auxins IBA and NAA (Stojičić, et al. 2008). A similar observation was also recorded on some *A. saccharum* embryos, especially those treated with high concentrations of TDZ; phenotypes included short hypocotyls and shoots, as well as enlarged shoot apical meristems.

Among the cytokinin treatments, although the range of conversion frequencies increased, the different types of cytokinins did not promote conversion to the same extent. Depending on the seed lot, the highest stimulation was either observed in the treatment with TDZ at 0.01 mg/L or in one of the three BAP treatments. Moreover, TDZ at 0.05 mg/L consistently induced lower conversion rates compared to the reduced TDZ concentration (Figure 4.3). According to Huetteman and Preece (1993), the TDZ concentrations used in the study, 0.01 mg/L or 45.5 nM and 0.05 mg/L or 227.3 nM, were within the recommended range of 1 nM to 10 µM. Moreover, treatments with TDZ, regardless of the

concentration, produced stunted seedlings with small leaves, which could be a sign that the concentration was too high. TDZ has been reported to lead to the formation of short shoots in several species including *Cercis Canadensis* var. *alba* L. (Yusnita et al. 1990) and *Quercus robur* L. (Chalupa 1988), and was also detected in *A. saccharum* in the current study on shoot organogenesis (Chapter 2). Huetteman and Preece (1993) suggested that this concern associated with TDZ may be overcome upon transfer to a hormone-free medium, as has been performed with *Populus* (Russel and McCown 1986) and *Rhododendron* (Preece and Imel 1991). Based on the reported observations, the conversion of *A. saccharum* embryos may be improved by applying lower TDZ concentrations within the range of 1-10nM. The TDZ concentration at 10 nM has been suggested for shoot production from nodal cultures of *Acer saccharinum* by Preece et al. (1991a, 1991b).

The embryo conversion rates in treatments with zeatin at 0.5 and 1.5 mg/L were not significantly different from some of BAP treatments (0.5 and 1.0 mg/L) in seed lot 20061304; however, the positive effect by zeatin decreased significantly in seed lots 20061307 and 20061309 (Figure 4.3). The reason for the low responses to zeatin in some seed lots is still unclear since zeatin is generally categorized as a highly-active cytokinin due to its physiology. Nikolic et al. (2006) described that the cytokinin-inactivation enzyme, also known as cytokinin oxidase/dehydrogenase (CKX), has a lower affinity for zeatin in most tissues; hence zeatin is conserved longer and generally exerts a higher biological activity.

Furthermore, the combination of BAP at 1.5 mg/L and TDZ at 0.01 mg/L (BAP+TDZ) induced different conversion responses among the three seed lots (Figure 4.3). The highest conversion response to this hormone treatment was observed in seed lot 20061307, where it was among the highest and statistically similar to the means of BAP treatments as well as TDZ at 0.01 mg/L. In seed lot 20061304, conversion in BAP+TDZ treatment was the lowest and was significantly different from all other cytokinin

treatments. Besides, some embryos in the BAP+TDZ treatment had been induced to form multiple shoots. This may be considered as a side effect to the high activity of the combined cytokinins, which also serve as a potential for regeneration via shoot organogenesis. Additionally, Nicolic et al. (2006) also presented that some of the cytokinin-treated *Lotus corniculatus* L. seeds were induced to form multiple shoot production. ZEA was found to be the most effective to stimulate the onset of organogenesis in seedlings of *L. corniculatus*; however, the highest number of regenerates per seedling was observed in treatments with TDZ.

The shoot proliferation observed in the current study only involved a small percentage of the total embryos. The multiple shoots were found to develop around the apical meristem (Figure 4.4). The induction of shoot proliferation is a well-known effect of cytokinins, which is commonly applied in the practices of micropropagation, such as shoot organogenesis (Brown and Thorpe 1986). All shoots formed in the BAP+TDZ treatment emerged from the axillary buds of the main shoot. Huetteman and Preece (1993) reported that shoot proliferation often times can be significantly improved by adding a low concentration of auxin or a second cytokinin into the culture medium. The induction of axillary shoot proliferation observed in the embryo culture of the current study may have been due to the combination of BAP and TDZ. Future attempts to induce shoot proliferation of *A. saccharum* embryo explants should consider using two types of cytokinin, possibly BAP and TDZ and perhaps at a lower TDZ concentration to avoid the formation of abnormal phenotypes.

Several suggestions to consider for subsequent embryo conversion study on *A. saccharum* include soaking whole seeds prior to sterilization, extending the duration of moist chilling and dark treatment, maintaining a cool temperature during the initial incubation period, and increasing the amount of carbohydrate source i.e. sucrose in the culture medium. Stratification or moist chilling is a common treatment to induce the breaking of dormancy and depending on the level of dormancy,

approximately 40-90 days of stratification at 0-5°C is suggested for *A. saccharum* seeds (Olson and Gabriel 1974). In the current study, moist chilling treatment was applied after the surface-sterilization procedure for approximately 18-24 hours. Soaking the seeds prior to sterilization and extending the moist chilling period may be helpful to improve conversion. Webb et al. (1973) found that *Acer saccharum* seeds soaked for 48 hours exhibited higher germination than those soaked for 0 or 24 hours. A study on four native Australian *Cyperaceae* species found that incubation at 15°C significantly increased the germination and growth of zygotic embryos in vitro as compared to 25°C (Panaia et al. 2009). Another study with *Acer pensilvanicum* reported that germination was slower but considerably higher in seeds incubated at 5°C for 16 hours of dark and 15°C for 8 hours of light than at 20°C for 16 hours of dark and 30°C for 8 hours of light (Bourgoin and Simpson 2004). The authors suggested that the warm temperature may have induced secondary dormancy since they observed that only seeds that started to germinate during the stratification period can continue germination at the high temperature conditions. Therefore when optimizing conditions for conversion, the conditions under which the seeds germinate and grow in nature should be taken into consideration.

Many *Acer* species produce dormant seeds that require a stratification period in order to germinate. Removal of the pericarp or the seed coat has been reported to speed the imbibition process of *Acer saccharum* (Webb and Dumbroff 1969) and *Acer ginnala* seeds (Dumbroff and Webb 1970). Among the *A. saccharum* seeds, the modification in the protective structures also reduced stratification requirements to as short as three weeks (Webb and Dumbroff 1969). The current study used isolated embryos thus pericarp was removed. Additionally, heavy contamination was detected by Webb and Dumbroff (1969) and when complete removal of testa of *A. saccharum* seeds was attempted to increase germination. The high contamination problem was also observed in the current study, which had been minimized by washing testa-covered dry embryos in 20% solution of commercial Javex® bleach for twenty minutes, followed by three rinses using sterile autoclaved water. To facilitate imbibition,

embryos were kept moist by keeping them with sterilized moistened cheesecloth in a sealed Petri dish while stratified overnight at 4°C. Imbibition may be improved by soaking seeds for 24 hours prior to sterilization; this may also increase the efficiency of the sterilization procedure. In addition, the application of gibberellic acid and kinetin during the initial soaking considerably reduced the stratification requirements for *A. saccharum* 'naked' seeds (Webb and Dumbroff 1969). The same authors also found that *A. saccharum* seeds germinated best at 5°C. Hence, to accommodate both stratification and optimum germination, isolated embryo cultures can be incubated in dark at 5°C for the first two weeks of culture, followed with a light treatment at 15-20°C to stimulate seedling growth during the remaining culture period. In addition, hormone treatments may be initiated at the imbibition stage by soaking seeds in a hormone solution accompanied with gentle agitation.

In the study, the medium was supplemented with 1% sucrose as carbohydrate source. This amount did not seem to be limiting the seedling growth; however there may be an advantage in increasing the amount of carbohydrate source to 3 or 4%. A study with isolated *Pinus heldreichii* embryos compared the influence of various forms of carbohydrate source, including sucrose, glucose, fructose and maltose (Stojičić et al. 2008). The study implemented five concentrations for each type of carbohydrates; 1, 2, 3, 4, and 5% and found that sucrose was the superior carbohydrate source among the four tested carbohydrates and that 3-4% of sucrose considerably improved the seedling dry weight compared to 1-2% sucrose.

In conclusion, the study shows that the conversion of *A. saccharum* embryos to small plantlets can be achieved within a 6-week period and the frequency of conversion may be improved by supplementing the culture medium with cytokinins, such as 6-benzylaminopurine (BAP) at 0.5-1.5 mg/L or thidiazuron (TDZ) at 0.01 mg/L. Based on the current observations, transfer of embryos onto a hormone-free medium may benefit seedling growth, particularly those that exhibit stunted appearance

or form shoots without any root. A lower concentration of TDZ may be applied if transfer of embryos to a secondary medium is not preferred. The concentrations of TDZ are recommended to remain within the range of 1-10 nM for inducing embryo conversion. Moreover, our study also detected the potential of applying a combination of BAP and TDZ to induce multiple shoot formation from *A. saccharum* embryos.

5.0 CHAPTER 2: IN VITRO SHOOT ORGANOGENESIS

5.1 Results

5.1.1 Bud explants

Sugar maple (*Acer saccharum*) buds were collected between April and May of 2008. During the collection period, the weather slowly warmed up and some buds reactivated and started to show new growth. This was shown by the increase in size and bulging appearance. At first bud explants were sterilized in 10% bleach solution for four minutes. A Plant Preservative Mixture™ (PPM) wash was implemented after detecting high contamination rate. The final sterilization procedure for bud explants began with a PPM™ wash for two hours, followed with a sterile-water rinse then transfer to a hormone-free medium (Appendix II) to preserve the moisture. The plate was sealed and kept at room temperature overnight. The next day, the explants were washed with 70% ethanol for 30 seconds then 20% bleach for 25 minutes and rinsed three times using autoclaved distilled water. At this time, all the scales were removed, and then the explants were re-sterilized with 10% bleach solution for 1 minute, then three rinsing steps using autoclaved distilled water, before being plated onto the appropriate medium.

Several days after first plating, the area around each bud explant darkened; thus the explants were transferred to fresh media to minimize the effect of the dark exudates. Initially all bud explants were first placed on induction medium (Appendix II) for four weeks; however, they failed to generate the expected results, consequently they were transferred to the multiplication medium (Appendix II). Subsequently buds were put on multiplication medium from the first plating, skipping the induction medium. The bases of the explants swelled and began to produce callus after approximately two to three weeks into the culture period. The basal callus slowly hardened and accumulated chlorophyll. After approximately 16 weeks, one of the rejuvenating buds was noticed to have developed shoots from

three separate points (Figure 5.1). This, however, was not observed in the other explants, in the following four weeks. The explant with the three shoots was dissected into three separate explants and these were transferred to the elongation medium (Appendix II). After four weeks, although shoot elongation was not observed, the culture was placed on the rooting medium (Appendix II) to induce root formation, which would complete the regeneration process. After a period of six weeks root formation was still not observed; while the explants started to wilt and their shoots shrivelled. This experiment demonstrated the feasibility of producing shoots from buds of sugar maple. However, the response was extremely low, indicating sub-optimal culture conditions.

5.1.2 Embryo explants

In 2007, a pilot project was carried out using fresh *A. saccharum* seeds collected from various trees in the city. From each seed, two types of explants were used; cotyledon and an embryo axis segments. At about two weeks into the culture period, both types of the explants began to expand and produce callus. Callus production generally started from the wounded edge of an explant. In addition, they slowly lost their chlorophyll and turned yellowish white since they were kept in dark. Although both explant types produced callus, the embryo axis seemed to respond better to the treatment for shoot production. The first medium used was the induction medium, which contained 1 µM of BA and 0.1 µM of TDZ (Appendix III). At about week 6, one of the embryo axis explants produced multiple shoots (Figure 5.2). This explant was collected from a tree on Talon Bay, Winnipeg; the same source of the bud explant that had the multiple shoots.

In 2008, the study was expanded to include more genotypes, using the seeds from the NTSC. Note that these seeds were mature dormant seeds collected from various locations in eastern Canada between 2002 and 2006. Similarly, the study utilized the same two types of explants from each seed and plated them on induction medium containing 225.25 µg/L of BA and 22.03 µg/L of TDZ (Appendix III).

After four weeks, the explants swelled and expanded. Callus production was evident; however, shoot development had not been observed. The treatments had failed to stimulate shoot proliferation in these explants after 28 weeks in culture. Seed production of the tree on Talon Bay, Winnipeg was absent in the fall of 2008; as a consequence, efforts to replicate the 2007 result were unsuccessful. Cultures of fresh immature embryos from the other locations also failed to proliferate.

5.1.3 Shoot tips and petioles

Sterilization procedures for shoot tips and petioles also needed optimization due to contamination problems. The structure of a shoot tip, particularly the area around the axillary buds, made sterilization of this type of explants rather difficult. The small space between the petiole and the main stem, occupied by a lateral bud, is a perfect hiding place for microorganisms. Initially the shoot tips were surface sterilized using 70% ethanol for 30 seconds followed with 0.6% sodium hypochlorite for 15 minutes. After the first trial batch, the concentration of sodium hypochlorite was doubled to 1.2% and duration was increased to 20 minutes. For petioles, additional sterilization also included a 4-hour wash with 4% PPM (Plant Preservation Mixture™) solution. Shoot tip explants were cultured in half-strength Murashige-Skoog medium with 2.5 µM of benzylaminopurine (BAP); while the petiole explants were placed on half-strength Murashige-Skoog medium with 0.04, 0.1 or 0.2 µM of thidiazuron (TDZ; Appendix III).

The shoot tip explants began to swell and increase in size about two weeks after plating. After four weeks in culture, some nodal and shoot tip explants had small amount of callus around the cut ends, and the buds started to break and shoot growth was observed. The total number of shoot segments plated was 212. More than half were contaminated and those that survived failed to produce adventitious shoots. In the petiole cultures, callus production was rare and conserved to edges of petioles. Some explants turned brown or became necrotic after seven weeks in culture without any sign

of shoot formation. Some remained green but failed to produce shoots after ten weeks. There were a total of 805 petiole segments, which were divided evenly into the three levels of TDZ treatment. The overall survival rate was about 75%. Moreover, similar to the observation during the initial period of bud cultures, the area around some of the petiole segments became slightly dark, particularly around explants that were failing. However, this was observed at approximately four weeks into in the culture period. The remaining living explants were subcultured onto new media. Furthermore, the amount of callus produced by petiole explants in the various levels of TDZ was not noticeably different and shoot formation was absent in all of the petiole cultures at the end of week 12. Slowly the petioles started to lose their chlorophyll and finally died.

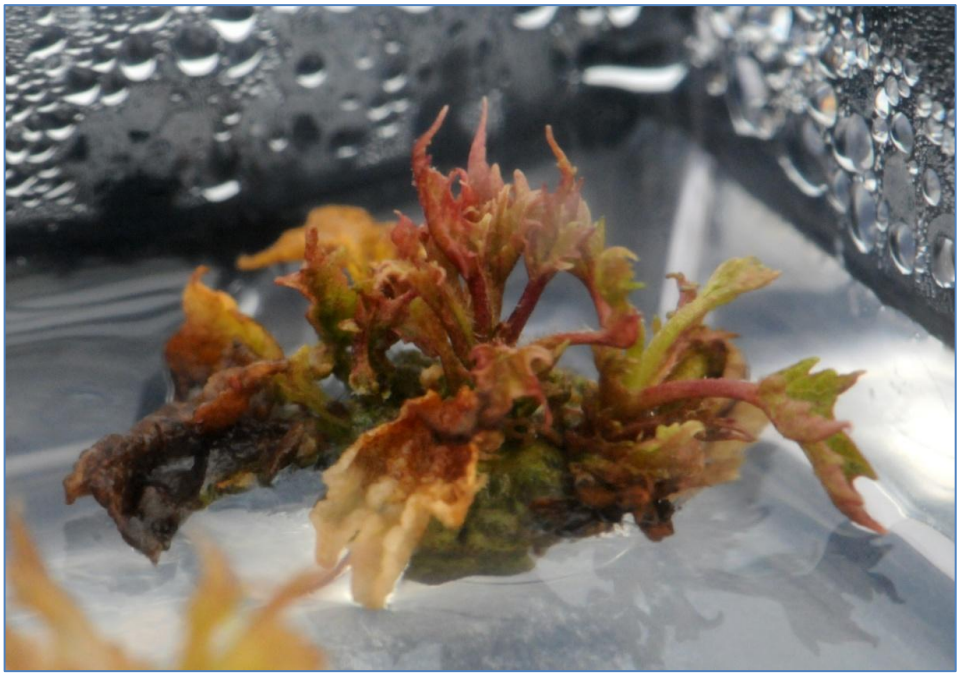


Figure 5.1 Multiple shoots growing from a single *Acer saccharum* shoot bud explant, derived from a rejuvenating bud collected in May 2008 from a mature tree (Talon Bay, Winnipeg).

Figure 5.2 Sugar maple (*Acer saccharum*) embryo axis on induction medium for 45 days showing the development of multiple shoots; explant was harvested off of a fresh seed collected from a residential tree in October of 2007.

5.2 Discussion

When culturing bud explants, the media around the bud darkened several days after the initial plating. The explants were transferred to fresh media to avoid any detrimental effect caused by the substance. Similar observation was also reported in other studies using bud explants of other species (Durkovic 2003; Biroscikova et al. 2004; Bowen-O'Connor 2007), where explants were transferred to new media and after approximately 10 days phenol-free cultures were obtained. Cultures of petiole segments also exhibited darkening of medium around some of the explants. On this case, however, the explants were not transferred to new media immediately since it was observed that the dark exudates were produced by petioles that were dying. It was unclear if the exudates observed in bud cultures were the same as those produced by the petiole segments.

The current study showed that the type of initial explant contributed greatly in the success of shoot induction and proliferation. The use of petiole segments as explant in this study proved to be the most inefficient. After two weeks in culture, the petiole segments swelled and some started forming callus. Callus production was limited to area around the wounded edges and the amount was also very low. At the end of the 12-week period, no shoot was induced from the petiole cultures. Similar observation was also reported by Durkovic (2003) on petiole segments of *Acer caudatifolium* Hayata or kawakami maple that were cultured on Woody Plant Medium (WPM) containing 1.0 mg/L BAP plus 0.05 mg/L NAA. Nevertheless, shoot multiplication was observed from basal callus of juvenile plumule and hypocotyls segment of sycamore maple or *Acer pseudoplantanus* when these explants were treated with 0.1 µM TDZ and 1.0 µM BAP (Wilhelm 1999). Many studies also have successfully produce multiple shoots from petiole cultures, and Durkovic et al. (2005) reported that petiole cultures of Formosan sweetgum (*Liquidambar formosana* Hance) required a longer time to achieve the typical multiple shoot culture compared to axillary bud cultures. The different responses may be due to genotypic difference,

the age of the donor plant, as well as culture medium composition including the concentrations of the growth regulators.

Another explant type used in the study was shoot tips or nodal segments. After several weeks in culture, bud break was observed and it was followed with shoot growth. The growing shoot however did not elongate. Also, there was no adventitious shoot formation by the callus produced around the wounded edges of the explants; thus shoot multiplication was not achieved after ten weeks in culture. In this study, the contamination rate was particularly high among the shoot tip explants and the stronger sterilization procedure only reduced the contamination rate to 25%. Another study by Vengadesan and Pijut (2009) used nodal segments of *Quercus rubra* L. as initial explants for shoot induction and harvested shoot components of in vitro-grown seedlings; thus avoided the high contamination problems. In addition, Mansouri and Preece (2009) collected nodal and shoot tip explants by inducing growth of softwood shoots from large stem segments of *Acer saccharinum* L. The practice of inducing shoot growth from a stem segment is also known as shoot forcing. In the study, the forcing was facilitated by treating the stem segments with 20% latex paint containing BA and/or gibberellic acid (GA_3) at concentrations ranging between 0.3 and 30 mM. The highest concentration, 30 nM, also converts to 6.8 g/L of BA and 10.4 g/L of GA_3. Shoot forcing allows for continued growth of softwood shoots beyond the regular growing season in the field or greenhouse. The study concluded that stem forcing treatment with BA showed the most potential to enhance in vitro response of softwood shoot explants; besides, later in the season shoot forcing may be enhanced by the addition of GA_3 to the existing BA treatment.

From the bud culture, only one of approximately 100 surviving buds formed multiple shoots. Shoot multiplication was observed from a reactivating bud explant. The dormant bud cultures had very low survival rate, partly due to contamination problem, and those that survived failed to form multiple

shoots. Many of the dormant bud explants became necrotic before reaching the 4-week mark. Wann and Gates (1993) successfully induced shoot proliferation in cultures of red maple (*Acer rubrum*) buds from dormant and greenwood cuttings. Other species such as Norway maple (*Acer platanoides*; Linden and Riikonen 2006) and wych elm (*Ulmus glabra*; Biroscikova et al. 2004) had been micropropagated using dormant buds as explants. The low rate of shoot multiplication may be contributed by several factors, including the age and genotype of the donor tree, the culture media composition and growth conditions, as well as the developmental stage of the explants when collected.

In this study, the treatments applied to the dormant mature embryo segments, axis and cotyledons, failed to induce shoot proliferation. Both types of explants increased in size and produced callus while in culture; yet no adventitious shoot was detected after 16 weeks. The basal callus hardened after two subcultures and only small amount of callus continued to be produced. Some explants looked slightly translucent and this may be a side effect of TDZ, as described by Huetteman and Preece (1997). The developmental stage of the explant source or the physiological state of the embryo may have played an important role in the induction of shoot proliferation from explants of zygotic embryo origin. The preliminary study successfully produced multiple shoots from callus cells of an embryo axis explant derived from an immature seed. These shoots, however, did not elongate after they were transferred to the shooting and maintenance medium which had a lower TDZ concentration and no growth regulator, respectively (Appendix III). When they were transferred to rooting medium, basal callus turned green and no root formation was detected after four weeks. Successful shoot proliferation from embryo segments was achieved in *Acer pseudoplantanus* by Wilhelm (1999) using multiplication medium composing of MS medium supplemented with 1 µM of BA and 0.04 µM of TDZ, and their rooting was increased by short pulses of auxin treatment followed with transfer to hormone-free medium with 1% activated charcoal. The use of activated charcoal in the rooting medium, after a short pulse of auxin may be implemented for future studies with *A. saccharum* microshoots.

The importance of selecting the appropriate explant types was demonstrated by Durkovic (1996) in a study comparing the culture responses of Norway maple (*Acer platanoides* L.) axillary buds, petiole segments, greenwood cuttings and leaf discs. Although callus was produced in greenwood cutting and petiole segment cultures, no shoot formation was observed. Only axillary buds achieved complete regeneration process including acclimatization of the regenerates to ex vitro conditions. Another study on Norway maple found that although both apical and axillary buds proved to be suitable explants for micropropagation, axillary buds showed slight advantage over apical buds from lower levels of TDZ (Linden and Riikonen 2006). Huetteman and Preece (1993) mentioned that seed explants are frequently used for in vitro propagation of various tree species because of their relatively high regeneration success rates. Wilhelm (1999) collected segments of plumule, hypocotyl and radicle from zygotic embryos of *Acer pseudoplantanus*. The plumule and hypocotyls achieved shoot multiplication; however, the radicle only showed root elongation and/or white callus growth. These differences in responses among the various explant types may partly due to the diversity in tissue organization within each explant. For example, a bud explant contains a shoot apex ready for growth, whereas the other explants, such as the embryo axis segment in the current study, the apical meristem was removed prior to plating, and thus new meristems need to be formed to generate adventitious shoots.

The use of TDZ for shoot induction of explants from various tree species, including other maple species has been widely documented with varying success (Preece et al. 1991; Huetteman and Preece 1993; Wann and Gates 1993; Wilhelm 1999; Linden and Riikonen 2006; Bowen-O'Connor et al. 2007; Mansouri and Preece 2009). In this study, elongation of the induced shoots was absent even after the explant was transferred to media with a different type of cytokinin compound at a lower concentration (Appendix II) in the case of bud cultures, and to a hormone-free medium (Appendix III) for the embryo axis cultures. Bowen-O'Connor et al. (2007) reported the expansion of *A. Grandidentatum* bud explants without shoot elongation when the explants were placed on DKW medium containing 0.001-0.1 μM TDZ

and suggested that TDZ although induced cell proliferation, did not promote cell elongation. The same conclusion on TDZ activity was made by Vengadesan and Pijut (2009) when the cotyledonary nodal cultures of northern red oak (*Quercus rubra* L.) treated with TDZ along with BA produced high number of shoot buds which failed to elongate. An elaborate review on TDZ outlined some of the concerns associated with the hormone which also include shoot fasciation, vitrification or hyperhydricity and inhibition of shoot elongation (Huetteman and Preece 1993). These effects were thought to result from the high cytokinin activity possessed by the compound, as well as its chemical structure that include phenyl groups.

A study by Bowen-O'Connor et al. (2007) tested the response of nodal segments of bigtooth maple (*Acer grandidentatum* Nutt.) when cultured on several types of tissue culture media; Murashige-Skoog (MS), Linsmaier-Skoog (LS), Driver and Kuniyuki Walnut (DKW) and Woody Plant Medium (WPM). The authors observed significant differences in the culture responses, such as rate of sprouting and number of shoots proliferated, among the different media and suggested DKW as the preferred medium for micropropagation of *A. grandidentatum*. Moreover they also recommended media other than MS for propagation of hard maples, which are known to be difficult to propagate in vitro. Hard maples include members of *Saccharina* Pax. section of the Aceraceae, such as bigtooth maple and sugar maple (Bowen-O'Connor et al. 2007). On the contrary, Brassard et al. (2003) reported successful multiplication and regeneration of sugar maple bud explants on MS media. The same response to MS nevertheless, was not observed in this study; although various factors, including genotypic differences, might have contributed to the limited response of these explants to the culture treatments.

Induction of bud explants to produce multiple shoots had been demonstrated with relatively high success rates in other maple species, such as in *A. saccharinum* (Mark and Simpson 1994), *A. caudatifolium* Hayata (Durkovic 2003), *A. rubrum* (Wann and Gates 1993), *A. platanoides* (Durkovic

1996, Linden and Riikonen 2006), and *A. saccharum* (Brassard et al. 2003). In the case of sugar maple, Brassard et al. (2003) showed that shoot organogenesis from shoot bud explant was possible although regeneration rate was still low. The age of the source tree may have contributed to the recalcitrant response as shown in the wych elm (*Ulmus glabra* Huds.) study by Biroscikova et al. (2004). The study showed that dormant buds of the older trees (over 70 years) had much less vigour in their organogenic ability compared to the progeny trees (15 years of age). As in the current study, similar recalcitrant responses were observed in the wych elm study on explants collected from the older trees, in that very few explants produced multiple shoots and their rooting was poor. In summary, the age of the donor plant may influence the explants in vitro responses and younger or more juvenile trees generally have better organogenic capacity. In addition, Cao et al. (2002) reported a significant improvement in in vitro regeneration rate of leaf explants of previously-recalcitrant highbush blueberry (*Vaccinium corymbosum* L.) cv. Bluecrop by implementing a two-step pre-treatment. It consisted of a treatment for four days in a medium with 5 µM TDZ and 2.6 µM NAA and followed with three days in another medium containing 7 µM zeatin riboside and 2.6 µM NAA. The short pulse of high-concentration cytokinins, especially TDZ, may also be beneficial for inducing shoot proliferation in *A. saccharum* explants.

To summarize, regeneration of *Acer saccharum* via shoot organogenesis is possible as demonstrated in the current experiment with bud and embryo explants, as well as by Brassard et al. (2003). However, the treatments used for shoot tip and petiole explants in this study failed to stimulate shoot proliferation. Several factors recommended for future experiments on sugar maple in vitro propagation include the use of juvenile trees as donor plants, short pulses of hormones as preconditioning treatments, selection of actively-growing or ready-for-growth explants, which can be provided via shoot forcing, as well as optimization of medium composition and growth conditions. The use of media designed for woody species, such as WPM and DKW, should also be considered. The concentrations of hormones also need some adjustments; the use of BA and TDZ is recommended.

6.0 CHAPTER 3: ROOTING OF STEM CUTTINGS

6.1 Results

6.1.1 Experimental year 2008

The first experiment in 2008 assessed the influence of several factors including cutting type, cultivar, collection timing and rooting hormone. The experimental design consisted of four parameters as follows; two cutting types, two cultivars, three collection times, and nine hormone treatments. Root formation was evaluated at the end of the rooting period, which was set to 12 weeks. The cuttings were harvested at the end of the 12th week, which fell on September 25, October 2 and 7, in the order of planting dates for the first, second and third collections. Although some still maintained green leaves, most of the cuttings had lost their leaves. Initially each cutting was tested for rooting by pulling them gently. Those that failed to root usually turned brown or black and became dry, these dead cuttings were discarded. Of the rooted cuttings, some had green leaves, red leaves, and some had brown leaves (Figure 6.1). After removing the dead, non-rooted cuttings, the remaining viable cuttings were harvested by digging them out of the sand using a garden fork. This was done carefully to minimize the amount of roots lost during the extraction process. Each rooted cutting was put into an individually-sealed plastic bag to maintain moisture.

Statistical analysis found that all factors tested had significant effects ($p \leq 0.05$) on rooting of stem cuttings of *A. saccharum.* In addition, a significant interaction effect on rooting was observed between cultivar and collection time. To show the interaction effect, rooting percentages across collections are presented for each cultivar. Of all the factors, only collection time showed considerable influence on the quality of roots produced on the rooted cuttings. Root quality was measured by several root parameters, namely number of primary roots, length of longest root, fresh weight and dry weight of roots. Roots were carefully rinsed to remove excess sand prior to weighing (Figure 6.2). Although only

collection time significantly affected root quality as an independent factor, some interactions were found to have significant effects on some of the root parameters.

6.1.1.1 Effect of cutting types

Root formation was significantly affected by the type of cutting, based on the presence or absence of an apical meristem (p=0.0002). Cuttings without an apical meristem or medial-type cuttings (28.6% of 476 cuttings) had significantly higher chance of forming roots than those of terminal-type (17.4% of 833 cuttings; Figure 6.3). Some cuttings did not survive the entire rooting period and had dried and turned brown or black when the rooting assessment was performed. These cuttings were mostly of the terminal type that had small young leaves. The differences between the two cutting types in root quality parameters were not significant (p>0.05; Table 6.1)

6.1.1.2 Effect of genotypic variation between cultivars

Likewise, cuttings collected from different sugar maple cultivars also displayed considerable variation in rooting frequencies (p=0.0056; Figure 6.4).Rooting was less among ‘Jefcan’ cuttings (16.3%) compared to ‘Bailsta’ cuttings (24.8%). The rooting frequency of ‘Jefcan’ cuttings was calculated based on a total of 514 cuttings, while there were a total of 795 cuttings of ‘Bailsta’. Genotypic differences between ‘Bailsta’ and ‘Jefcan’ did not induce significant differences in root quality parameters. The mean values for primary root count, root length, and fresh and dry root weights were not significantly different between cultivars (p>0.05; Table 6.1).

Figure 6.1 Sugar maple (*Acer saccharum*) cuttings in sand 12 weeks after planting. Most had lost their leaves, only some retained their green leaves. Cuttings were collected in July 2008 and treated with various hormones to induce rooting.

Figure 6.2 Rooted *Acer saccharum* 'Jefcan' cutting that had developed three primary roots and some lateral roots. The cutting still had green leaves attached after being rooted for 12 weeks in the sand bed.

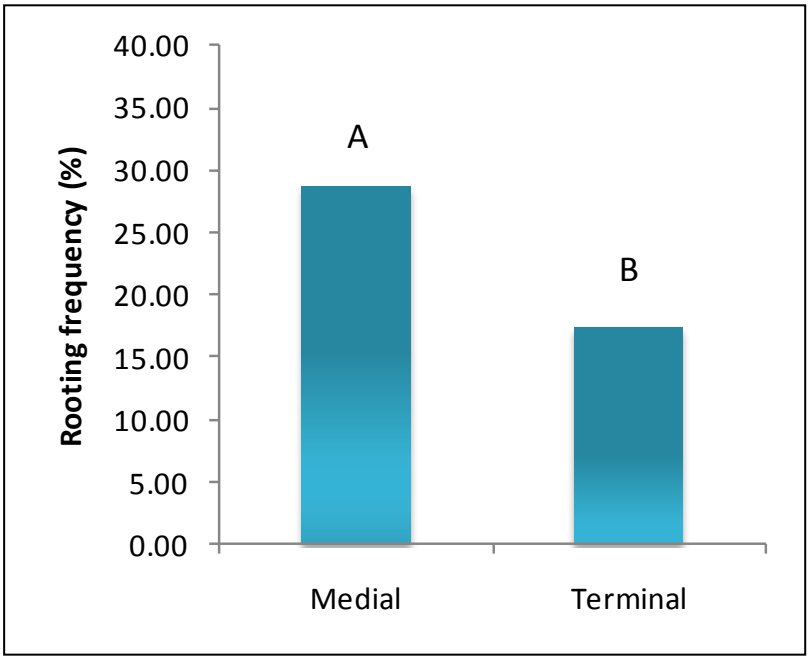


Figure 6.3 Effect of cutting type on the rooting frequencies of *Acer saccharum* 'Bailsta' and 'Jefcan' cuttings of medial and terminal types from 2008 experiment. Each bar represents the particular treatment's rooting percentage and the upper-case letters indicate that means were significantly different from each other at p≤0.05.

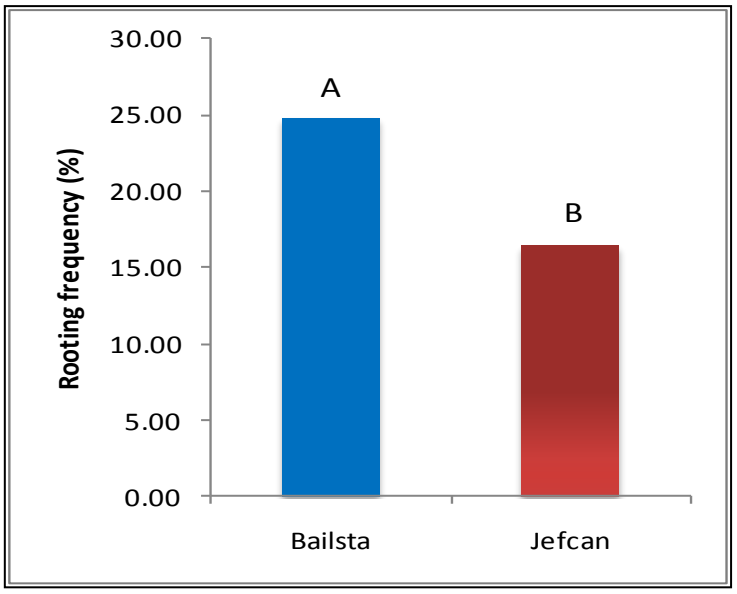


Figure 6.4 Effect of cultivar on rooting capacity of *Acer saccharum* cuttings of 'Jefcan' and 'Bailsta' cultivars. Each bar represents the particular treatment's rooting percentage and the upper-case letters indicate that means were significantly different from each other at p≤0.05.

Table 6.1 Average numbers of primary roots, lengths of longest root, fresh and dry weights of roots of *Acer saccharum* 'Jefcan' and 'Bailsta' cuttings rooted in 2008 experiment in each level of each factor (abbreviations: IBA: indole-3-butyric acid, NAA: naphthaleneacetic acid, the numbers represent the concentration of each compound in g/L)

	Number of primary roots	Length of longest root (cm)	Fresh weight of roots (mg)	Dry weight of roots (mg)
Collection (GDD)				
1 July 2 (495)	3.2 a	14.5 a	900.3 a	115.6 a
2 July 9 (578)	2.3 b	7.4 b	258.4 b	31.3 b
3 July 14 (640)	2.1 b	5.7 b	151.1 b	29.9 b
Rooting hormone				
StimRoot®#2	2.0 a	12.8 a	392.1 a	57.4 a
IBA-5	2.8 a	12.1 a	591.1 a	80.4 a
IBA-10	2.0 a	7.5 a	291.7 a	46.1 a
NAA-5	2.5 a	7.7 a	261.9 a	34.4 a
NAA-10	2.1 a	6.2 a	250.3 a	28.5 a
IBA-5/NAA-5	3.0 a	11.0 a	532.4 a	66.3 a
IBA-5/NAA-10	2.5 a	7.6 a	350.8 a	41.5 a
IBA-10/NAA-5	2.9 a	8.4 a	490.8 a	74.0 a
IBA-10/NAA-10	2.7 a	9.6 a	332.9 a	59.2 a
Cultivar				
Bailsta	2.6 a	9.8 a	468.4 a	60.4 a
Jefcan	2.4 a	8.6 a	300.6 a	45.8 a
Cutting type				
Medial	2.6 a	9.3 a	462.4 a	63.2 a
Terminal	2.3 a	9.1 a	305.6 a	43.4 a

6.1.1.3 Effect of rooting hormone application

In the 2008 experiment, there were nine rooting hormone treatments. The rooting success ranged from 13.5 to 32.9% and hormone effect was statistically significant ($p<0.0001$; Figure 6.5). The treatments with IBA at 5 g/L (IBA-5) and combination of IBA and NAA at 5 g/L (IBA-5/NAA-5) gave the highest rooting frequencies (31.7-32.9%; Figure 6.5). The rooting percentages of the commercial rooting hormone, StimRoot®#2, and the formulation of NAA at 10 g/L (NAA-10) and a combination treatment of IBA at 10 g/L and NAA at 10 g/L (IBA-10/NAA-10) were among the lowest. The poor performance of StimRoot®#2 was not expected since its auxin composition, equivalent to 4 g/L of IBA, is very similar to IBA 5 g/L. Moreover, all other treatments were the intermediates, being not significantly different from both groups; the highest- and the lowest-rooting treatment groups. In addition, among all nine hormone treatments, the average numbers of primary roots formed per rooted cutting were not significantly affected by the composition and concentration of hormone (Table 6.1). Across all treatments, on average two to three primary roots were found on each rooted cutting, irrespective of hormone types and levels.

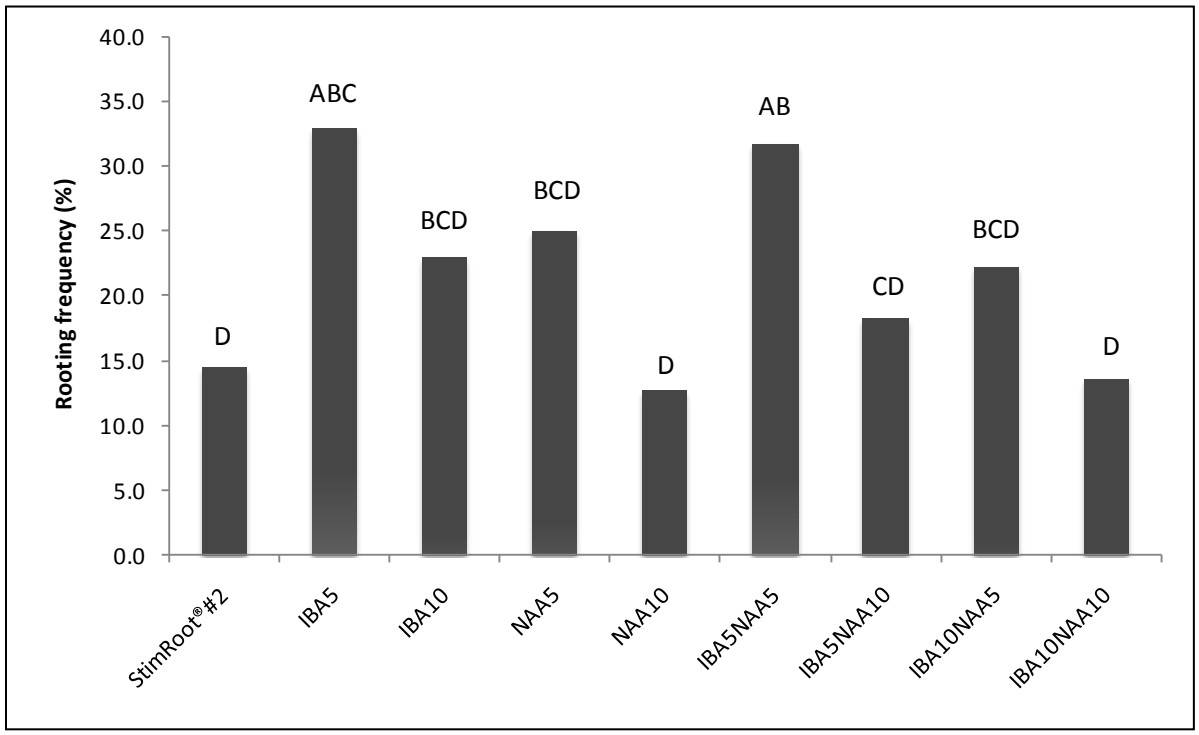


Figure 6.5 Rooting frequencies of *Acer saccharum* cuttings collected in July 2008 and treated with eight rooting hormone formulations and one commercial rooting hormone, StimRoot®#2. Each bar represents the particular treatment's rooting percentage and the same upper-case letters indicate means not significantly different from each other at $p \leq 0.05$.

6.1.1.4 Effects of collection timing

Since the interaction between collection time and cultivar effects was found to be significant ($p=0.0124$), the effect of collection time is reported separately for each cultivar (Figure 6.6). The highest rooting frequency was observed among 'Bailsta' cuttings from the first collection and lowest among 'Jefcan' cuttings from the second collection. The rooting percentage of 'Bailsta' cuttings, as shown in the graph (Figure 6.6), decreased gradually over the collection period, from 30.1% to 20.9% then to 15.3%. Among 'Jefcan' cuttings, a different trend was observed. Rooting frequency of 'Jefcan' cuttings decreased between the first and second collections (18.8 to 8.1%); and was restored with a slight improvement in the third collection (19.4%). Moreover, the large reduction in rooting frequency among 'Jefcan' cuttings between the first and the second collections was found to be statistically insignificant ($p>0.05$).

In the beginning of the section it was mentioned that only collection time significantly affected the quality of roots produced by rooted cuttings as an individual factor ($p<0.0001$; Table 6.1). In all root parameters, the collection time factor showed significant effects, and the same trend was observed throughout, where the first collection produced the largest root system based on number of primary roots, length of longest roots, as well as fresh and dry weights of the whole root system (Table 6.1). Moreover, the differences between the second and third collections were statistically insignificant ($p>0.05$) in all of the root quality parameters.

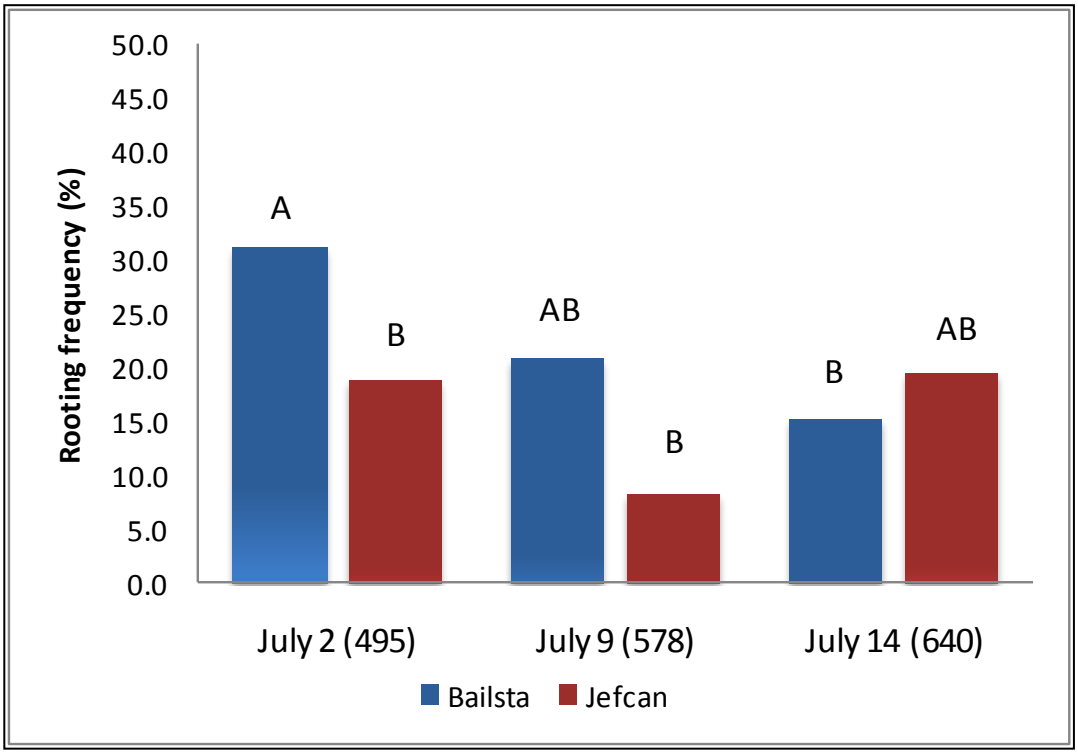


Figure 6.6 Rooting frequencies of *Acer saccharum* cuttings from cultivars 'Jefcan' and 'Bailsta', across three collection times (described by date of collection; while the values in brackets represent the accumulated growing degree days – GDDs) in the 2008 experiment. Each bar represents the particular treatment's rooting percentage and the same upper-case letters indicate means not significantly different from each other at $p \leq 0.05$.

6.1.1.5 Interaction effects on the quality of roots formed by the cuttings

The interactions between hormone and cultivar, as well as between collection time and cutting type, were shown to be significant on the mean number of primary roots.The significant interactions between collection time and cutting type on number of primary roots ($p=0.0067$) and on fresh weight of roots ($p=0.0439$) are presented in Figures 6.7 and 6.8. In both parameters, medial cutting type displayed superiority over the terminal cutting type in the first and third collections. On the other hand, the opposite was observed in the second collection, where terminal cutting type had higher average values than the medial cutting type in terms of number of primary roots as well as fresh weight of roots. Nonetheless, the differences in root parameters between the two cutting types in the second and third collections were statistically insignificant ($p>0.05$) and only in the first collection did the two cutting types show significant differences in the quality of roots they produced. The number of primary roots produced by rooted medial cuttings from the third collection was lower than that from the first collections, yet they were not significantly different from each other (Figure 6.7).

In addition, the interaction effect between rooting hormone and cultivar was also reported to be significant on the mean number of primary roots ($p=0.0087$; Figure 6.9). Overall, the cultivar 'Jefcan' responded well to the treatment with IBA at 5 g/L; whereas 'Bailsta' rooted cuttings were more likely to produce higher numbers of primary roots when treated with NAA, regardless of the concentration (Figure 6.9). Moreover, among the combination treatments, the number of primary roots was less influenced by the genotypes.

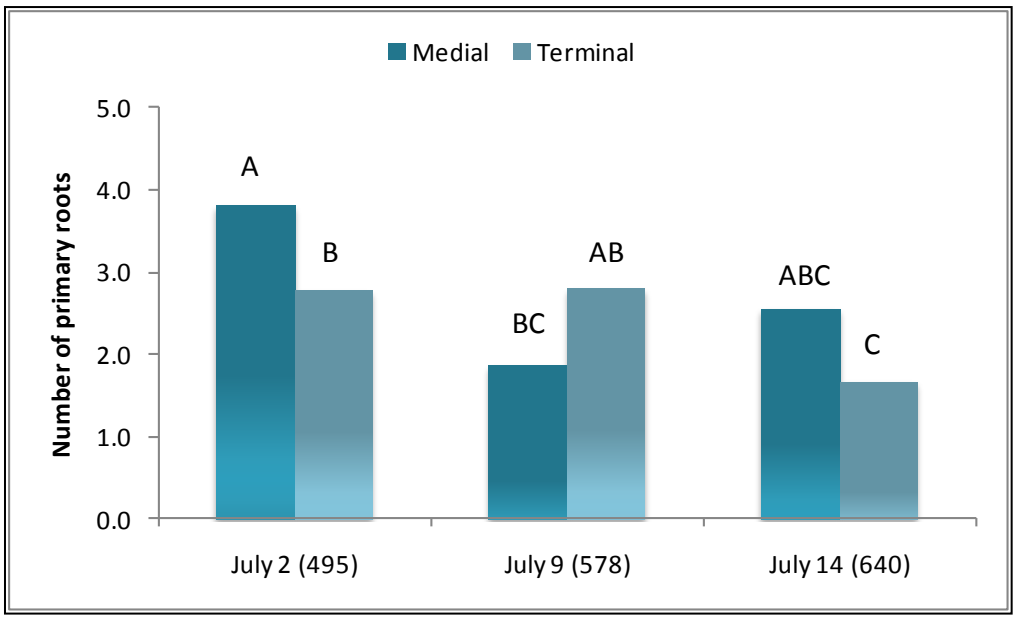


Figure 6.7 The average number of the primary roots of rooted cuttings of terminal and medial types over the three collections in 2008 experiment with *Acer saccharum* 'Bailsta' and 'Jefcan' cuttings. (Values in brackets represent the accumulated growing degree days – GDDs). Each bar represents the particular treatment's average value and the same upper-case letters indicate that values are not significantly different from each other at p≤0.05.

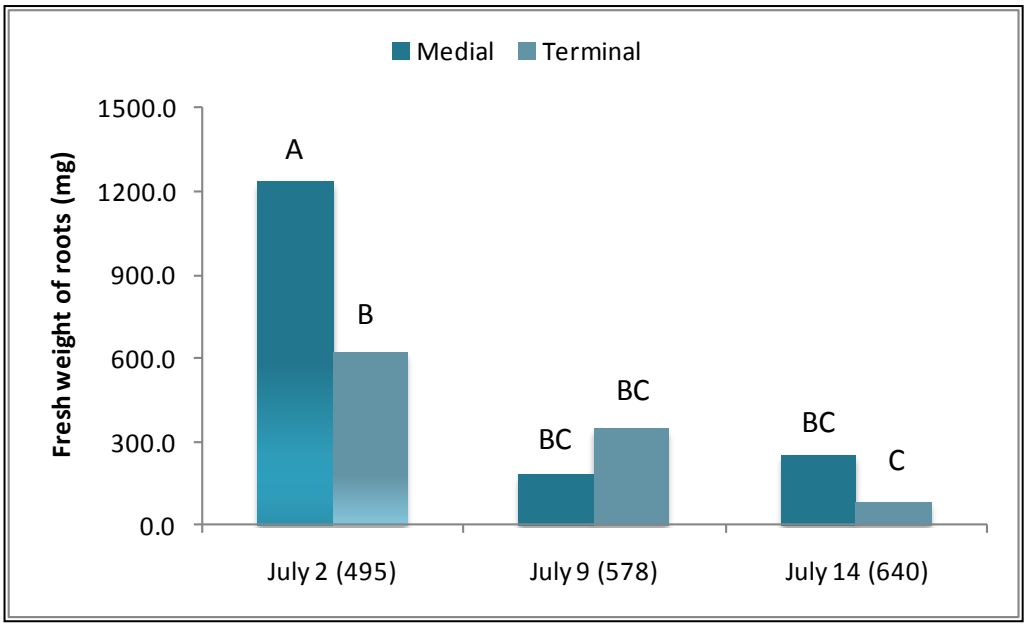


Figure 6.8 The fresh weight of roots of rooted cuttings of terminal and medial types over the three collections in the 2008 experiment with *Acer saccharum* 'Bailsta' and 'Jefcan' cuttings. (Values in brackets represent the accumulated growing degree days – GDDs). Each bar represents the particular treatment's average value and the same upper-case letters indicate that values are not significantly different from each other at p≤0.05.

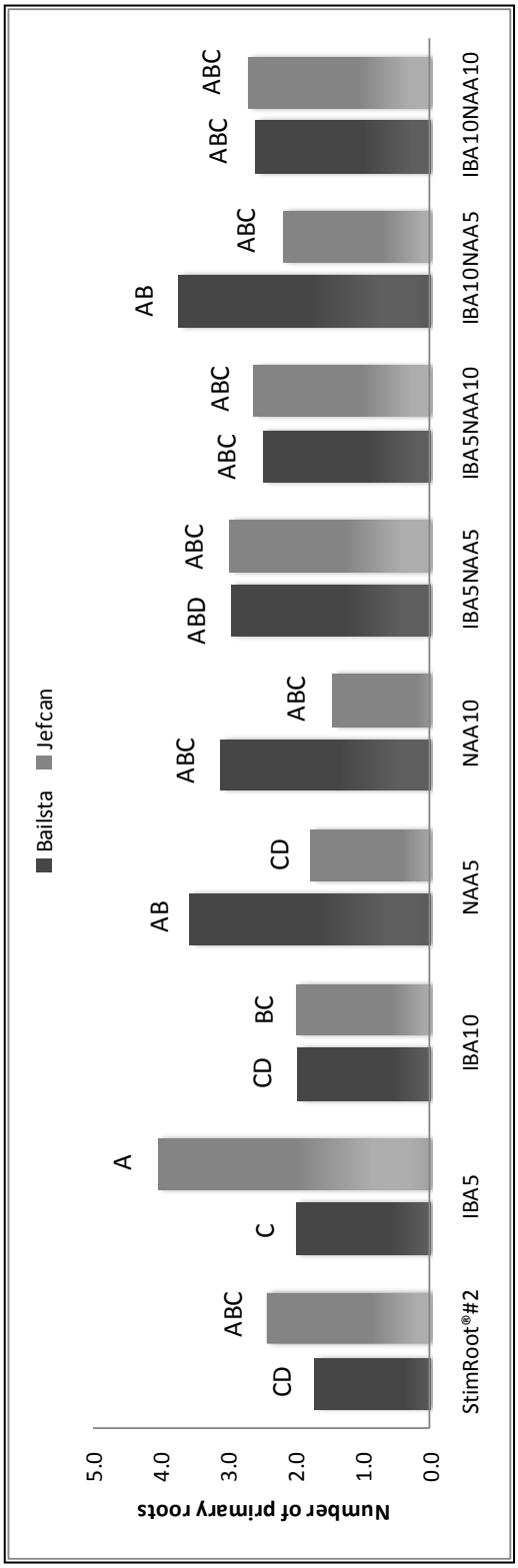


Figure 6.9 The average number of primary roots of *Acer saccharum* 'Jefcan' and 'Bailsta' rooted cuttings when treated with various rooting hormones (abbreviations: IBA: indole-3-butyric acid, NAA: naphthaleneacetic acid, the numbers represent the concentration of each compound in g/L). Each bar represents the particular treatment's average value and the same upper-case letters indicate that values are not significantly different from each other at p≤0.05.

6.1.2 Experimental year 2009

In 2009, in order to test for several parameters, the study comprised a number of experiments that were interconnected. Due to extremely rare rooting incidences among the cuttings of the first collection time, the results were not included for statistical analysis. To examine the collection time effect, the rooting results from cuttings of field-grown 'Jefcan' trees from the second, third and fourth collections that were planted at Glenlea site were used; the same set of data was used to test the effects of rooting hormone application. The data consisted of rooting responses from 525 cuttings. Aside from the main rooting hormone treatments, the effect of acidic pH on rooting, by treating cuttings with distilled water at pH 4.0, which was similar to the pH of the IBA-5 solution, was also investigated. The data used for this test was those of field-grown 'Jefcan' cuttings from the second and third collections that were in control (distilled water at neutral pH) and pH treatments, bringing a total of 130 cuttings. The data from the cuttings planted at the two sites were used to assess the influence of rooting conditions. The third site, the university greenhouse, was not included in statistical analysis. The results were pooled from four hormone treatments; control IBA-5, IBA-10/NAA-5 and StimRoot® #2, of the third collection; hence the effect was tested based on results of 210 cuttings. Moreover, the experiment studied the influence of age and growing environments by comparing rooting results of cuttings collected from field-grown to container-grown 'Jefcan' trees. The container trees were approximately two years younger than the field trees and they were protected from winter damage by flax and poly covering from November to April. The test compared the results of 140 cuttings. Additionally, the 2009 experiment also assessed the effects of genotypic variation among *A. saccharum* cultivars. Three cultivars that were examined in the study included 'Jefcan', 'Bailsta' and 'Green Mountain'. These cuttings were collected from container-grown trees and there were a total of 180 cuttings, which were equally divided among control and IBA-5 treatments.

6.1.2.1 The effect of timing of cutting collection

As previously mentioned, rooting of cuttings from the first collection time (June 17) was extremely low. These cuttings were planted in 1:1 peat and perlite mix and kept in a misting box in a greenhouse. During the rooting period, many cuttings had fungal contamination. Most of their leaves turned brown and fell off after several weeks. Due to the nature of the misting box, the temperature and humidity levels inside were often observed to be quite high on hot sunny days. At the end of the 12^{th} week, none of the cuttings had green leaves attached. Of the total 200 cuttings, only three cuttings formed roots, two cuttings were from IBA-5 treatment and one from the control treatment (distilled water). Several cuttings had formed callus at the cutting base but did not develop any root. The number of roots formed on the rooted cuttings was also very low, where only one cutting from the IBA-5 treatment had two primary roots.

Similar to the observations recorded from the 2008 experiment, at the end of the rooting period some cuttings had died and turned brown or black while others had formed roots and some still retained their leaves. At the Glenlea site the cuttings were removed from the sand beds by digging around them using a garden fork. The cuttings from the second, third and fourth collections that were planted in sand beds at the Glenlea greenhouse rooted considerably better compared to those from the first collection. Individually, rooting of the second, third and fourth collections were 32.5%, 25.6% and 40.5% respectively (Figure 6.10). Combining all the collections, root formation occurred on 33.9% of the cuttings. Furthermore, analysis using Proc Genmod in SAS® revealed that the timing of cutting collection contributed substantial influence on the rooting frequency ($p=0.0184$; Figure 6.10). Rooting frequency decreased between the second and third collections, although the difference was found to be not significant ($p>0.05$). Rooting frequency increased drastically for cuttings from the fourth collection time (40.5%), and that became the highest among the four collections. In addition, collection time considerably affected the number of primary roots formed by the rooted cuttings within the 12-week

rooting period ($p<0.0001$). The average number of primary roots formed by cuttings from the fourth collection time was the highest at 6.3 roots per cutting, which was significantly different from that of collection two (3.7) and collection three (2.7; Figure 6.11).

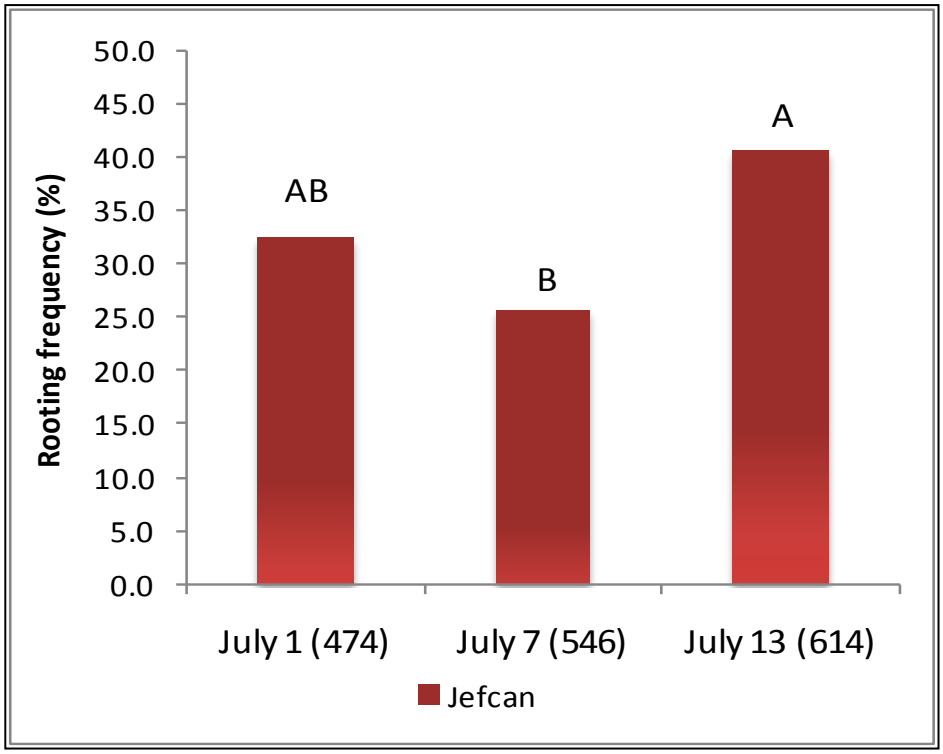


Figure 6.10 Effect of collection time on the number of *Acer saccharum* 'Jefcan' cuttings that formed roots. (Values in brackets represent the accumulated growing degree days – GDDs). Each bar represents the particular treatment's rooting percentage and the same upper-case letters indicate that values are not significantly different from each other at $p \leq 0.05$.

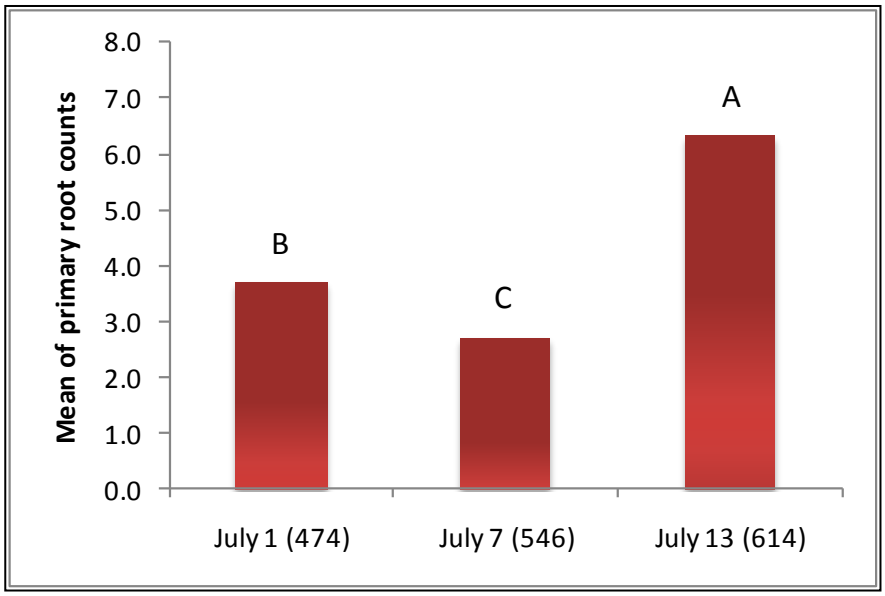


Figure 6.11 Effects of collection time on the number of primary roots formed by rooted *Acer saccharum* 'Jefcan' cuttings over a period of 12 weeks. (Values in brackets represent the accumulated growing degree days – GDDs). Each bar represents the particular treatment's average value and the same upper-case letters indicate that values are not significantly different from each other at p≤0.05.

6.1.2.2 The effect of rooting hormone application

6.1.2.2.1 Results from the cuttings taken from the field-grown trees

Rooting percentages varied among the six treatments applied to the field-grown 'Jefcan' cuttings planted in the Glenlea sand bed. The highest rooting frequency was detected among cuttings in the control treatment (47.6%; Figure 6.12). The treatments with IBA at 5 g/L and StimRoot®#2 yielded lower percentages of rooted cuttings although statistically, these rooting frequencies along with that of control were not significantly different from each other (Figure 6.12). The cuttings in the Dyna Gro Root Gel® treatment rooted at 30.5%, which also belonged to the same mean group. The lowest rooting frequency at 13.3% was observed in treatment with IBA 10 g/L and NAA 5 g/L and was significantly different from all the other treatments. With regards to mean number of primary roots, the differences among the treatments were statistically insignificant (p=0.1991).

In addition to the main hormone treatment, a side study looked into the effect of pH on rooting. Distilled water with pH adjusted to 4.0 was used to treat cuttings prior to planting. Root formation and the number of primary roots formed by the rooted cuttings were compared to those of neutral pH control. Table 6.2 shows significant differences on rooting between pH treatment and the control treatment. Cuttings treated with acidic water were less likely to form roots than those treated with neutral distilled water. Moreover, the average number of primary roots formed by the cuttings treated with acidic pH water was not significantly different from those produced by rooted cuttings of neutral control treatment (Table 6.2).

6.1.2.2.2 Results from the cuttings taken from the container-grown trees

Cuttings collected from container-grown trees were divided into two hormone treatments; IBA at 5 g/L and distilled water as control. The results are presented in Table 6.3. When treated with IBA at 5 g/L, 51 of 90 cuttings formed roots; whereas from the control, 47 of 90 cuttings rooted. The difference in rooting was not significant ($p>0.05$). Nonetheless, the mean number of primary roots formed by the rooted cuttings was considerably affected by the application of auxin. Rooted cuttings in the IBA-5 treatment had a significantly higher mean number of primary roots compared to those in control (Table 6.3).

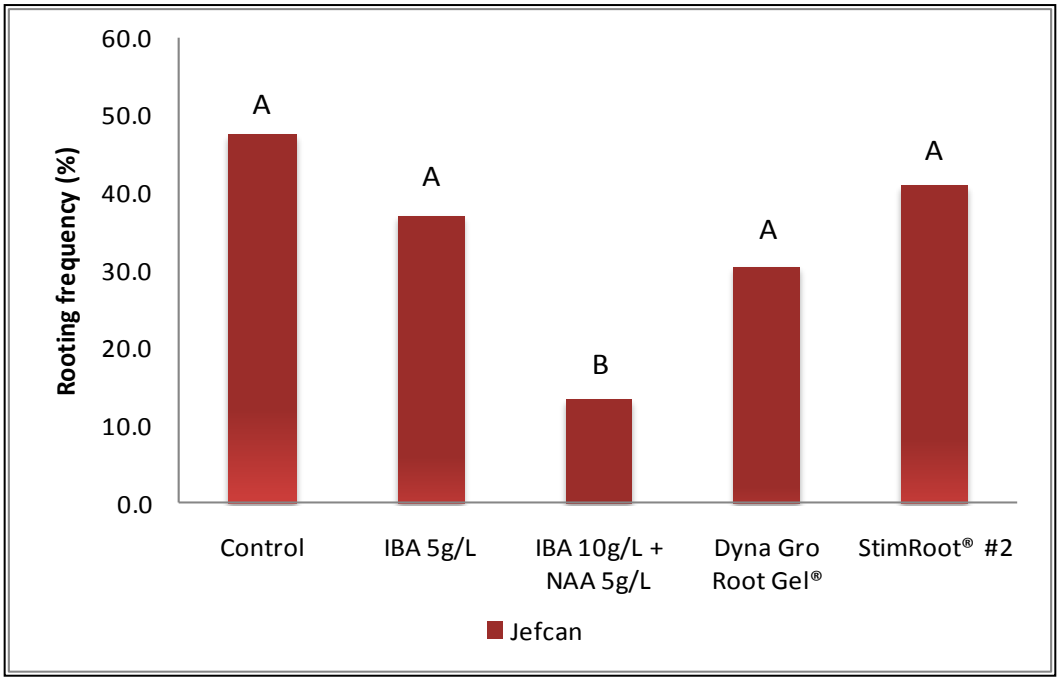


Figure 6.12 Frequency of rooting of Acer saccharum 'Jefcan' cuttings treated with various rooting hormones (control: distilled water, IBA: Indole-3-butyric acid, NAA: naphthaleneacetic acid; Dyna Gro Root Gel® and Stim-Root®No.2 are commercial rooting hormones). Each bar represents the particular treatment's rooting percentage and the same upper-case letters indicate that values are not significantly different from each other at p≤0.05.

Table 6.2 The effect of acidic pH (4.0), as compared to neutral water (control), on rooting of *Acer saccharum* 'Jefcan' cuttings and the average number of primary roots per rooted cutting.

Treatment	Total number of cuttings	Number of rooted cuttings	Rooting (%)	Number of primary root
Control	65	31	47.69 a	3.5 a
pH 4.0	65	21	32.31 b	2.4 a

Values of number of primary roots are the mean (±SE) of *n* number of rooted cuttings. Values represented by same lower case letters are not significantly different at p ≤ 0.05.

Table 6.3 Rooting results of *Acer saccharum* cuttings from container-grown trees of cultivars 'Bailsta', 'Green Mountain' and 'Jefcan', treated with distilled water (control) and solution of indole-3-butyric acid at 5 g/L.

Hormone	Total number of cuttings	Number of rooted cuttings	Rooting (%)	Number of primary roots
Control	90	47	52.2 a	2.2 b
IBA 5 g/L	90	51	56.7 a	7.1 a

Values of number of primary roots are the mean (±SE) of *n* number of rooted cuttings. Values represented by same lower case letters are not significantly different at p ≤ 0.05.

6.1.2.2.3 Results from the different rooting conditions

Rooting of cuttings at the Morden site were similar to the trend observed in the main hormone experiment. Nonetheless, there was a significant interaction between rooting conditions and hormone treatments on the mean number of primary roots formed by the rooted cuttings ($p=0.0041$). Figure 6.13 shows the average numbers of primary roots formed by cuttings treated with various hormones at the two sites. Significant rooting condition effect was detected in the treatment with StimRoot®#2. The other hormone treatments did not show significant difference between the two rooting conditions. The results from IBA-10/NAA-5 treatment were not included since none of the cuttings in this particular treatment at the Glenlea site formed any roots. The mean number of primary roots of rooted cuttings of IBA-10/NAA-5 treatment at Morden site was 3.9 roots per cutting and was an intermediate between those of control and IBA-5.

6.1.2.3 The effect of different rooting systems and conditions

Rooting response of cuttings from the first collection time was exceptionally low; only 3 out of 240 cuttings planted formed roots after the 12-week rooting period. Many cuttings from this collection lost their leaves early in the period, within approximately three weeks of planting. At the time of harvest, almost all cuttings had lost their leaves and most had died. Some cuttings formed callus at their basal end, but failed to develop roots. Moreover, the cuttings from the third collection time were divided into two groups and were distributed to the two sites, Glenlea and Morden, to determine the effect of the different rooting conditions. From a total of 210 cuttings, 124 were successfully induced to form roots (Table 6.4). Furthermore, 25 of 100 cuttings were rooted at the Glenlea site (25%); while 99 of 110 cuttings formed roots at the Morden site (90%). Evidently, the difference was substantial and it was statistically significant at $p\leq0.001$. Some cuttings from the Glenlea site were noted to form roots slightly above the cut end and the part of stem below the adventitious roots decayed. Since the cuttings

from the Morden site were not removed from the rooting medium, it was not possible to determine whether this observation was specific to the Glenlea site.

In addition to the rooting medium difference, the Morden greenhouse was noticeably cooler and less humid compared to the Glenlea greenhouse. The greenhouse in Morden was also positioned in a more shaded area. Figures 6.14 and 6.15 illustrate the successful rooting at the Morden site. The predominant effect continued to the number of primary roots formed per rooted cutting. The rooting conditions significantly affected the number of primary roots, where cuttings at Morden site produced a more profuse root system than those at Glenlea ($p<0.0001$; Table 6.4).

6.1.2.4 The effect of genotypic variation among the three cultivars

In general root formation occurred at 54.44%, which equalled 98 rooted cuttings out of the total 180 cuttings from all three cultivars combined. Within each cultivar, 'Jefcan', 'Bailsta', and 'Green Mountain' cuttings produced roots at the rates of 68.3, 51.7, and 43.3%, correspondingly (Figure 6.16). The difference in rooting frequency between 'Jefcan' and 'Bailsta' was not significant at $p \leq 0.05$, and similarly between 'Bailsta' and 'Green Mountain'. Rooting among 'Jefcan' cuttings, however, was significantly higher than among 'Green Mountain' cuttings. Besides, substantial differences were recorded among the mean numbers of primary roots formed by cuttings of the three cultivars (Figure 6.17). Among the three, 'Jefcan' cuttings produced the highest number of primary roots (5.7 roots per cutting) on average, compared to 'Bailsta' (3.8) and 'Green Mountain' (3.2) cuttings. Taken as a whole, cuttings from container-grown 'Jefcan' trees were more likely to form roots and generate higher number of primary roots than those from the other two cultivars, 'Bailsta' and 'Green Mountain'.

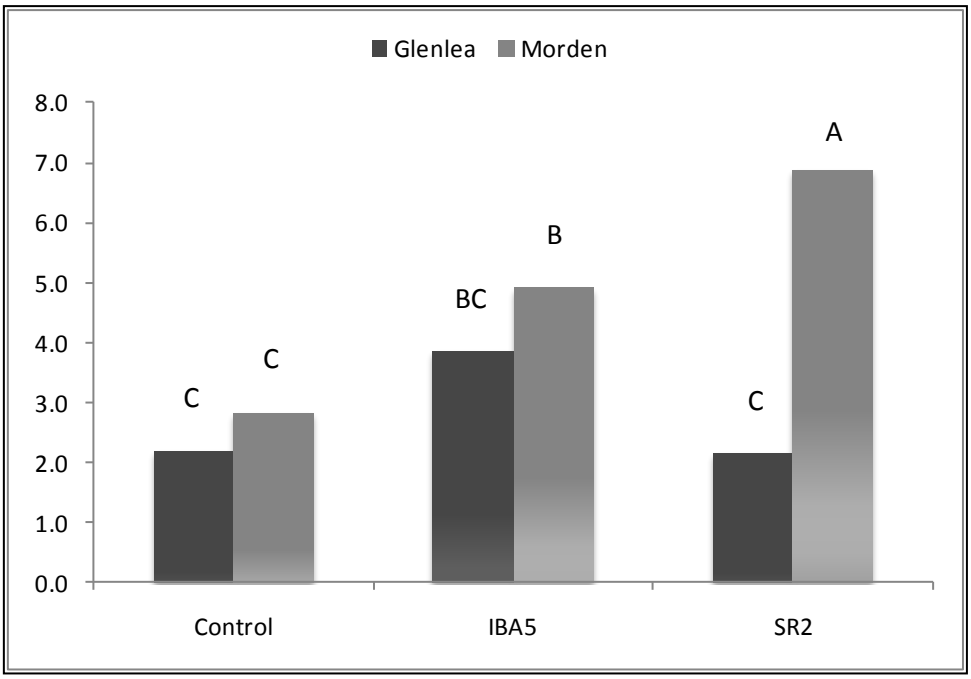


Figure 6.13 The average numbers of primary roots formed by *Acer saccharum* 'Jefcan' cuttings in different hormone treatments grown under different rooting conditions at Glenlea and Morden. Each bar represents the particular treatment's rooting percentage and the same upper-case letters indicate values are not significantly different from each other at p≤0.05.

Table 6.4 *Acer saccharum* 'Jefcan' cuttings collected on July 7, 2009 were rooted at different locations; Glenlea and Morden, Manitoba. Each site had different rooting conditions; including humidity, temperature, and rooting medium composition (further details in materials and method section). Rooting was assessed after 12 weeks.

Site	Total number of cuttings	Number of rooted cuttings	Rooting (%)	Number of primary roots
Glenlea	100	25	25.0 b	2.9 ± 0.4 b
Morden	110	99	90.0 a	5.0 ± 0.3 a
Overall	210	124	59.1	4.6 ± 0.3

Values of number of primary roots are the mean (±SE) of number of rooted cuttings. Values represented by same lower case letters are not significantly different at p ≤ 0.05.

Figure 6.14 A rooted cutting of *Acer saccharum* 'Jefcan' in a plug of peat moss mix, successfully formed roots after a 12-week rooting period in a greenhouse with controlled misting system. The cutting was collected on July 7, 2009 and was treated with distilled water and inserted into the rooting medium.

Figure 6.15 The bottom view of a styrofoam tray of *Acer saccharum* 'Jefcan' cuttings at Morden site. Cuttings were collected in July 2009 and rooted over a period of 12 weeks. Roots grew through the bottom holes as their development progressed, and majority of the holes had visible roots at the end of the rooting period.

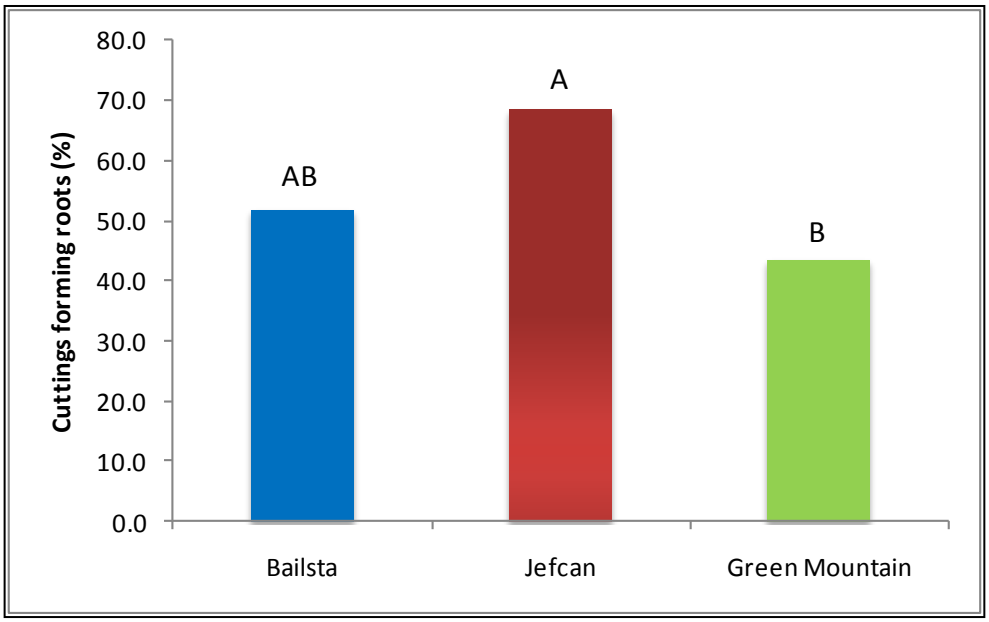


Figure 6.16 Effects of genotypic variation between three cultivars of *Acer saccharum* on rooting frequencies (%) of cuttings collected on July 13, 2009 from 3-to-4-year-old trees grown in containers. Each bar represents the particular treatment's rooting percentage and the same upper-case letters indicate values are not significantly different from each other at p≤0.05.

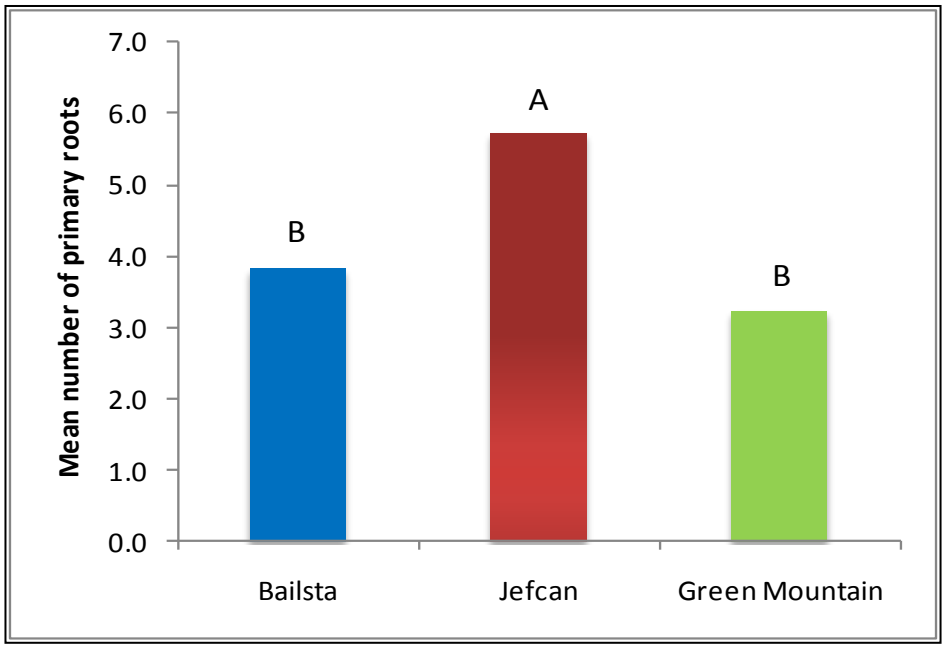


Figure 6.17 Effects of genotypic variation on the average number of primary roots formed by rooted cuttings of three cultivars of *Acer saccharum* within 12 weeks of rooting period. Each bar represents the particular treatment's rooting percentage and the same upper-case letters indicate values are not significantly different from each other at p≤0.05.

6.1.2.5 Testing the effect of growing conditions and age difference of source tree

In order to test if differences in age and growing conditions of the source trees influenced the cutting's rooting response, the results from cuttings taken from container-grown 'Jefcan' trees were compared to those taken from field-grown 'Jefcan' trees. Also, only cuttings of field-grown 'Jefcan' trees from the fourth collection treated with control and IBA at 5 g/L were used for this purpose. Thus, the effect was evaluated on a total of 140 cuttings. Rooting of field and container cuttings were 45% and 68.3% respectively (Table 6.5), and they were confirmed to be significantly different at 1% confidence level. On the other hand, the effect of growing conditions and age of source trees was statistically insignificant and that cuttings from both types of source trees produced on average seven primary roots per rooted cutting ($p>0.05$; Table 6.5).

6.1.2.6 Examining the relationship between leaf parameters and rooting response of cuttings

For the purpose of examining the relationship between leaf factors and the cutting's rooting response, data from the field-grown 'Jefcan' cuttings, comprising 695 cuttings, were combined. These were pooled from all collection times. Leaf size was measured as leaf area and was done by scanning the leaves to produce digital images, which were then analyzed using the software APS Assess® version 2.0 (American Pathological Society, 2008). The statistical analysis found that the average leaf area of rooted cuttings (51.6±0.8 cm^2) was significantly lower than that of non-rooted cuttings (55.1±0.7 cm^2, Table 6.6). Moreover, a correlation test performed on the pooled data indicated a significant correlation between leaf area and the number of primary roots produced per rooted cutting (Table 6.7). Nevertheless, the correlation coefficient (-0.2125) indicated a very weak correlation. Hence, leaf area would be an ineffective indicator of a cutting's primary root counts even though a significant negative correlation was detected between the two parameters.

In addition to leaf size, color saturation and intensity were also tested against rooting responses of cuttings. Both of these parameters were also measured from leaf digital images using APS Assess® version 2.0 (American Phytopathological Society, 2008). Similar to the leaf area, the average color saturation values and color intensity values of second-youngest-node leaves that belonged to rooted cuttings, 140.5±0.8 and 94.0±0.4 respectively were significantly different from those that failed to form roots, 137.9±0.9 and 92.3±0.3 respectively ($p \leq 0.05$, Table 6.6). The range of average color saturation was 87 to 182; while the range of average color intensity was 80 to 120. As for their correlation to the number of primary roots produced by the rooted cuttings, leaf color saturation did not show any significant correlation ($p > 0.05$) towards primary root count of rooted cuttings, whereas leaf color intensity had slightly positive correlation (0.1709), which was significant at $p \leq 0.05$ (Table 6.7). Nonetheless, the correlation was too weak to provide any acceptable measure of indication for the number of primary roots formed by a particular cutting.

Table 6.5 The effect of age and growing conditions of the stock plants on rooting and the number of primary roots of *Acer saccharum* 'Jefcan' cuttings collected in mid-July.

Source tree	Total number of cuttings	Number of rooted cuttings	Rooting (%)	Number of primary roots
Container-grown	60	41	68.3 a	6.8 a
Field-grown	80	36	45.0 b	7.0 a

Values of number of primary roots are the mean (±SE) of number of rooted cuttings. Values represented by same lower case letters are not significantly different at $p \leq 0.05$.

Table 6.6 The average leaf area, leaf color saturation and color intensity of rooted and non-rooted cuttings of *Acer saccharum* 'Jefcan' collected in July 2009 (n_R represents the number of rooted cuttings, n_N represents the number of cuttings failed to form roots).

Leaf factor	n_R	Mean value among rooted cuttings	n_N	Mean value among non-rooted cuttings
Area	298	51.6 ± 0.8 a	397	55.1 ± 0.7 b
Color - Saturation		140.5 ± 0.8 a		137.9 ± 0.9 b
Color - Intensity		94.0 ± 0.4 a		92.3 ± 0.3 b

Values represented by same lower case letters are not significantly different at $p \leq 0.05$.

Table 6.7 Results from correlation analysis between the number of primary roots per cutting and leaf area, leaf color saturation and leaf color intensity (n_R represents the number of rooted cuttings).

Leaf factor	n_R	Pearson correlation coefficient	p-value
Area	298	-0.2125	0.0002
Color - Saturation		-0.0664	0.2533
Color - Intensity		0.1709	0.0031

6.1.3 Effects of variation between years

Due to the selection process of the cutting materials for the 2009 experiment, its results had a higher range of rooting frequencies compared to the previous year. However, when the results of the same cutting type (medial), cultivar ('Jefcan'), and hormone treatments were compared between the years, the differences in rooting response was found to be insignificant ($p>0.05$, Figure 6.18). Statistical analysis also showed no significant interactions between year and hormone, and between year and collection time, meaning that the responses of the collection timing and hormone treatments did not differ significantly between years.

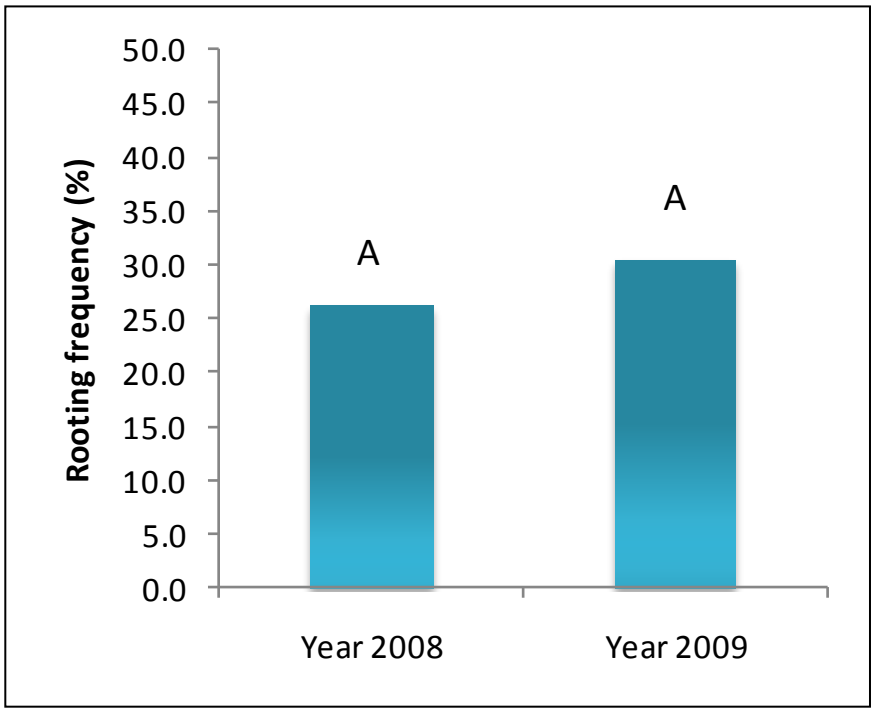


Figure 6.18 Rooting response of *Acer saccharum* 'Jefcan' cuttings collected in July of 2008 and 2009 and planted in sand beds at Glenlea site. Each bar represents the particular treatment's rooting percentage and the same upper-case letter(s) above each bar indicates means are not significantly different from each other at $p\leq0.05$.

6.2 Discussion

6.2.1 Effects of cutting type based on the presence of apical meristem

The results of cutting experiment in 2008 revealed a significant difference (p=0.0002) in rooting frequency between apical and medial cutting types (Figure 6.3). In light of this conclusion, the following year's experiment focused on rooting responses of medial-type cuttings. Mixed results have been reported in terms of rooting abilities of apical or terminal and medial or basal cutting types in various species (Preece et al. 1991a; Allegre et al. 1998; Kibbler et al. 2004; Spethman 2007; Vakouftsis 2009). In *A.saccharum*, cutting type has not been investigated although several authors (Donnelly 1977; Alsup et al. 2003; Tousignant et al. 2003)) have recommended a number of physiological and morphological guidelines for achieving high rooting percentages. Stem base water content, leaf morphology and terminal bud developmental stage are some parameters that had been suggested for recognizing the appropriate times for harvesting *A. saccharum* cuttings.

Many studies have found that terminal cuttings had higher rooting than medial cuttings and this phenomenon may be explained by the higher levels of endogenous root-promoting substances in terminal cuttings since it comprised the terminal bud, or by the assumption that tissues of terminal cuttings were more juvenile and less differentiated, hence more readily induced to form roots (Hartmann et al. 1997; Alegre et al. 1998). The contrasting result of the current study may be due to the fact that medial cuttings are more likely to have mature or well-developed leaves. According to Kibbler et al. (2004), cuttings with mature leaves had the advantage of forming roots faster than cuttings with immature leaves. The differences are found in the way these leaves utilize resources. Young, immature leaves generally act as net importers of nutrients and growth regulators (Fischer and Hansen 1977); thus limiting the auxin supply to the proximal end of the cutting for adventitious root formation.

Moreover, the overall developmental stages of medial-type cuttings are more advanced than the terminal-type cuttings. This may be accounted for improved cutting survival and rooting frequency. For example, immature leaves are more likely to have thin epidermis and lack secondary thickening thus more susceptible to desiccation (Kibbler et al. 2004). Furthermore, Kibbler et al. (2004) found that *Backhousia citriodora* cuttings took approximately 8 to 24 weeks to develop adventitious roots, and for this reason selecting cutting materials based on factors that increase cutting survival is crucial. Moreover, *A. saccharum* cuttings also require an extended period of time to develop roots; hence improving cutting survival may help increase the rooting frequency. In addition, the superiority of medial-type cuttings observed in the current study may be limited to certain genotypes of *A. saccharum*, or may be counterbalanced by selecting terminal cuttings that are more advanced in the developmental stage.

6.2.2 Effects of genotypic variation among cultivars

The influence of genotype on rooting ability of cuttings was demonstrated in this study by the significant variation in rooting frequencies among cultivars; 16.3-24.8% in the 2008 experiment ($p=0.0056$) and 43.3-68.3% in the 2009 experiment ($p=0.0167$). Similarly, the importance of clonal effects in various species has been highlighted in several studies (Wilkins et al. 1995; Alegre et al. 1998). Moreover, *A. saccharum* in particular has been frequently reported to display significant clonal effects in response to rooting of cuttings (Dunn and Townsend 1954; Gabriel et al. 1961; Donnelly 1977, Rioux et al. 2003; Tousignant et al. 2003). The effect was demonstrated in a study that collected *A. saccharum* cuttings from 24 trees and detected that rooting percentages of individual trees ranged from 5 to 100% (Tousignant et al. 2003).

Between the two experimental years, although cultivar effects were always significant, the responses were not consistent. In 2008, ‘Bailsta’ cuttings (24.8%) rooted at a significantly higher

frequency than ‘Jefcan’ cuttings (16.3%; Figure 6.4). Nonetheless, rooting of ‘Jefcan’ cuttings (68.3%) in the 2009 experiment dominated over ‘Bailsta’ (51.7%) and ‘Green Mountain’ (43.3%, Figure 6.16). Further statistical analysis of the 2009 results revealed that the difference between ‘Jefcan’ and ‘Bailsta’ was not significant at 5% confidence level, and only ‘Green Mountain’ cuttings rooted at significantly lower frequency compared to ‘Jefcan’. Furthermore, the cultivar effect of the 2009 experiment was tested among cuttings collected from younger trees grown in containers; while the 2008 experiment compared data from cuttings of well-established trees growing on the field. Also, the rooting percentages of 2008 experiment were based on combined results of terminal and medial cuttings, whereas in 2009 only medial cuttings were used in the study. In addition, the inconsistent results may be stimulated by year, cutting type and source tree variation. Greenwood et al. (1976) suggested that clonal variation in rooting response among genotypes of *A. saccharum* may not be related to endogenous auxin content but due to cutting base ability to accumulate auxin. Moreover, high rooting potential of cuttings from certain genotypes may be attributed to their efficiency at synthesizing or activating rooting cofactors (Kling et al. 1988) or essential enzymes (Haissig 1974).

Up to the current date, rooting studies on *A. saccharum* never used particular cultivars; thus this is the first report on rooting of cuttings on *A. saccharum* cultivars. This study concluded that, although cultivar effect was always significant, the actual response of each cultivar relating to the other(s) may vary between years and/or source tree age and growing environment. Further studies are needed to determine the optimum conditions and treatments for each cultivar to achieve efficiency levels required for commercial production. As for the cultivar ‘Jefcan’, the 2009 rooting study at Morden site had successfully displayed high rooting response with the application of IBA at 5 g/L or StimRoot®#2 (Figure 6.13).

6.2.3 Effects of collection timing

In the current study, rooting frequencies of *A. saccharum* cuttings were considerably influenced by the timing of cutting collection, both in 2008 ($p<0.0001$) and 2009 ($p=0.0184$). Alsup et al. (2003) collected cuttings of *A. saccharum* twice monthly starting from mid April through early October of 1999, and observed that only those collected in May and early June formed roots. Hardwood cuttings of *A. saccharum* failed to root and those collected later in the growing season, after the greenshoot started to lignify and turn brown, rarely produced roots or would form roots at low frequencies (Alsup et al. 2003; Tousignant et al. 2003).

The results of 2008 rooting experiment demonstrated a gradual decline in rooting efficiency; hence the first collection yielded the highest rooting frequency of 25.7% representing all cutting types and cultivars and hormone treatments. Since the interaction between collection time and cultivar was significant ($p=0.0124$), the data was presented separately for each cultivar (Figure 6.6), which showed that although rooting of 'Bailsta' decreased over time; 'Jefcan' cuttings had a remarkable increase in rooting between the last two collections, from 8.1% to 19.4%. Although the increase was not statistically significant, the rooting frequency of 'Jefcan' from the third collection was not significantly different from rooting of 'Bailsta' cuttings from the first collection, which was the highest across cultivars and collections. Further testing is still required, but it is suspected that 'Jefcan' cultivar has a wider span of optimum collection time than 'Bailsta'.

The rooting efficiency of the first collection time in the 2009 experiment, which was performed at 264 GDDs, was extremely low and possibly due to factors other than collection time (refer to the section on rooting conditions). In the 2009 experiment, when the result of the first collection was excluded, the lowest rooting frequency was observed among cuttings of the third collection time (25.6%). The decrease from the previous collection was not statistically significant ($p>0.05$), thus rooting

frequency of the third collection time was not significantly different from that of the second collection (Figure 6.10). Nonetheless, a significant increase was detected between the third and fourth collection times. In 2008, an increase was also observed in rooting frequencies of 'Jefcan' cuttings of the medial type between the second and the third collection times. Moreover, the low-rooting collection times were done at approximately the same time based on the calendar dates (July 9, 2008 and July 7, 2009) and accumulated GDDs (578 in 2008 and 546 in 2009). These characteristics were also similar between the collections with the increased rooting in 2008 and 2009 (July 13, 2008 at 640 GDDs and July 14, 2009 at 614 GDDs). Meteorological data were acquired from the Weather Station at Portage La Prairie, where the cuttings were collected, however the data did not show any marked differences in terms of daily average temperature and precipitation between collection times. It was unclear what caused the increased rooting at the last collection, yet these results show that the optimum window of *A. saccharum* 'Jefcan' cutting collection may extend beyond 600 GDDs.

Furthermore, variation in rooting response of *A. saccharum* cuttings between years was reported by Alsup et al. (2003) and Rioux et al. (2003). However, the current study found that the rooting results of comparable cuttings in the 2008 and 2009 experiments were not significantly different from each other (Figure 6.18; p=0.4833). Other than heat accumulation as measured by growing degree days or GDDs, some authors recommended using terminal bud developmental stage, leaf morphology and stem base water content to designate the optimum time of collection for *A. saccharum* cuttings (Donnelly 1977; Tousignant et al. 2003). Moreover, Tousignant et al. (2003) suggested the accumulation of 270 degree days above 5°C, or the presence of one to three pairs of terminal bud scales, as well as stem base water content between 55-75% to achieve high rooting frequency in *A. saccharum* cuttings. In addition, terminal leaf morphology was recognized by Donnelly (1977) to be associated with rooting potential of cuttings. The author observed that greenwood cuttings with mature leaves and visible terminal bud accomplished the best rooting. The author also reported that a significant increase in

starch contents coincided, approximately, with the maximum rooting response. The marked increase of starch content represented the time when active growth ceased, developing leaves matured and maximum shoot elongation was reached; thus the plant begun to accumulate photosynthates, including starch. Besides, Donnelly (1977) mentioned that warm spring weather may influence the optimum cutting collection period. Also, since GDD is calculated based on the average of the maximum and minimum daily temperatures as compared to a base temperature, in this case 5 °C, it does not take into account the fluctuation or the consistency of heat distribution over time. The use of plant morphological conditions, such as leaf or bud developmental stage, may entail smaller variation and should be optimized to achieve consistent rooting results.

Additionally, in the 2008 experiment, the significant effect of collection time was also observed in the quality of root system developed by the cuttings. Timing of collection was the only main factor that had significant effects on number of primary roots ($p=0.0051$), length of the longest root ($p<0.0001$) and fresh ($p<0.0001$) and dry weights ($p<0.0001$) of the whole roots. In contrast, the study by Alsup et al. (2003) did not record any significant effect by timing of collection on root number and root length of *A. saccharum* cuttings. The significant effect of collection time on root quality was repeated in 2009 experiment as measured by the number of primary roots (Figure 6.11). The difference in responses observed in this study, as compared to other studies, was possibly due to clonal effects, or a combination of several factors, including rooting hormone treatment. The amount of accumulated growing degree days (GDDs) of the cutting collections in 2008 and 2009 followed the suggestion of Tousignant et al (2003) to begin collection after approximately 270 degree days above 5°C had accumulated in order to achieve high rooting percentages in *A. saccharum* cuttings. Since the earliest collection of the current study already had over than 400 GDDs, future studies on the same *A. saccharum* cultivars should examine rooting response of cuttings collected earlier in the season to determine the beginning of the optimum collection period of these cultivars.

6.2.4 Effects of rooting hormone application

The ability of auxin to promote root formation in cuttings has been well documented and the endogenous auxin effect may be enhanced by supplying auxin exogenously (Hartmann et al. 1997). Moreover, IBA is the most widely used form of auxin in cutting propagation (Nickell 1982). The application of auxin in various forms and concentrations has been reported to increase rooting response and improve root quality in many species (Wiesman and Lavee 1995; Wilkins et al. 1995; Alegre et al. 1998; Ercişli et al. 2002; Alsup et al. 2003; Bredmose et al. 2004; Fogaca and Fett-Neto 2005; Vakouftsis et al. 2009). The application of different types of rooting hormones to the proximal end of the cuttings was performed in 2008 and 2009 experiments and significant hormone effects on rooting frequencies were observed in both years (Figures 6.6 and 6.12).

The 2008 experiment found significant variation in rooting frequencies of cuttings treated with various hormone treatments. Although both IBA-5 and IBA-5/NAA-5 achieved the highest rooting, only IBA-5 was repeated in 2009 due to limited resources. The other treatment, IBA-10/NAA-5, was chosen to confirm the type of auxin and its concentration level to achieve optimum rooting and high quality roots in *A. saccharum* 'Jefcan' cuttings. In future research, it would be interesting to compare responses of other cultivars to IBA-5 and IBA-5/NAA-5 treatments and if these treatments would maintain their superiority over StimRoot®#2. According to the 2009 results, IBA-5 did not significantly increase the 'Jefcan' cutting response as compared to StimRoot®#2, both in terms of rooting frequency and the number of primary roots. Hence the treatment of IBA-5/NAA-5 is the only one that remains to be tested on 'Jefcan' cultivar.

In 2009, all hormone treatments, except for IBA-10/NAA-5, had similar rooting efficiencies ranging from 30.5-41.0% (Figure 12), suggesting that the combination of IBA at 10 g/L and NAA at 5 g/L is suboptimal compared to the lower IBA concentrations (4-5 g/L) of the other treatments. Moreover,

the control treatment produced the highest rooting frequency of 47.6%, which was not significantly different from the rooting of hormone treatments IBA-5 and StimRoot®#2. Similarly, Rioux et al. (2003) conducted several experiments with *A. saccharum* cuttings to test the efficiency of several rooting chemicals and found in some of the experiments that rooting of cuttings treated with various chemicals did not differ significantly from the control.

Furthermore, the slightly lower rooting among IBA-treated cuttings compared to control cuttings may be associated with the ethanol component of the IBA solution. Metaxas et al. (2008) reported that the high percentage of ethanol used to dissolve IBA may be detrimental to *Arbutus unedo* cuttings since a significantly lower cutting survival was observed among cuttings in the ethanol control than the water control. Moreover, survival of cuttings treated with IBA dissolved in ethanol was slightly but significantly lower than those in K-IBA (water-based) treatment. Although our study did not record noticeable differences in cutting survival between control and IBA treatment, the use of water-soluble IBA can be a subject of investigation for further improvement of *A. saccharum* cutting survival and rooting potential.

Additionally, Fogaca and Fett-Neto (2005) observed higher in vitro rooting frequencies of *Eucalyptus saligna* and *E. globulus* shoot cuttings when treated with the same concentration of IBA and indole acetic acid (IAA) than of NAA or indole acetic acid aspartate (IAAasp). The authors suggested that the enhanced performance of IBA may be related to its higher stability relative to IAA, which is more susceptible to enzymatic degradation (Ludwig-Muller 2000). The ability of IBA to be converted in vivo into IAA may also facilitate slow release of auxin, which contributes to the greater effect on rooting. Furthermore, NAA, being a very stable auxin, might have persisted longer in plant tissues in its free form and hindered root emergence, as demonstrated by De Klerk et al. (1997) in rooting of *Malus domestica* 'Jork 9' that favoured IAA treatment over NAA, especially in a continuous exposure system. In the

current study, based on the 2008 rooting results, NAA at 5 g/L gave similar rooting frequency as IBA at the same concentration (Figure 6.5), and may suggest that this level was still within the safe concentration range of NAA for rooting of *A. saccharum* cuttings using the quick dip method. Furthermore, the 2009 result of the Dyna-gro® root gel, which consists of IBA at 5 g/L and NAA at 2.5 g/L, shows a rooting frequency (30.5%) that was not significantly different from IBA-5 and StimRoot®#2, indicating that *A. saccharum* cuttings may tolerate NAA, in spite of its persistence, within the range of 2.5-5 g/L. In the case of IBA-10/NAA-5 treatment in the 2009 experiment, the poor rooting response might have been due to the high concentration of IBA, while accompanied with the persistent NAA.

Between the two years, StimRoot®#2 produced different rooting results. Rooting frequency of StimRoot®#2 in 2008 (14.5%) was among the lowest, but in 2009 cuttings treated with StimRoot®#2 rooted at 41.0%, which was the highest among the hormone treatments (excluding control). The differences in efficiency were not as noticeable in other hormone treatments. Furthermore, statistical analysis showed that overall change in rooting frequencies of IBA-5, IBA-10/NAA-5, and StimRoot®#2 between 2008 and 2009 experiments were not significant ($p>0.05$). Alegre et al. (1998) also reported that auxin treatments, irrespective of dose, had increased rooting and cutting survival of *Dorycnium pentaphyllum*; nonetheless the effect was species specific, since the study also found that the same IBA treatments failed to display any significant effects on rooting response and survival of *Dorycnium hirsutum* cuttings.

In the main hormone experiment of 2009, auxin application did not significantly affect the quality of roots, as measured by number of primary roots ($p=0.5206$), length of the longest root ($p=0.0795$) and fresh ($p=0.6433$) and dry ($p=0.7465$) weights of roots in 2008 and number of primary roots ($p=0.1991$). Nonetheless, the hormone effect was found to be significant on number of primary roots among control and IBA-treated cuttings collected from the container-grown trees ($p<0.0001$) as

well as among cuttings rooted at Morden site (p=0.0006). It is proposed that the effect of IBA on root quality may be more pronounced among the juvenile cuttings of *A. saccharum* and/or that the rooting conditions at the Glenlea site masked the effect of auxin in promoting root growth. In addition, Bredmose et al. (2004) suggested that *Rosa hybrida* cuttings may benefit from higher concentration of IBA when rooted at low temperatures, which indicated that auxin may interact with other factors, such as temperature.

The negative effect of acidic environment to adventitious rooting formation was demonstrated by the experiment comparing the treatment of cuttings with pH-4.0 distilled water to the neutral control. The pH at 4.0 was chosen based on the pH of the solution of IBA at 5 g/L. In addition, the average numbers of primary roots of the two treatments were not significantly different between the two treatments. Although pH of the culture medium has been shown to positively influence rooting of apple microcuttings (Harbage et al. 1998), the current study found that treatment of *A. saccharum* 'Jefcan' stem cuttings with water at pH 4.0, in the absence of auxin, did not increase root formation. These results confirmed that the effect shown by the auxin treatment with IBA-5 was not attributed to the pH component of the solution, but to the effects of the hormone itself. In addition, the reduction in rooting efficiency in acidic pH treatment as compared to neutral control suggests that adjusting the pH of IBA-5 solution to neutral pH may result in an increase in rooting frequency.

6.2.5 Effects of rooting medium and conditions

Several factors that were incorporated into the differences between the three sites included humidity, temperature, light, and rooting substrate. The rooting result of cuttings in the misting box was not included in the statistical analysis. Rooting was particularly low at this site due to occasional high temperatures, possibly above 30°C, that resulted from accumulated heat during sunny days. The construction of the misting box did not include ventilation, thus heat and humidity often continued to

build up causing an enormous amount of stress to the plants. In addition, the use of peat-perlite mix combined with a fog system might have been unsuitable. According to Vakouftsis et al. (2009), survival of *Cupressus macrocarpa* 'Goldcrest' cuttings was significantly higher when perlite was used alone as rooting substrate, compared to mixtures of peat and perlite at 1:1 ratio (v/v). Moreover, the same study found that mist propagation gave better overall rooting than a fog system among *C. macrocarpa* 'Goldcrest' cuttings. In the end, the humidity and temperature stresses impacted the cuttings substantially and their effects were lethal in the majority of the cuttings. In this particular part of the experiment, the factors affecting rooting response were mainly humidity and temperature of the propagation space.

Between Glenlea and Morden sites, the combination of factors produced a significant difference in rooting response, both in terms of rooting frequency ($p<0.0001$) and the quality of roots as measured by the number of primary roots (0.0007). According to Dirr and Heuser (1987) an ideal rooting medium depends on the species, cutting type, season and propagation system. Sand is an inexpensive option but the results may be inconsistent, whereas the more-costly peat-perlite mixes often make excellent rooting components. Ercişli et al. (2002) outlined several properties of rooting medium to consider, such as water retention capacity and the amount of large pores, which provide aeration and drainage. The use of sand as a rooting medium may be considered non-ideal because of its high bulk density providing limited gas exchange. In addition, Wojtusik et al. (1994) found that cuttings rooted in sand formed shorter roots than those rooted in perlite/vermiculite mixtures, possibly attributed to the higher amount of force required for penetration.

Metaxas et al. (2008) reported insignificant differences in rooting potential and root quality of *Arbutus unedo* cuttings rooted in perlite, peat-perlite mixture and sand; nonetheless, the cuttings rooted in sand suffered 65% survival loss, which was more than double of the losses in other rooting

media. Moreover, MacDonald (1986) described that poor cutting survival is commonly caused by poor drainage of the rooting medium causing reduced oxygen, which is essential for respiration. Another sign of poor drainage is the formation of roots near the surface of the medium, which is more likely to have less moisture and more oxygen. This sign was observed in some of the cuttings planted at the Glenlea site, where roots emerged from the part of stem slightly above the cut end and the basal end below the adventitious roots rotted. The deterioration of the stem may have been due to inadequate oxygen supply for the high respiration rates associated with the rooting process. In addition, the poor aeration at the Glenlea site may be attributed to the compaction of the sand, which is relatively heavy, particularly when wet, reducing the amount of large pores. Furthermore, the rooting medium's water retention capacity and aeration properties determine the water content of the substrate (MacDonald 1986), which is indirectly proportional to the amount of water absorbed by cuttings (Grange and Loach 1983). In addition, as observed among the cuttings planted at the Glenlea and Morden sites, the rooting medium interacted with the hormone treatments (Figure 6.13) possibly because water uptake is related to auxin uptake. This explained the difference in mean primary root numbers of rooted cuttings treated with StimRoot®#2 at the two sites (Figure 6.13). Since StimRoot®#2 is in talc form, water uptake could be essential for its effect.

Aside from the obvious promoting on rooting frequency and mean number of primary roots, the use of peat plugs at the Morden site as a rooting medium may considerably facilitate the process of storing rooted cuttings over the winter season and transplanting them in the following spring. Since the root systems are enclosed by the rooting medium, they are exposed to a smaller degree of stress and are less likely to be damaged as compared to bare-rooted cuttings harvested from the sand beds at the Glenlea site. Although further examination is needed, cuttings with bare roots are likely to have a lower percentage of survival than the cuttings rooted in peat plugs. In addition, for the nursery application, the use of peat plugs can also greatly increase the efficiency of transplanting since it will allow for machine-

assisted transplanting process. Furthermore, the portability feature of the plug-tray system for cutting propagation would be beneficial particularly when one is working with several species with different root formation rates. Even within species, each cultivar or genotype may root differently. Since misting is usually reduced as the root development progresses, certain cuttings that reach the threshold earlier can be moved to a lower-misting area without risking the other cutting's rooting process.

Moreover, many studies have investigated the importance of rooting conditions, such as light intensity and propagation temperature, on rooting ability of cuttings of many species (Moe 1973; Zhang et al. 1997; Allegre et al. 1998; Bredmose et al. 2004, Druege and Kadner 2008; Rasmussen et al. 2009). Moe (1973) observed improved rooting by increasing Photosynthetic Photon Flux Density (PPFD); however a rooting study on miniature rose cuttings by Bredmose et al. (2004) found no significant differences when PPFD was increased from 46 to 72 µmol $m^{-2}s^{-1}$. As for optimum temperature for root formation, responses varied with species but generally cutting survival is improved at cooler temperatures. Nonetheless, growth, including root formation, may be slower or inhibited at these low temperatures (Rasmussen et al. 2009). Overall, cutting propagation of many temperate species benefit most from an optimum temperature range of 18-25°C (Kester 1970).

Additionally, supplementation of bottom heat may also be valuable for the stimulation of adventitious root development (Dirr and Heuser 1987; Hartmann et al. 1997). It must be practiced with caution since over-heated rooting substrate may be produce inhibitory effects as reported by Zhang et al. (1997) where rooting of 'Autumn Flame' red maple and 'Indian Summer' freeman maple decreased from 74% to 29% and 25% as the rooting substrate temperature increased from 24 to 30 and 33°C respectively. Moreover, maintaining low air temperature while keeping warm substrate temperature had been reported to benefit rooting of pelargonium cuttings (Druege and Kadner 2008). The study compared rooting of pelargonium cuttings that were rooted at 10°C air temperature, with root zone

temperature maintained at 20°C, to those grown at 20°C, in both air and root zone. The authors found that lower air temperatures significantly increased tissue carbohydrate levels, particularly sugars, which were positively correlated with reduced leaf senescence. This in turn was implicated in increased survival rate of the cuttings. Furthermore, Veierskov (1988) concluded that higher sugar availability in the leaves stimulates basipetal transport of carbohydrates towards the cutting's stem base, in which carbohydrates would be quickly metabolized due to the higher root zone temperature. Although the Morden site did not provide bottom heat, the lower air temperature had improved cutting survival, which may contribute to higher rooting frequency and better quality of root system. The combination of several factors that were different at the two sites did not allow for specific conclusion on the individual factors; however the result of the current study showed that *A. saccharum* cuttings benefitted greatly from shaded, cool propagation environment in conjunction with a rooting substrate that had optimum moisture retention capacity and provided sufficient aeration, such as mixtures of perlite and peat.

6.2.6 Effects of age and growing environmental conditions of stock plants

Rooting response of cuttings collected from trees of different ages and growing conditions were compared. Cuttings from the younger trees ,which were grown in nursery containers with special winter protection treatment, rooted at a significantly higher rate compared to those taken from the older trees growing on the field without any winter protection ($p=0.0057$). Richer et al. (2003) studied the effect of partial and total light exclusion or etiolation of stock plants on rooting responses of *A. saccharum* cuttings collected from these plants. The initial study used cuttings from two-year-old treated stock plants and found significant differences in rooting, where cuttings from etiolated stock plants, both partial and total, showed considerably higher rooting frequencies than control. Nonetheless, the effect disappeared when similar treatments were applied to nine-year-old stock plants. The study suggested that maturation of *A. saccharum* trees can be associated with reduction in rooting potential of the cuttings.

The decrease in rooting ability of cuttings as trees mature has also been reported in other species (Greenwood and Hutchison 1993). The study comparing treatments of light exclusion by Richer et al. (2003) also demonstrated that surrounding conditions may manipulate the physiology of the trees, which can be implicated in the rooting capacity of the cuttings. Trees grown in the field were more likely to be exposed to harsh conditions as a combination of wind, temperature and light, which may impose some degree of stress on the trees. On the other hand, container-grown trees were more sheltered and suffered less environmental stress. The exposure to stressful conditions may influence the hormonal balance of the plant's cells and tissues, which might have been implicated in the reduction of rooting capacity of the cuttings of these trees. Due to limited plant resources, we were not able to test for age and growing environment independently. In conclusion, as a combined effect, differences in age and growing conditions of the stock plants, may influence rooting of *A. saccharum* 'Jefcan' cuttings.

6.2.7 Relationship between rooting responses and leaf size of the second-youngest node

The current study measured leaf area of a leaf from the node below the terminal or apical meristem and found that the average leaf area of the rooted cuttings (51.6 cm^2) was significantly lower ($p=0.0016$) than that of the non-rooted cuttings (55.1 cm^2). In other words, root formation was more likely to occur in cuttings with smaller leaf area than those with larger leaves. The range of leaf area measured in the current study was 13.8-105.6 cm^2, with an overall average of 53.7 cm^2. Hartmann et al. (1997) provided a general rule for achieving high rooting frequency with *Acer* cuttings, which was to collect the cuttings when the terminal bud has been formed but before the terminal leaves reach maturity. A contrasting suggestion was made by Donnelly (1977), which was to collect *A. saccharum* cuttings when the terminal leaves have matured as judged by the size and color of leaves. In addition, Rioux et al. (2003) and Hartmann et al. (1997) pointed out that *A. saccharum* leaves were particularly susceptible to water stress. Hence, smaller leaves may benefit from lower transpiration rates, which could be associated with higher cutting survival.

Moreover, leaf area was also found to be negatively correlated with the number of primary roots (r^2=-0.21246), meaning that cuttings with larger leaves were more likely to produce lower number of primary roots. The leaf parameter was analyzed by pooling the data from the field 'Jefcan' cuttings that were planted at the Glenlea site. In addition, Druege et al. (2000) and Bredmose et al. (2004) concluded that rooting ability is related to the amount of stored and/or supplied carbohydrates and phytohormones. Bredmose et al. (2004) observed that increased cutting size positively influenced the time to visible root. Also, Costa and Challa (2002) noted that the root weights of *Rosa hybrida* cuttings were positively related to leaf area of the cuttings. In the current study, although the correlation was found to be significant (p=0.0002), it was also very weak. Further studies on the effect of leaf size on rooting responses of *A. saccharum* cuttings are still needed.

6.2.8 Relationship between rooting responses and leaf color of the second-youngest node, as measured by color saturation and intensity

Statistical analysis on the leaf color data revealed significant differences between the means of leaf color saturation and intensity of rooted and non-rooted cuttings. Although the differences between these means were not large (Table 6.6), the significance showed that cuttings with second-youngest-node leaf having higher reading of leaf color saturation and intensity were more likely to produce roots than those with lower leaf color saturation and intensity reading. Implementation of this parameter for selection of cuttings may be facilitated by the development of simple and rapid method of scanning and measuring leaf color parameters. Furthermore, Murakami et al. (2005) provided an instructional guideline for leaf color assessment using digital images. This method provided an alternative means to quantify leaf color in relation to aspects of plant health while eliminating the need for special equipment and hazardous chemicals. As a quick and accurate method of quantifying foliar color, van den Berg and Perkins (2004) evaluated the use of a portable chlorophyll meter to estimate chlorophyll of *A. saccharum* leaves and concluded that CCM-200 (Opti-Sciences, Tyngsboro, MA) can be used effectively

as quick, non-destructive method to provide a relative estimate of total chlorophyll in most sugar maple leaves. This method may be more readily applied in the propagation industry, if relationship between relative chlorophyll content of leaf and rooting capacity of the cutting were found to be strong and significant.

In terms of root quality, leaf area saturation was not significantly correlated to mean primary root counts. Correlation test between leaf color intensity and mean number of primary roots was significant ($p=0.0031$); however, similar to the case of leaf area, the correlation coefficient was too far from 1 or -1 signifying weak correlation. Thus leaf color saturation and intensity are not capable of providing reliable indication of potential root quality of rooted cuttings. Testing the relationship between chlorophyll indexes of *A. saccharum* leaves and rooting ability of the cuttings, as well as how this relationship changes over the growing period may provide some insights into the potential of applying such method of measurement of plant physiological conditions in relation to cutting propagation. The portable feature of the device evaluated by van den Bert and Perkins (2004) would work well with the nature of the nursery industry.

6.2.9 Additional discussion

In a study by Wilkins et al. (1995), several red maple and freeman maple cultivars were found to vary in their susceptibility to leaf desiccation injury during cutting propagation. Based on the significant positive correlation between functional leaf surface and the final root or shoot dry mass, the authors concluded that minimizing foliar damage during rooting period, including the period of root growth, may contribute greatly to the growth of propagules, especially the roots. Hence, the quality of roots produced by the rooted cuttings would be improved. It is recommended to maintain acceptable humidity even after cuttings have developed roots, particularly for *A. saccharum*, considering its vulnerability to water stress (Hartmann et al. 1997; Rioux et al. 2003).

Other than environmental stress, shoots of cuttings may be under internal strain due to the application of auxin, which at high concentrations had been reported to negatively influence shoot growth, resulting in the occurrence of leaf senescence (De Klerk et al 1999). This detrimental effect was suggested to be linked to auxin effect in promoting ethylene synthesis. Besides, the addition of ethylene inhibitor, silverthiosulfate (STS) along with auxin was observed to significantly improve the appearance of rooted microcuttings upon transfer to ex vitro. Therefore it is important to select for conditions and characteristics that increase cutting survival.

In addition, the application of auxin together with other compounds, such as plant growth retardants and auxin conjugation inhibitor, may allow for more effective rooting response (Ludwig-Muller 2000). Plant growth retardants, such as triadimefon, when combined with IBA, resulted in more effective induction of adventitious root formation in mungbean cuttings (Pan and Gui 1997). Several factors are known to regulate the activity of exogenously-applied auxin. Differences in auxin metabolism have been connected with the variation in rooting ability of some species (Epstein and Ludwig-Muller 1993). For instance, some difficult-to-root cultivars of *Prunus avium* have been shown to conjugate IBA more rapidly than the easy-to-root counterparts (Epstein et al 1993). Although other factors, such as uptake and transport, can also account for the variation in rooting behaviour, future research on difficult-to-root cultivars may benefit from combining auxin treatment with these chemicals.

Since the optimum collection period of *A. saccharum* cuttings may be limited to the active growing season, an alternative method of obtaining such material at other times of the year would be beneficial. Henry and Preece (1997) induced shoot growth from dormant stem segments of Japanese maple (*A. palmatum* Thunb.), red maple (*A. rubrum* L.) and sugar maple (*A. saccharum* Marsh.). The efficiency of shoot formation by the dormant stem segments was highly variable among species and clones. Their reports showed that *A. palmatum* and *A. rubrum* achieved the highest shoot production

(53-60%) while only 20% of *A. saccharum* stem sections formed shoots. In addition, rooting capacity of these forced shoots varied greatly. Nonetheless, the study proved that this method is feasible though optimization is required; particularly for *A. saccharum*. The low rooting frequency among *A. saccharum* softwood cuttings in the study might have been due to the low auxin treatment (0.1% IBA, equivalent to 1 g/L); increasing the auxin level may be able to improve rooting response of these cuttings.

Moreover, shoot production efficiency may be improved by treating the dormant stem segments with plant growth regulators that promote shoot growth. Mansouri and Preece (2009) forced shoot growth from *A. saccharinum* stem segments to be used as explants for in vitro propagation. Although the hormone treatment did not significantly influence the number of shoots produced from the *A. saccharinum* stem segments, the shoots produced by treated segments showed better growth and improved in vitro response. In addition, the use of a combination of BA and GA_3 to treat stem segments was recommended. Optimization may also include other types of shoot-growth promoting hormones, or other forms of cytokinins. Also, the concentration may need to be increased; the highest concentration used in the *A. saccharinum* study (Mansouri and Preece 2009) was 30 mM of BA and GA_3, which are equivalent to 6.8 g/L of BA and 10.4 g/L of GA_3.

In addition, Kibbler et al. (2004) reported several characteristics of cuttings of the recalcitrant *Backhousia citriodora* that significantly improved their rooting capacity. These characteristics included mature leaves, wide stem (>3 mm) and actively growing axillary buds. Besides, further testing revealed that the removal of axillary buds considerably accelerated root formation in cuttings collected in autumn (actively-growing buds) and winter (inactive or dormant buds) seasons. These results indicated that the improved rooting associated with cuttings that have actively growing axillary buds can be attributed to the physiological state of the plants when axillary buds were growing vigorously (Kibbler et al 2004), and that axillary buds, both active and dormant, posed some inhibitory effects on rooting of *B. citriodora*

cuttings (Kibbler et al. 2002; Kibbler et al. 2004). The acceleration of root development would be a great advantage for cutting propagation of *A. saccharum*, in particular when the rooting environment is not optimal for cutting survival, since generally *A. saccharum* cuttings require a prolonged period of time to produce roots. Future research on *A. saccharum* cutting propagation should consider evaluating the effect of the removal of axillary buds on rooting responses of *A. saccharum* cuttings.

Overall, this study has found that *A. saccharum* 'Jefcan' cuttings can be successfully rooted at high frequencies (>90%), whether or not auxin was applied, when planted in rooting medium that provides optimum aeration and moisture, while maintained in an environment that promotes cutting survival. The optimum window of cutting collection was not clearly defined, but based on the results across the collection times, *A. saccharum* 'Jefcan' can achieve optimum rooting when collected between 400 and 600 accumulated GDDs. Although the initial part of the study showed that terminal-type cuttings did not respond to rooting as well as medial-type cuttings did, it would be appropriate to test whether the presence of the apical meristem or the immaturity of the tissue elicited the effect. In addition, since clonal effects among *A. saccharum* genotypes are known to be significant, several parameters may need to be optimized for the propagation of other *A. saccharum* cultivars. We suggest that 'Bailsta' cuttings be treated with NAA or NAA in combination with IBA, to achieve the most favourable rooting response.

7.0 GENERAL DISCUSSION AND CONCLUSIONS

Based on the results of the current study, *Acer saccharum* can be propagated using several methods, namely shoot organogenesis, embryo conversion and stem cutting propagation. Some methods are more efficient than the others; nonetheless, responses may vary among genotypes as demonstrated in all of the methods of propagation tested in the study. The results of the in vitro shoot organogenesis study using bud and embryo explants showed that shoot multiplication was feasible although several conditions still need to be optimized. Selection of suitable explant type at the appropriate collection time was found to be critical to the success of in vitro regeneration. Immature embryos from seeds collected at the end of the growing season prior to maturation and drying showed vigorous growth and respond well in culture. Reactivating buds collected in early spring were found to respond best; whereas shoot tips and petiole explants may need to be obtained from juvenile trees. Explant collection during the period of active growth is recommended for improved overall in vitro response.

The need for a long stratification period to overcome seed dormancy has been amended through the application of growth regulators, which then induced embryo conversion in isolated *A. saccharum* embryos. The method aimed for the full conversion of isolated embryos into whole plantlets, having shoot and root systems. The greatest effect was observed in treatments with cytokinin BAP at 0.5-1.5 mg/L or TDZ at 0.01 mg/L. A lower concentration of TDZ is suggested since some abnormal phenotypes were recorded among the seedlings. In addition, combined use of BAP and TDZ at 1.5 mg/L and 0.01 mg/L was shown to induce axillary shoot proliferation, a potential for shoot organogenesis.

Among the three techniques, this study found the highest efficiency in using cutting propagation system. Various factors were tested on several sugar maple genotypes, particularly that of ‘Jefcan’, which is also known as Unity® (trade name). Since root formation may take several weeks in *A.*

saccharum cuttings; factors that increase cutting survival, such as cool temperatures and well-developed leaves, will likely improve rooting response. Moreover, leaves of *A. saccharum* are particularly susceptible to desiccation stress hence reducing leaf area may also increase survival and rooting frequency. The application of rooting hormone was shown to be unnecessary for high rooting potential; nonetheless, the quality of roots formed by cuttings treated with exogenous auxin was significantly enhanced. Cuttings with low numbers of primary roots may not survive transplanting; therefore, auxin supplementation may be used to increase the production quality.

In conclusion, although our study found that medial-type cuttings performed significantly better than the terminal counterpart, we could not identify the cause, whether the presence of shoot apical meristem or other characteristics of terminal-type cuttings. For now, the conclusion is that cuttings with immature leaves rarely survive the rooting period; thus we recommend avoiding their use. Additionally, depending on the cultivar, cutting collection may begin as early as 300 accumulated GDDs. As for 'Jefcan', collections beginning at 500 GDDs were able to reach high rooting frequencies, when provided with supporting rooting conditions. 'Bailsta' and 'Green Mountain' cuttings rooted at high frequencies (approximately 50%) when collected at 600 GDDs; however based on the 2008 results, it is suspected that 'Bailsta' may achieve higher rooting potential when collected earlier and cutting collection is suggested to begin at 300 GDDs. Since 'Green Mountain' had similar response to 'Bailsta' in the 2009 experiment, both are suspected to behave similarly; thus early collection should also be considered when working with this cultivar. As for rooting hormone application, various treatments of 'Jefcan' cuttings demonstrated insignificant differences as compared to control. However, due to significant effects by genotypes, the importance of exogenous auxin supplementation in other cultivars of *A. saccharum* still needs to be investigated. Moreover, according to the 2008 results on root quality, 'Bailsta' cultivar may benefit more from NAA-type auxin than IBA-type. Although 'Green Mountain' cuttings from container-grown trees demonstrated negligible difference in rooting potential between

control- and auxin-treated cuttings, the effect may be conserved among cuttings of container-grown trees, which are more juvenile and less stressed. These conclusions and suggestions are summarized in Figure 7.1.

Suggestions for future research in cutting propagation of *A. saccharum* include optimization of collection time and hormone treatments for other genotypes and characterization of cuttings that result in high cutting survival and rooting potential. In addition, another key step in the propagation system takes place when the regenerated plantlets or the rooted cuttings are transplanted for further growth. Cuttings with bigger, more profuse root systems have a higher chance of survival. The in-vitro regenerated plantlets require sufficient growth to be achieved prior to transfer into soil and ex vitro conditions. The transition process should be carried out gradually to ensure survival of these plantlets. Research into factors that influence survival during transplanting would be appropriate.

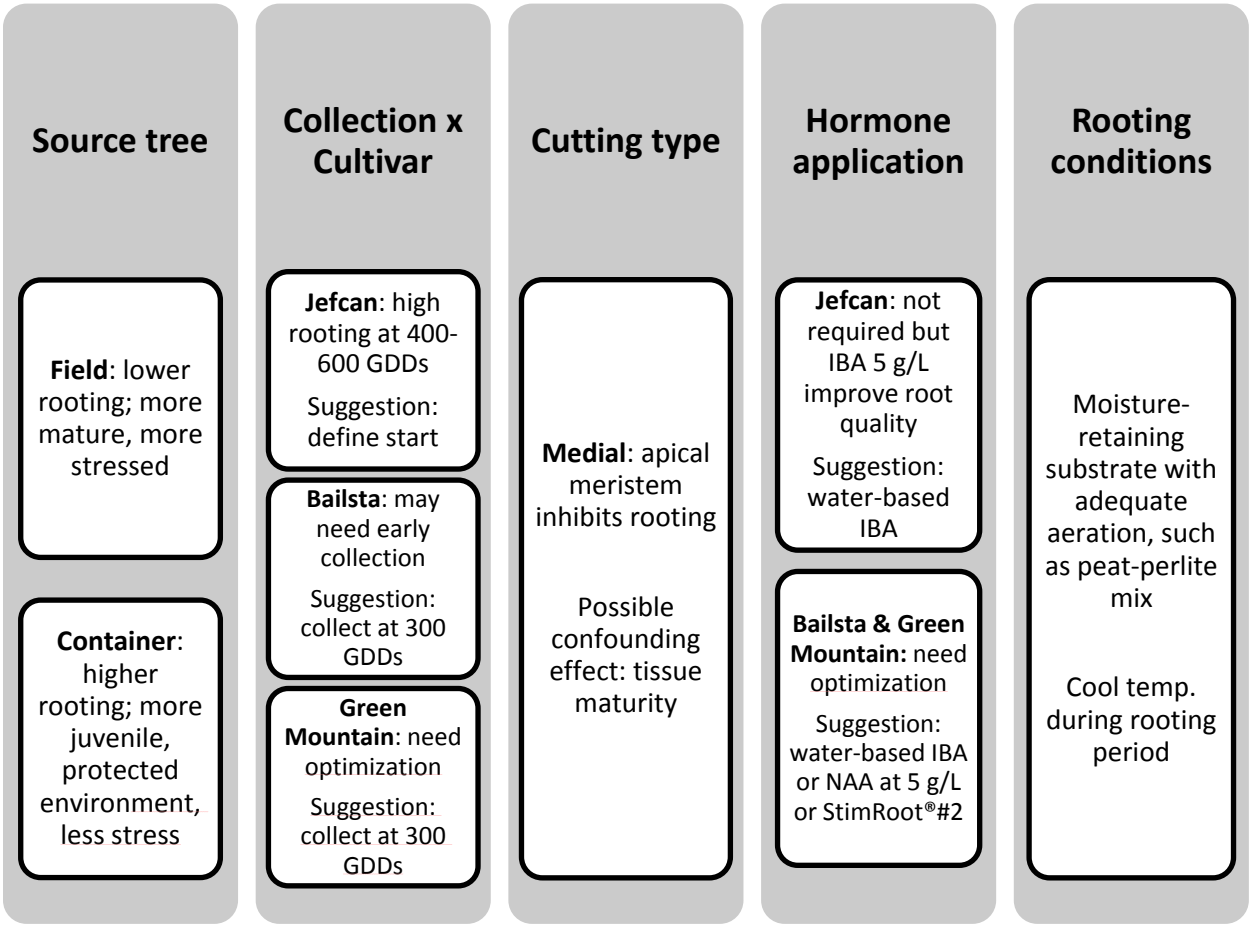


Figure 7.1 Summary of the conclusions along with some suggestions on source tree, collection period, cutting type, hormone treatment and rooting condition for the three cultivars of *Acer saccharum*; 'Bailsta', 'Green Mountain' and 'Jefcan'

8.0 LITERATURE CITED

Abeles, F.B, Morgan, P.W. and Saltveit Jr., M.E. 1992. Ethylene in plant biology. 2nd ed. Academic Press: San Diego

Acquaah, G. 2005. Horticulture: Principles and practices. 3rd ed. Pearson Prentice Hall, Upper Saddle River, NJ. 822 pp.

Alegre, J., Toledo, J.L., Martinez, A., Mora, O. and De Andres, E.F. 1998. Rooting ability of *Dorycnium* spp. under different conditions. Sci Hort. 76: 123-129.

Alsup, C.M., Cole, J.C. and Claypool, P.L. 2003. Timing and IBA application affect rooting of *Acer saccharum* Marsh. stem tip cuttings. Prop Ornam Plants 3(1): 42-46.

Alsup, C.M., Cole, J.C. and Claypool, P.L. 2004. Stem cuttings of Caddo sugar maple trees differ in rooting potential. Acta Hort. 630:263-269.

Arteca, R.N. 2006. Introduction to horticultural science. Thompson Delmar Learning, Clifton Park, NY. p. 215-217.

Baskin, C.C. and Baskin, J.M. 2004. p. 162-179.Determining dormancy-breaking and germination requirements from the fewest seeds. In: Ex-situ Plant Conservation: Supporting Species Survival in the Wild. Guerrant, E.O., Havens, K., Maunder, M. eds. Island Press, Washington, DC.

Bewley, J.D. and Black, M. 1994. Seeds: Physiology of development and germination. 2nd ed. Plenum Press, New York, NY. 445 pp.

Bewley, J.D. and Black, M. 1978. Physiology and Biochemisty of seeds in relation to germination. Vol.1: Development, germination, and growth. Springer-Verlag, Berlin, Heidelberg, New York. 306 pp.

Biroscikova, M., Spisakova, K., Liptak, S., Pichler, V. and Durkovic, J. 2004. Micropropagation of mature wych elm (*Ulmus glabra* Huds.). Plant Cell Rep. 22:640-644.

Bourgoin, A. and Simpson, J.D. 2004. Soaking, moist-chilling, and temperature effects on germination of *Acer pensilvanicum* seeds. Can J For Res. 34:2181-2185.

Bowen-O'Connor, C.A., Hubstenberger, J., Killough, C., VanLeeuwen, D.M. and St. Hilaire, R. 2007. In vitro propagation of *Acer grandidentatum* Nutt. In Vitro Cell Dev Biol – Plant 43: 40-50.

Brassard, N., Richer, C., Tousignant, D. And Rioux, J.A. 2003. Multiplication végétative de l' *Acer saccharum*. Contribution à la micropropagation. Can J For Res. 33:682-690.

Bredmose, N., Kristiansen, K. and Nielsen, B. 2004. Propagation temperature, PPFD, auxin treatment, cutting size and cutting position affect root formation, axillary bud growth and shoot development in miniature rose (*Rosa hybrida* L.) plants and alter homogeneity. J Hort Sci Biotechnol. 79 (3): 458-465.

Brian, P.W., Hemming, H.G. and Lowe, D. 1960. Inhibition of rooting of cuttings by gibberellic acid. Annals Bot. 24:407-419.

Brickell, C.D. 2004. The international code of nomenclature for cultivated plants. 7th ed. International society of horticultural plants, Leuven, Belgium. 123 pp.

Brown, L. 2008. Applied principles of horticultural science. 3rd ed. Butterworth-Heinemann, Burlington, MA. p.23.

Brown, J.W.S., Ersland, D.R. and Hall, T.C. 1982. Molecular aspects of storage protein synthesis during seed development. In: The Physiology and Biochemistry of Seed Development, Dormancy and Germination. Khan, A.A. ed. Elsevier Biomedical Press, New York, NY.

Brown, D.C.W., Thorpe, T.A. 1986 Plant regeneration by organogenesis. p.49-65. In: Cell culture and somatic cell genetics of plants, vol. 3. Vasil, I.K. ed. Academic Press, New York.

Bukovac, M. J. 1963. Induction of parthenocarpic growth of apple fruit with gibberellin A3 and A4. Bot Gaz. 124:191–195.

Burd, S.M. and Dirr, M.A. 1977. Propagation of selected *Malus* taxa from softwood cuttings. Proc Int Plant Prop Soc. 27:427-31.

Cao, X.L., Hammerschlag, F.A. and Douglas, L. 2002. A two-step pretreatment significantly enhances shoot organogenesis from leaf explants of highbush blueberry cv. Bluecrop. Hort Sci. 37(5):819-821.

Chalupa, V. 1988. Large scale micropropagation of *Quercus robur* L. using adenine-type cytokinins and thidiazuron to stimulate shoot proliferation. Biol Plant (Praha) 30:414-421.

Chapman, D.J. and Hoover, S. 1981. Propagation of shade trees by softwood cuttings. Proc Int Plant Prop Society 31:507-11.

Chen, S.S.C. and Chang, J.L.L. 1972. Does gibberellic acid stimulate seeds germination via amylase synthesis? Plant Physiol. 49: 441-442.

Clouse, S.D. and Sasse, J.M. 1998. Brassinosteroids: Essential regulators of plant growth and development. Ann Rev Plant Phys Plant Mol Biol. 49: 427-451.

Cooper, J.P. 1958. The effects of gibberellic acid on a genetic dwarf in *Lolium perenne*. New Phytologists 57(2):235-238.

Costa, J.M. and Challa, H. 2002. The effect of the original leaf area on growth of softwood cuttings and planting material of rose. Sci Hort. 95:111-121.

Cutler, H. G. 1991. Brassinosteroids through the looking glass. In: Brassinosteroids: Chemistry, bioactivity, and application. Cutler, H.G., Yokota, T., Adam, G. eds. ACS Symposium Series, 474. Washington: J Am Chem Soc. 334-345.

De Klerk, G. J., Brugge, J. T. and Marinova, S. 1997. Effectiveness of indoleacetic acid, indolebutyric acid, and naphthaleneacetic acid during adventitious root formation *in vitro* in *Malus* 'Jork 9'. Plant Cell Tiss Org Cult. 49:39-44.

De Klerk, G. J., W. Van der Krieken, J. C. De Jong. 1999. Review: The formation of adventitious roots: New concepts, new possibilities. In Vitro Cell Dev Biol – Plant 35:198-199.

Dedio, W. and Clark, K.W. 1971. Influence of cytokinins on isoflavone and anthocyanin synthesis in red clover seedlings. Pest Science 2(2):65-68.

Deikman, J. and Hammer, P.E. 1995. Induction of anthocyanin accumulation by cytokininins in *Arabidopsis thaliana*. Plant Physiol. 108(1):47-57.

Dirr, M.A. and Heuser, C.W., Jr. 1987. The reference manual of woody plant propagation: from seed to tissue culture. Varsity Press, Athens, GA. 239 pp.

Donnelly, J.R. 1977. Morphological and physiological factors affecting formation of adventitious roots of sugar maple stem cuttings. United States Department of Agriculture Forest Service Research Paper NE-365. 5 pp.

Druege, U., Zerche, S., Kadner, R. and Ernst, M. 2000. Relation between nitrogen status, carbohydrate distribution and subsequent rooting of *Chrysanthemum* cuttings as affected by pre-harvest nitrogen supply and cold-storage. Annals Bot. 85:687-701.

Druege U. and Kadner, R. 2008. Response of post-storage carbohydrate levels in pelargonium cuttings to reduced air temperature during rooting and the relationship with leaf senescence and adventitious root formation. Postharv Biol Tech. 47:126-135.

Dumbroff, E.B. and Webb, D.P. 1970. Factors influencing the stratification process in seeds of *Acer ginnala*. Can J Bot. 48: 2009-2015.

Dunn, S. And Townsend, R.J. 1954. Propagation of sugar maple by vegetative cuttings. J For. 52:678-679.

Dure, L. S. 1993. The lea proteins of higher plants. In: Control of Plant Gene Expression. Verma, D.P.S. (ed.). CBC Press: Boca Raton, FL. p. 325-335.

Durkovic, J. 1996. In vitro regeneration of Norway maple (*Acer platanoides* L.). Biol Plant. 38 (2):303-307.

Durkovic, J. 2003. Regeneration of *Acer caudatifolium* Hayata plantlets from juvenile explants. Plant Cell Rep. 21:1060-1064.

Durkovic, J., Pichler, V. and Lux**,** A. 2005. Micropropagation with a novel pattern of adventitious rooting in Formosan sweetgum. Can J For Res. 35 (12):2775-2780.

Environment Canada. 2006. Nature: Environmental changes and the sugar maple industry [Internet]. EnviroZine: 64. [cited on 2 December 2010] Available from: http://www.ec.gc.ca/EnviroZine/english/issues/64/nature_e.cfm.

Epstein, E. and J. Ludwig-Muller. 1993. Indole-3-butyric acid in plants: occurrence, biosynthesis, metabolism and transport. Phys Plant. 88:382-389.

Epstein, E., Zilkah, S., Faingersh, G. and Rotebaum, A. 1993. Transport and metabolism of indole-3-butyric acid in sterile easy-to-root and difficult-to-root cuttings of sweet cherry (*Prunus avium* L.). Acta Hort. 329:292-295.

Ercişli, S., Anapali, O., Esitken, A. and Sahin, U. 2002. The effects of IBA, rooting media and cutting collection time on rooting of kiwifruit. Gartenbauwissenschaft 67(1):34-38.

Fahn, A. 1982. Plant Anatomy. 3rd ed. Pergamon Press: Oxford, NY. 544 pp.

Fernández-Lorenzo, J.L., Iglesias-Díaz, M.I. and Gutiérrez-Araujo, O. 2000. Micropropagation of a selected rootstock of *Acer palmatum*. Acta Hort. 536:347-354

Finch-Savage, W.E. and Leubner-Metzger, G. 2006. Seed dormancy and the control of germination. New Phytol. 171:501–523

Fischer, P. and Hansen, J. 1977. Rooting of chrysanthemum cuttings: influence of irradiance during stock plant growth and of decapitation and disbudding of cuttings. Sci Hort. 7:171-178.

Fogaca, C.M. and Fett-Neto, A.G. 2005. Role of auxin and its modulators in the adventitious rooting of *Eucalyptus* species differing in recalcitrance. Plant Growth Reg. 45:1-10.

Foley, M.E. 2005. What is afterripening? [Abstract]. J Am Soc Hort Sci. 40(4):956.

Forest Genetics Council of British Columbia. Glossary of Forest Genetics Terms [Internet]. [Cited on 15 December 2009] Available from: http://www.fgcouncil.bc.ca/doc-glos.html

Gabriel, W.J., Marvin, J.W. and Taylor, F.H. 1961. Rooting greenwood cuttings of sugar maple–effect of clone and medium. US Department of Agriculture, Northeastern Forest Experiment Station, Upper Darby, PA. Research Note NE-144.

George, E.F., Hall, M.A. and De Klerk, G.J. 2007. Plant propagation by tissue culture. Vol 1 The background. 3rd ed. Springer: Dordrecht, Netherlands. p. 227-281.

Goldberg, R.B., Depaiva, G. and Yadegari, R. 1994. Plant embryogenesis – zygote to seed. Science 266(5185):605-614.

Grange, R. I. and Loach, K. 1983. The water economy of unrooted leafy cuttings. J Hort Sci. 58:9-17.

Gray, D. J. and Purohit, A. 1991. Somatic embryogenesis and development of synthetic seed technology. Crit Rev Plant Sci. 10:33-61.

Greenwood, M.S., Atkinson, O.R. and Yawney, H.W. 1976. Studies of hard- and easy-to-root ortets of sugar maple: Difference not due to endogenous auxin content. Plant Prop. 22:3-6.

Greenwood, M.S. and Hutchison, K.W. 1993. Maturation as a developmental process. p.14-33. In: Clonal Forestry. I. Genetics and biotechnology. Ahuja, M.R. and Libby, W.J. eds. Springer-Verlag, Berlin, Germany.

Haber, A.H. and Luippold, H.J. 1960. Separation of mechanisms initiating cell division and cell expansion in lettuce seed germination. Plant Phys. 35:168-173.

Haissig, B.E. 1974. Influences of auxins and auxin synergists on adventitious root primordium initiation and development. New Zealand J For Sci. 4:311-323.

Harbage, J.F., Stimart, D.P. and Auer, C. pH affects 1H-indole-3-butyric acid uptake but not metabolism during the initiation phase of adventitious root induction in apple microcuttings. J Amer Soc Hort Sci. 123(1):6-10.

Harlan, J.R. 1992. Crops and man. 2nd ed. American Society of Agronomy : Crop Science Society of America Inc.: Madison, WI. 284 pp.

Hartmann, H.T., Kester, D.E., Davies, F.T., Jr. and Geneve, R.L. 1997. Plant propagation: Principles and practices. 6th ed. Prentice Hall, Upper Saddle River, NJ. 770 pp.

Hazra B.G. and Pore, V.S. 1998. Brassinosteroids, new class of phytohormones. J Indian Chem Soc. 75(10-12):746-757.

Henry, P.H. and Preece, J.E. 1997. Production and rooting of shoots generated from dormant stem sections of maple species. Hort Sci. 32(7):1274-1275.

Hillhorst, H.W.M. and Karssen, C.W. 1992. Seed dormancy and germination: the role of abscisic acid and gibberellins and the importance of hormone mutants. Plant Growth Reg. 11:225-38.

Horsley, S.B., Long, R.P., Bailey, W., Hanlett, R.A. and Wargo, P.M. 2002. Health of Eastern North American sugar maple forest and factors affecting decline. Northern J Appl For. 19: 34-44.

Huetteman C.A. and Preece J.E. 1993. Thidiazuron: a potent cytokinin for woody plant cell tissue culture. Plant Cell Tiss Org Cult 33:105-121.

KeÇpczyński, J. and KeÇpczyńska, E. 1997. Ethylene in seed dormancy and germination. Phys Plant. 101(4):720-6.

Kendrick, J. 2004. Tapping the Manitoba maple – a Prairie cottage industry [Internet]. Statistics Canada: Canadian Agriculture at a Glance census year 2001. [cited on 1 December 2009]. Available from: http://www.statcan.gc.ca/pub/96-328-m/2004032/4194001-eng.pdf.

Kester, D.E. 1970. Temperature and plant propagation. Proc Int Plant Prop. Soc. 20:153-163.

Khan, A.A. 1971. Cytokinins: Permissive role in seed germination. Science 171: 853-859.

Khripach, V., Zhabinskii, V., and de Groot, A. 2000. Twenty years of brassinosteroids: Steroidal plant hormones warrant better crops for the XXI century. Annals Bot. 86:441–447.

Kibbler, H., Johnson, M.E. and Williams, R.R. 2004. Adventitious root formation in cuttings of *Backhousia citriodora* F. Muell 1. Plant genotype, juvenility and characteristics of cuttings. Sci Hort. 102: 133-143.

Kibbler, H., Williams, C.M., Williams, R.R. and Johnson, M.E. 2002. Inhibition of adventitious rooting in *Backhousia citriodora* F. Muell. cuttings correlate with the concentration of essential oil. J Hort Sci Bio. 77:705-711.

King, R.W. 1982. Abscisic acid in seed development. In the physiology and biochemistry of seed development, dormancy and germination. Khan, A.A. ed. Elsevier biomedical Press, New York, NY.

Kling, G.J., Meyer, M.M., Jr. And Seigler, D. 1988. Rooting cofactors in five *Acer* species. J Amer Soc Hort Sci. 113:252-257.

Lin, X. and Leung, D.W.M. 2002. Culture of isolated zygotic embryos of *Pinus radiata* D. Don. Part I: Factors influencing *in vitro* germination and growth of isolated embryos. In Vitro Cell Dev Biol – Plant 38:191-197.

Linden, L. and Riikonen, A. 2006. Effects of 6-benzylaminopurine, thidiazuron, and type of explant on in vitro development of *Acer platanoides* L. Prop Ornam Plants 6(4):201-204.

Ludwig-Muller, J.L. 2000. Indole-3-butyric acid in plant growth and development. J Plant Growth Reg. 32:219-230.

MacDonald, B. 1986. Practical woody plant propagation for nursery growers. Timber Press, Portland, OR. 660 pp.

Mansouri, K. and Preece, J.E. 2009. The influence of plant growth regulators on explant performance, bud break, and shoot growth from large stem segments of *Acer saccharinum* L. Plant Cell Tiss Org Cult. 99:313-318.

Mark, T.R. and Simpson, S.E. 1994. Factors affecting shoot development in apically dominant *Acer* cultivars *in vitro*. J Hort Sci. 69: 543-551.

Maximova, S.N., Young, A., Pishak, S. and Guiltinan, M.J. 2008. Field performance of *Theobroma cacao* L. plants propagated via somatic embryogenesis. In Vitro Cell Dev Biol – Plant 44(6):487-493.

McCullough, D. and Usborne, R. Emerald ash borer: Frequently asked questions [Internet]. Emerald ash borer; c2008 [cited 17 September 2009]. Available from http://www.emeraldashborer.info/faq.cfm#6.

Metaxas, D., Syros, T. and Economou, A. 2008. Factors affecting vegetative propagation of *Arbutus unedo* L. by stem cuttings. Prop Ornam Plants 8(4):190-197.

Miles, D.F., TeKrony, D.M. and Egli, D.B. 1988. Changes in viability, germination, and respiration of freshly harvested soybean seed. Crop Sci. 28:700-704.

Moe, R. 1973. Propagation, growth and flowering of potted roses. Acta Hort. 31:35-50.

Mok, M.C. 1994. Cytokinins in plant development – An overview (Chapter 12). p.155-166. In: Cytokinins chemistry, activity and function. Mok, D.W.S., Mok, M.C. eds. CRC Press, Boca Raton, Florida.

Murakami, P.F., Turner, M.R., van den Berg, A.K. and Schaberg, P.G. 2005. An instructional guide for leaf color analysis using digital imaging software. USDA Forest Service, Northeastern Research Station, Newton Square, PA. General Technical Report NE-327.

Nickell, L. G. 1982. Plant growth regulators. Agricultural uses. Springer, New York, pp.4-5.

Nicolic, R., Mitic, N., Miletic, R. and Neskovic, M. 2006. Effects of cytokinins on *in vitro* seed germination and early seedling morphogenesis in *Lotus corniculatus* L. J Plant Growth Reg. 25:187-194.

Olson, D.F. Jr., Gabriel, W.J. 1974. *Acer* L., maple. P. 187-194. In: Seeds of woody plants in the United States. Schopmeyer CS, tech. coord. Agric. Handbk. 450. USDA Forest Service, Washington, DC.

Ozeki, Y. and Komamine, A. 1986. Effects of growth regulators on the induction of anthocyanin synthesis in carrot suspension cultures. Plant Cell Phys. 27(1):1361-1368.

Pan, R. and Gui, H. 1997. Physiological basis of the synergistic effects of IBA and triadimefon on rooting of mung bean hypocotyls. Plant Growth. Reg. 22:7-11.

Panaia, M., Bunn, E., Turner, S.R. and McComb, J. 2009. Incubation temperature critical to successful stimulation of in vitro zygotic embryo growth in four Australian native Cyperaceae species. Plant Cell Tiss Org Cult. 97:197-202.

Paton, D.M., Willing R.R., Nichols, W. and Pryor, L.D. 1970. Rooting of stem cuttings of eucalyptus: A rooting inhibitor in adult tissue. Aus J Bot. 28:175-183.

Pech y Aké, A., Maust, B., Orozco-Segovia, A. and Oropeza, C. 2007. The effect of gibberellic acid on the in vitro germination of coconut zygotic embryos and their conversion into plantlets. In Vitro Cell Dev Biol – Plant. 43:247-253.

Poland, T.M. and McCullough, D.G. 2006. Emerald Ash borer: Invasion of the urban forest and the threat to North America's ash resource. J For. 104(3):118-124.

Preece, J.E. and Imel, M.R. 1991. Plant regeneration from leaf explants of *Rhododendron* 'P.J.M. Hybrids'. Sci Hort. 48:159-170.

Preece J.E., Huetteman C.A., Ashby W.C. and Roth P.L. 1991a. Micro- and cutting propagation of silver maple. I Results with adult and juvenile propagules. J. Am Soc Hort Sci. 116(1):142-148.

Preece J.E., Huetteman C.A., Ashby W.C. and Roth P.L. 1991b. Micro- and cutting propagation of silver maple. II. Genotype and provenance affect performance. J. Am Soc Hort Sci. 116(1):149-155.

Quist, T. HORT 201 Plant propagation – Growth regulators in propagation [Internet].Department of horticulture and landscape architechture, Purdue University. [cited 29 April 2009]. Available from http://www.hort.purdue.edu/hort/courses/HORT201/Lec/020209GrowthRegulatorsProp.pdf.

Rasmussen, A., Smith, T.E. and Hunt, M.A. 2009. Cellular stages of root formation, root system quality and survival of *Pinus elliottii* var. *elliottii* × *P.caribea* var. *hondurensis* cuttings in different temperature environments. New Forests 38:285-294.

Raven, P.H., Evert, R.F. and Eichhorn, S.E. 1999. Biology of plants. 6th ed. W.H. Freeman and Co. /Worth Publishers, New York, NY.

Richer, C., Tousignant, D., Rioux, J.-A., Mottard, J.-P. and Brassard, N. 2003. Exclusion partielle ou totale de la lumière sur les tiges d'*Acer saccharum* avant le bouturage. Can J Plant Sci. 83:869–872.

Rioux, J.-A., Richer, C., Lamy, M.-P., Tousignant, D. and Brassard, N. 2003. Propagation végétative de l'érable à sucre : effetsde divers traitements hormonaux ou chimiques sur l'enracinement de boutures prélevées sur plusieurs arbres. Can J Plant Sci. 83:851–858.

Rogan, P.G. and Simon, E.W. 1975. Root growth and the onset of mitosis in germinating *Vicia faba*. New phytol. 74:273-275.

Ronald, P. Emerald ash borer background information. Landscape Manitoba Newsletter – Spring 2006. [cited on 22 September 2009]. Available from: http://www.canadanursery.com/Storage/11/743_News-Spring-06-2.pdf.

Rosier, C.L., Frampton, J., Goldfarb, B., Wise, F.C. and Blazich, F.A. 2005. Stumping height, crown position, and age of parent tree influence rooting of stem cuttings of fraser fir. Hort Sci. 40(3):771-777.

Russel, J.A. and McCown, B.H. 1986. Thidiazuron-stimulated shoot differentiation from protoplast-derived calli of *Populus*. VI Intl Congr Plant Tiss Cell Cult Abstracts p.49.

Schleiden, M.J. 1938. Beitrage zur phytogenesis. Muller Arch Anat Und Physiol. 4:137-176.

Schwartz, B.W., Yeung, E.C., Meinke, D.W. 1994. Disruption of morphogenesis and transformation of the suspensor in abnormal *suspensor* mutants of *Arabidopsis*. Development 120:3235–3245.

Shih, M.D., Hoekstra, F.A. and Hsing, Y.I.C. 2008. Late embryogenesis abundant proteins. p. 211-255. In: Advances in Botanical Research vol. 48. Kader, J.-C. and Delseny, M. eds. Academic Press, San Diego, CA.

Simon, E.W. 1984. Early events in germination. p.77-116.In Seed Physiology; Vol. 2: Germination and Reserve Mobilization. R.M. Murray, ed. Academic Press, New York, NY.

Smith, R.H. 1992. Plant tissue culture techniques and experiments. Academic Press, San Diego, CA. p.57.

Spethmann, W. 2007. Increase of rooting success and further shoot growth by long cuttings of woody plants. Prop Ornam Plants 7(3):160-166.

Statistics Canada. 2008. Service Buletin: Production and value of honey and maple products 2008 [Internet]. Catalogue no. 23-221-X. [cited on 17 November 2009] Available from: http://www.statcan.gc.ca/pub/23-221-x/23-221-x2008000-eng.pdf.

Steeves, T.A. and Sussex, I.M. 1989. Patterns in plant development. 2nd ed. Cambridge University Press, Cambridge, NY. 388 pp.

Steward, F.C., Mapes, M.O. and Mears, K. 1958. Growth and organized development of cultured cells. II. Organization in cultures grown from freely suspended cells. Amer J Bot. 45:705-708.

Stirk, W.A., Gold, J.D., Novak, O., Strnad, M. and van Staden, J. 2005. Changes in endogenous cytokinins during germinatin and seedling establishment of *Tagetes minuta* L. J. Plant Growth Reg. 47:1-7.

Stojičić, D., Janošević, D., Uzelac, B. and S. Budimir. 2008. Factors influencing germination and growth of isolated embryos of Pinus heldreichii. Arch Biol Scie. 60(4):673-679.

Sugiyama, M. 1999. Organogenesis in vitro. Curr Op. Plant Biol. 2:61-64.

Swamy, K.N. and Seeta Ram Rao, S. 2006. Influence of brassinosteroids on rooting and growth of geranium (*Pelargonium* sp.) stem cuttings. Asian J Plant Sci. 5(4):619-622.

Taiz, L and Zeiger, E. 2002. Plant physiology. 3rd edition. Sinauer Associates, Inc., Sunderland, Massachusetts. p.494-496, 504-505.

Tousignant, D., Richer, C., Rioux, J.-A., Brassard, N., Mottard, J.-P. 2003. Vegetative propagation of sugar maple: Relating stem water content and terminal bud developmental stage to adventitious rooting of stem cuttings. Can J Plant Sci. 83:859-867.

USDA, Agricultural Research Services, National Genetic Resources Program. *Germplasm Resources Information Network - (GRIN)* [Online Database]. National Germplasm Resources Laboratory, Beltsville, Maryland. [cited on 13 December 2009]. Available from: http://www.ars-grin.gov/cgi-bin/npgs/html/taxon.pl?1227.

Vakouftsis, G., Syros, T., Kostas, S., Economou, A.S., Tsoulpha, P., Scaltsoyiannes, A. and Metaxas, D. 2009. Effect of IBA, time of cutting collection, type of cuttings and rooting substrate on vegetative propagation in *Cupressus macrocarpa* 'Goldcrest'. Prop Ornam Plants 9(2):65-70.

Van den Berg, A.K. and Perkins, T.D. 2004. Evaluation of a portable chlorophyll meter to estimate chlorophyll and nitrogen contents in sugar maple (*Acer saccharum* Marsh.) leaves. For Ecol Man. 200:113-117.

Veierskov, B. 1988. Relations between carbohydrates and adventitious root formation. In: Adventitious root formation in cuttings. Davis, T. D., Haissig, B.E., Sankhla, N. eds. Dioscorides Press: Portland, OR. p.70-78.

Vengadesan, G. and Pijut, P.M. 2009. In vitro propagation of northern red oak (*Quercus rubra* L.). In Vitro Cell Dev Biol. – Plant 45:474-482.

Wann, S.R. and Gates, E.E. 1993. Micropropagation of mature red maple (*Acer rubrum* L.) in Concurrent Session 2: Hardwood genetics and propagation of 23rd Southern Forest Tree Improvement Conference Proceedings in Atlanta, Georgia. June 14-17.

Webb, D.P. and Dumbroff, E.B. 1969. Factors influencing the stratification process in seeds of *Acer saccharum*. Can J Bot. 47:1555-1563.

Webb, D.P., Staden, J.V. and Wareing, P.F. 1973. Seed dormancy in *Acer*. J Exp Bot. 24:105-116.

Werner, T., Motyka, V., Strnad, M. and Schmülling, T. 2001. Regulation of plant growth by cytokinin. Proc Natl Acad Sci USA 98:10487–10492.

Wiesman, Z. and Lavee, S. 1995. Enhancement of IBA stimulatory effect on rooting of olive cultivar stem cuttings. Sci Hort. 62:189-198.

Wilhelm, E. 1999. Micropropagation of juvenile sycamore maple via adventitious shoot formation by use of thidiazuron. Plant Cell Tiss Org Cult. 57:57-60.

Wilkins, L.C., Graves, W.R., and Townsend, A.M. 1995. Development of plants from single-node cuttings differs among cultivars of red maple and freeman maple. Hort Sci. 30 (2):360-362.

Wojtusik, T., Boyd, M.T., and Felker P. 1994. Effect of different media on vegetative propagation of Prosopis juliflora cuttings under solar-powered mist. For Ecol Man. 67:267-271.

Yusnita, S, Geneve, R.L. and Kester, S.T. 1990. Micropropagation of white flowering eastern redbud (*Cercis Canadensis* var *alba* L.). J Env Hort. 8:177-179.

Zhang, H., Graves, W.R., Townsend, A.M. 1997. Water loss and survival of stem cuttings of two maple cultivars held in subirrigated medium at 24 to 33°C. Hort Sci. 32(1):129-131.

9.0 APPENDICES

Appendix I Media composition inducing conversion in sugar maple (*Acer saccharum*) embryos to produce whole plantlets (Abbreviations: IBA – indole-3-butyric acid, BAP – benzylamino purine, GA_3 – gibberellic acid, ZEA – zeatin, TDZ – thidiazuron).

Ingredient	Amount (mg/L)	Supplemented plant growth regulators (PGRs)					
		IBA	BAP	GA3	ZEA	TDZ	BAP+TDZ
Murashige-Skoog basal salt mixtures		0.5, 1.0 or 1.5 mg/L	0.5, 1.0 or 1.5 mg/L	0.1, 0.5 or 1.0 mg/L	0.5 or 1.5 mg/L	0.001 or 0.005 mg/L	1.5 mg/L of IBA and 0.001 mg/L of TDZ
NH_4NO_3	1650						
KNO_3	1900						
$CaCl_2 \bullet 2H_2O$	440						
$MgSO_4 \bullet 7H_2O$	370						
KH_2PO_4	170						
H_3BO_3	6.2						
$MnSO_4 \bullet H_2O$	16.9						
$ZnSO_4 \bullet H_2O$	8.6						
KI	0.83						
$NaMoO_4 \bullet 2\ H_2O$	0.25						
$CuSO_4 \bullet 5H_2O$	0.025						
$CoCl_2 \bullet 6H_2O$	0.025						
$FeSO_4 \bullet 7H_2O$	27.8						
$Na_2EDTA \bullet 2H_2O$	37.3						
LV vitamin stock							
Nicotinic acid	160						
Pyridoxine HCl	32						
Thiamine HCl	32						
LV iron stock							
FeSO4•7H2O	546						
$Na_2EDTA \bullet 2H_2O$	724						
Glycine	50						
Glutamine	50						
Serine	50						
Myo-inositol	100						
Phytagel	3500						
Sucrose	10000						

Appendix II Media composition for shoot organogenesis study using sugar maple (*Acer saccharum*) buds, dormant and active, collected between March and May of 2008 (Abbreviations: TDZ – thidiazuron, 2iP – 2-isopentenyl adenine, PAA – phenylacetic acid, NAA – naphthaleneacetic acid).

Ingredient	Amount (mg/L)	Type of medium			
		I. Induction	II. Multiplication	III. Elongation	IV. Rooting
Murashige-Skoog basal salt mixtures		No PGR added	0.01 mg/L of TDZ and 2 mg/L of 2iP	0.3 mg/L of PAA	1 mg/L of NAA and 0.3 mg/L of PAA
NH_4NO_3	825				
KNO_3	950				
$CaCl_2 \cdot 2H_2O$	220				
$MgSO_4 \cdot 7H_2O$	185				
KH_2PO_4	85				
H_3BO_3	3.1				
$MnSO_4 \cdot H_2O$	8.45				
$ZnSO_4 \cdot H_2O$	4.3				
KI	0.415				
$NaMoO_4 \cdot 2\ H_2O$	0.125				
$CuSO_4 \cdot 5H_2O$	0.0125				
$CoCl_2 \cdot 6H_2O$	0.0125				
$FeSO_4 \cdot 7H_2O$	13.9				
$Na_2EDTA \cdot 2H_2O$	18.65				
LV vitamin stock					
Nicotinic acid	160				
Pyridoxine HCl	32				
Thiamine HCl	32				
LV iron stock					
FeSO4•7H2O	546				
$Na_2EDTA \cdot 2H_2O$	724				
Glycine	50				
Glutamine	50				
Serine	50				
Myo-inositol	100				
Phytagel	3500				
Sucrose	15000				

Appendix III Media composition for various steps in shoot organogenesis of embryo explants harvested from sugar maple (*Acer saccharum*) seeds. The four types of medium represent the four stages in the process (Abbreviations: BAP – benzylamino purine, TDZ – thidiazuron, IBA – indole-3-butyric acid).

Ingredient	Amount (mg/L)	Type of medium			
		I. Induction	II. Shooting	III. Maintenance	IV. Rooting
Murashige-Skoog basal salt mixtures		225.25 µg/L of BAP and 22.03 µg/L of TDZ	8.81 µg/L of TDZ	No PGR added	25.0 mg/L of IBA
NH_4NO_3	1650				
KNO_3	1900				
$CaCl_2 \cdot 2H_2O$	440				
$MgSO_4 \cdot 7H_2O$	370				
KH_2PO_4	170				
H_3BO_3	6.2				
$MnSO_4 \cdot H_2O$	16.9				
$ZnSO_4 \cdot H_2O$	8.6				
KI	0.83				
$NaMoO_4 \cdot 2\ H_2O$	0.25				
$CuSO_4 \cdot 5H_2O$	0.025				
$CoCl_2 \cdot 6H_2O$	0.025				
$FeSO_4 \cdot 7H_2O$	27.8				
$Na_2EDTA \cdot 2H_2O$	37.3				
LV vitamin stock					
Nicotinic acid	160				
Pyridoxine HCl	32				
Thiamine HCl	32				
LV iron stock					
FeSO4•7H2O	546				
$Na_2EDTA \cdot 2H_2O$	724				
Glycine	50				
Glutamine	50				
Serine	50				
Myo-inositol	100				
Phytagel	3500				
Sucrose	30000				

Appendix IV Media composition for shoot organogenesis study using sugar maple (*Acer saccharum*) shoot tips and petiole segments collected in June 2008. (Abbreviations: BAP – benzylamino purine, TDZ – thidiazuron)

Ingredient	Amount (mg/L)	Explant type	
		Shoot tips	Petiole segments
Murashige-Skoog basal salt mixtures		2.5 µM of BAP	0.04, 0.1 or 0.2 µM of TDZ
NH_4NO_3	825		
KNO_3	950		
$CaCl_2 \cdot 2H_2O$	220		
$MgSO_4 \cdot 7H_2O$	185		
KH_2PO_4	85		
H_3BO_3	3.1		
$MnSO_4 \cdot H_2O$	8.45		
$ZnSO_4 \cdot H_2O$	4.3		
KI	0.415		
$NaMoO_4 \cdot 2\ H_2O$	0.125		
$CuSO_4 \cdot 5H_2O$	0.0125		
$CoCl_2 \cdot 6H_2O$	0.0125		
$FeSO_4 \cdot 7H_2O$	13.9		
$Na_2EDTA \cdot 2H_2O$	18.65		
LV vitamin stock			
Nicotinic acid	160		
Pyridoxine HCl	32		
Thiamine HCl	32		
LV iron stock			
FeSO4•7H2O	546		
$Na_2EDTA \cdot 2H_2O$	724		
Glycine	50		
Glutamine	50		
Serine	50		
Myo-inositol	100		
Phytagel	3500		
Sucrose	15000		