EXPLORING HOW TEMPERATURE AFFECTS DORMANCY INDUCTION AND COLD ACCLIMATION INITIATION IN HYBRID POPLAR

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ABSTRACT

Dormancy, cold hardiness and height growth were examined in four poplar clones exposed to four temperature conditions (13.5ºC/8.5ºC, 18.5ºC/3.5ºC, 18.5ºC/13.5ºC and 23.5ºC/8.5ºC day/night temperatures) under short photoperiod. The selected clones were 'WP-69' ('Okanese')- early acclimation, 'Walker' and 'Katepwa' - intermediate acclimation, and 'Prairie Sky'- late acclimation. Changes in physical water properties and mobility within the vascular tissue region, vascular transition region into the axillary bud and the upper axillary bud were assessed during endodormancy development using Magnetic Resonance Microimaging (MRMI).

In summary:

a) There were distinct differences between poplar clones during dormancy induction in response to temperature. For example, 'Katepwa', 'Walker' and 'WP-69' clones became endodormant but 'Prairie Sky' did not enter endodormancy. Endodormancy development and cold acclimation in 'WP-69' were less affected by temperature than 'Katepwa' and 'Walker' suggesting that genotypic variation exists in response to temperature change.

b) Growth cessation, not endodormancy, was a prerequisite for cold acclimation since cold hardiness increased in 'Prairie Sky' in the absence of endodormancy. However, increases in endodormancy coincided with increase in cold hardiness in other clones.

c) Low night temperatures (18.5ºC/3.5ºC) delayed endodormancy development and cold acclimation in all clones compared to the warm night temperature treatment (18.5ºC /13.5ºC). Night temperature was negatively correlated with time to growth cessation, and cold hardiness and positively correlated with dormancy development. Changes in night temperature may affect time to growth cessation, subsequently altering timing of cold acclimation and endodormancy development since growth cessation appeared to be a prerequisite for both processes.

d) ADC (Apparent Diffusion Coefficient), an indicator of water mobility within living tissues, was negatively correlated with endodormancy induction. Specifically, the transition region of vascular tissue between the stem and the lower axillary bud showed the highest correlation with endodormancy development. By contrast, decreases in T_1

relaxation times, an indicator of biophysical water properties, were inconsistent with changes in endodormancy levels in axillary buds. Thus, ADC appears to correspond more closely with endodormancy development than changes in T_1 relaxation times.

It is apparent that temperature impacts dormancy development in hybrid poplar. Underlying changes in water appear to correspond with changes in endodormancy. Under future warming scenarios, genotypes such as WP 69 (‘Okanese’) that are less sensitive to temperature and maintain a consistent, endodormancy induction pattern, may be better fit to changing climates.

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1.0 INTRODUCTION

Predicted future temperature increases could impact growing season length and temperature fluctuations during all seasons (IPCC, 2007), including autumn. Increases in temperature may affect overall minimum temperatures, which is a major limitation to the distribution of a plant species in temperate regions. Furthermore, rate of temperature change between seasons and the ability of a plant to acclimate is essential to survive the onset of winter conditions. During the growing season, cold hardiness of woody plants in the temperate regions are just below 0 ºC (Sakai, 1965; Burke *et al*., 1976). In autumn, to survive, plants in these regions must stop growth in advance of the onset of cold temperatures. Once growth cessation and dormancy have been initiated, plants begin to acclimate to survive cold conditions (Weiser, 1970). This period where no growth occurs, or dormancy, is an adapted response by woody plants, which synchronizes growth patterns with the environment and subsequently helps withstand the cold winter conditions characteristic of these regions. Woody plants must maximize growth while minimizing risk of damage or death during the transition periods between active growth and the dormant state. Understanding how temperature changes may affect dormancy and related processes is necessary to predict how woody plants will respond to changing seasons under conditions resulting from predicted increases in temperature.

Short photoperiod is considered to be the main factor regulating growth cessation and dormancy development in woody plants (Garner and Allard, 1923; Kramer, 1936; Wareing, 1956; Weiser, 1970; Olsen *et al*., 1997). However, other factors have also been reported to affect timing and rate of dormancy induction. These include temperature (Junttila, 1980; Junttila *et al.*, 2003; Svendsen *et al.*, 2007) and abiotic and biotic stresses (Stevenson, 1994; Chen and Li, 1978). Low temperatures have been shown to induce dormancy in northern ecotypes of *Cornus sericea* L. (Svendsen *et al.*, 2007) and *Populus tremula* x *Populus tremuloides* Michx. (Welling *et al*., 2002) even under long days. However, when studying temperature-short photoperiod interactions, warmer temperatures have been shown to induce earlier growth cessation and dormancy development (Fuchigami *et al.*, 1971; Fuchigami *et al.*, 1982; Stevenson, 1994; Heide, 2003; Junttila *et al.*, 2003). Palonen (2006) reported earlier growth cessation was observed under

warmer temperatures than cooler temperatures in raspberry (*Rubus idaeas* L.). Furthermore, deeper dormancy was induced in raspberry under warm temperatures (20ºC) compared to cool temperatures (4ºC) (Palonen, 2006). Contrasting dormancy development in response to temperature highlights the importance of identifying responses of hybrid poplar to temperature change. There may be species-specific or origin-specific responses to temperature that suggest certain species or ecotypes may tolerate temperature change better than others.

Fuchigami *et al*. (1982) suggests that development of significant cold hardiness is dependent on attainment of vegetative maturity, which can be defined as the point at which growth and bud break will not occur even if favourable environmental conditions are present. In contrast, Fuchigami *et al*. (1971) and Weiser (1970) suggest that growth cessation is the prerequisite for both dormancy development and significant cold acclimation. Determining the point at which increases in cold hardiness occur, and the prerequisites required for cold hardiness attainment, is necessary to identify the interactions between dormancy acquisition and cold hardiness.

Although assessment of whole-plant responses to temperature during dormancy induction identifies how different woody plants may respond to climate change, underlying physiological changes occurring within the plant regulates these responses. Differential physiological changes regulating growth, dormancy and cold hardiness are of particular importance in understanding how woody plants will tolerate temperature change during the autumn. No single approach has been successful in determining how dormancy is regulated. Although hormonal studies have dominated dormancy research, Tanino (2004) suggested that assessing the relationships between mass hormonal levels and dormancy regulation would not in itself be successful because there are processes unrelated to dormancy occurring at the same time that are also regulated by hormones. Other approaches have included examining gene expression (Chen *et al*., 2000) and protein changes (Arora *et al.*, 1992). Dormancy is a complex, dynamic, multi-faceted phenomonen (van der Schoot, 1996) and as such, a physiological approach that incorporates knowledge developed from previous studies is valuable in identifying the underlying mechanisms of bud dormancy.

Changes in biophysical properties of water during dormancy induction are a combination of many physiological changes associated with bud dormancy. These changes include saccharides (Cox and Stushnoff, 2003; Marquat *et al.*, 1999; Palonen, 1999), proteins (Arora *et*

al., 1992; Rowland *et al.*, 1996) and lipids (Izadyar and Wang, 1999). These physiological changes during dormancy induction can affect the biophysical properties of water. Water content also markedly decreases during dormancy induction (Faust *et al*., 1991; Welling *et al.*, 2002; Jeknic and Chen, 1999; Li *et al.*, 2003; Wake and Fennell, 2000). These changes in water content and biophysical properties of water can be measured using Nuclear Magnetic Resonance (NMR) imaging. Faust *et al.* (1991) identified changes in water status, using NMR in chilled and unchilled apple (*Malus sp*.). These changes in water status have also been observed in grape buds (Gardea *et al.*, 1994; Fennell *et al.*, 1996). Identifying changes in water and the regulation of water movement within woody plants may support emerging theories on dormancy mechanisms.

Hybrid poplar is a suitable system for studying how temperature affects dormancy-related processes. Poplar is an ideal model system for studying physiological processes in woody plants because of their rapid growth, simple propagation, and mapped genome sequence (Brunner *et al.*, 2004). Poplar has a large geographic range and high compatibility in interspecific hybridization. This produces high variability in growth and physiological characteristics in interspecific hybrids adapted to a particular climate. Poplar clones used in this study were selected from hybrid poplar clones that have been successfully grown on the Canadian prairies with similar growth rates, yet have contrasting cold acclimation patterns (Silim *et al.*, 2005, unpublished). 'WP-69' ('Okanese') acclimates early, 'Katepwa' and 'Walker' show intermediate acclimation trends and 'Prairie Sky' acclimates late in the fall and is susceptible to winter damage. The contrasting patterns make these clones suitable material to explore how ambient temperatures during the dormancy induction phase affects dormancy development and other related processes such as growth cessation and cold acclimation.

While other studies have explored how temperature affects dormancy, few have specifically looked at day-night temperature differences. This study aimed to determine how day-night temperatures affected dormancy development and cold acclimation in four contrasting poplar clones under short-day photoperiods in controlled environment chambers. In addition, changes in water status and water mobility during dormancy induction were examined. This project yielded significant insight into how temperature interacts with dormancy and related processes. It also contributed towards understanding the extent to which woody plants are able to tolerate temperature increases during the fall induction period. Using NMR imaging, the

connection between water mobility and biophysical properties of water andbud dormancy was established. NMR imaging is a useful tool for non-destructively assessing localized water in plant tissues.

2.0 LITERATURE REVIEW

2.1 Climate Change

Over the next 75 years, mean global temperature is forecast to increase between 1.1 and 6.4ºC (IPCC, 2007). This is partially a product of increasing greenhouse gases, such as carbon dioxide and methane (IPCC, 2007). Temperature increases will be more pronounced at northern latitudes and interiors of continents including North America (IPCC, 2007). The effect of temperature increases on ecosystem processes and agricultural productivity is not completely known. Motha and Baier (2005) suggested that agricultural productivity will decline in southern regions of the Great Plains but agricultural productivity may increase in northern regions. These predictions were based on a best-case scenario in which temperature increased by 1.5ºC by 2100. Further increases in temperature will have a negative effect on agricultural productivity in all regions because of increased incidence of drought, insects and disease (IPCC, 2007). Plant species have naturally shifted in response to temperature change in the past. However, domesticated plants may not maintain the same ability to tolerate temperature change.

One notable effect of climate change will be the increase in length of the growing season (Motha and Baier, 2005). Wheaton (2001) predicted mean temperature during autumn to be 3 to 5ºC higher over the next 75 years. The mean temperature (1961-1991) during September in Saskatoon (52º7'N) is 11ºC (Environment Canada, 2007). Specifically, changes during the autumn and spring affect growth patterns of plants because these temperature changes can affect the growing season. Woody plants used for shelterbelts are important for water conservation; improving biodiversity and providing snow cover during the winter on the Canadian prairies (Agriculture and Agri-Food Canada, 2007). It is unknown how these cultivars and selections of woody plants, with relatively low genetic variability, will tolerate temperature change. The ability of woody plants, particularly domesticated plants, to effectively respond to temperature change and changes in the growing season is important for agricultural production, forestry and horticulture.

2.2 Poplar Biology

The genus *Populus*, which contains many economically important species, belongs to the family Salicaceae. Salicaceae is a member of the class of angiosperms, Malpighales. The genus is divided into six subgroups: Leuce, Aigeros, Tacamahaca, Leucoides, and Turanga (Eckenwalder, 1996). Although classification within this genus is complex, there is a general agreement of approximately 30 species within the genus. There are two common classifications of *Populus*, poplar and aspen. Although hybridization is common within Populus, natural hybridization between poplar and aspen does not occur in nature.

The geographical range of *Populus* falls within the temperate regions in the northern hemisphere, however, a small population exists in the subtropical mountains of eastern and northern Africa (Bradshaw *et al.*, 2000). Individual poplar species commonly have wide geographical distributions, sometimes spanning across a large latitudinal range. This distribution produces ecotypes adapted to varied environments producing latitudinal, altitudinal and coastal-inland clines within species. For example, *Populus tremuloides* has populations extending east from Alaska to Eastern Canada and south to Mexico where it survives at high altitudes. Other examples include *P. tremula* in Europe and *P. balsamifera* in North America.

The genus *Populus* is adapted to survive many different climates with different environmental stresses including cold and drought. Poplars are dioecious and rely on wind for pollen and seed dispersal. They also reproduce asexually from roots and shoots allowing them to survive frequent fires and other disturbances. This ability to survive disturbances and wind-dispersed seed allows for rapid occupation of recently disturbed areas in both upland (Aspens) (Burns and Honkala, 1990) and lowland sites (Cottonwoods) (Braatne *et al.*, 1996). Species within *Populus* are often considered to be primary succession species because of their ability to thrive in high disturbance regions (Peterson and Peterson, 1994).

Populus are deciduous trees that regularly lose their leaves in the fall. New growth initially originates from a preformed shoot within the bud where growth is initiated in the spring and the meristem continues to expand and grow throughout the season until bud set in the fall (Bradshaw *et al.*, 2000). Flowering in poplar occurs relatively early compared to other woody plants with flowering occurring in as little as 3-6 years from seed (Brunner *et al.*, 2004). This is advantageous for studying phenology and rapid generation times are beneficial for breeding.

Poplar can exhibit a large degree of morphological plasticity where density significantly affects structure of the tree and canopy size and growth patterns (Peterson and Peterson, 1994). All poplar species are diploid (2n = 38) resulting in interspecific compatibility within subgroups and between some subgroups. Interspecific hybrids are common, both naturally and in conventional breeding programs. Interspecific hybrids demonstrate heterosis (hybrid vigour). Hybrid poplars generally have a more rapid growth rate than their parental species. Rapid growth rate commonly observed in hybrid poplar is a valuable trait for developing poplar as a short rotation woody crop. Maturity (20-30m tall trees) of hybrid poplar can be reached in as little as 8-20 years. In contrast, maturity time of *Populus tremuloides,* a common poplar in forests of Canada, is approximately 75 years (Peterson and Peterson, 1994).

The genome of *Populus trichocarpa* has recently been mapped (Brunner *et al.*, 2004), increasing the potential for identifying candidate genes that control physiological processes such as bud dormancy, cold acclimation, drought tolerance and wood formation. The genome size of *Populus trichocarpa* is only 4 times larger than *Arabidopsis* and is significantly smaller than other frequently studied woody plant species (Bradshaw and Stettler, 1993). A 'poplar chip' with 15,000 ESTs (Expressed Sequence Tags) for microarray analysis has been used to identify candidate genes that control physiological processes (Brunner *et al.*, 2004). Poplar is an effective plant system for physiological studies because of ease of propagation, rapid growth and establishment, availability of germplasm, high degree of variability, transformability and a significant amount of literature already present on poplar. A number of poplar-based physiological studies have been conducted, including genetic variability (Bradshaw and Stettler, 1993), wood formation (Mijnsbrugge *et al.*, 2000), drought stress (Street *et al.*, 2006), dormancy (Howe *et al.*, 2003) and cold hardiness (McCamant and Black, 2000), among others.

2.3 Bud Dormancy in Woody Plants

2.3.1 Definition of Dormancy

Woody plant distribution in cold temperate climates is primarily determined by the ability to survive temperatures below zero. For survival, woody plants within these regions have evolved the ability to stop growth in advance of onset of unfavourable conditions. Cessation of

growth is a response by woody plants to synchronize growth with environmental conditions. Furthermore, the ability to maintain cold hardiness under warm abnormal, warm conditions is dependent on the ability to maintain its dormant state under fluctuating temperatures (Tanino *et al.*, 1989). There is a trade-off between maximizing growth to be competitive and stopping growth to avoid damage from cold winter conditions. An accurate, complete definition of dormancy has been difficult to develop because the physiological, genetic and biochemical mechanisms of dormancy are still not fully understood. Doorenbos (1953) defined dormancy as "any case in which a tissue predisposed to elongate does not do so". While this definition was simple, more recent definitions focused on growth of the meristem (Fuchigami *et al.*, 1982; Lang *et al.*, 1987). Fuchigami *et al.* (1982) defined dormancy as a state where the bud is not growing while Lang *et al.* (1987) described dormancy as a state where there is no visible growth of any structure containing a meristem. These definitions, while correct, tend to imply that dormancy is a discrete stage rather than a dynamic, continuous phase that changes in response to environmental stimuli. These definitions do not take into account the physiological, biochemical and molecular changes associated with dormancy. Dormancy is a complex, dynamic, multi-faceted phenomenon (van der Schoot, 1996) with different stages. There are most likely multiple factors regulating dormancy status and transition between phases of dormancy in woody plants. Dormancy is a process regulated internally, initiated by environmental conditions where changes in gene expression promote physiological, biochemical, morphological and phenological changes within a meristem of a plant that result in growth cessation and inability to resume growth when favorable environmental conditions are present.

Dormancy is not a single process and therefore cannot be generalized into one stage. In an effort to describe and identify dormancy more accurately, researchers have divided the dormant phase of woody plants into different stages that correspond to the response of a plant (bud) to environmental conditions that promote growth. Samish (1954) defined three stages of dormancy, quiescence, rest and correlative inhibition. "Quiescence", initially suggested by Meyer and Anderson (1952; cited in Samish, 1954), is the stage of dormancy when no growth occurs as a result of unfavourable environmental conditions. "Rest", defined by Chandler (1925; cited in Samish, 1954), is the stage when no bud growth occurs even when environmental conditions are favourable. This is caused by internal factors within the bud itself. "Correlative

inhibition" is defined as the stage where factors within the plant, but outside of the bud, such as apical dominance prevent bud break.

Lang (1987) proposed new terminology in an attempt to establish more accurate terminology than the three terms described above. These three different stages are endo-, eco- and paradormancy (Lang, 1987). Endodormancy is the equivalent of "rest", ecodormancy is the equivalent of "quiescence" and paradormancy is equivalent to "correlative inhibition". These three states of dormancy are not fully independent of each other. A tree is in a state of endodormancy until it fulfills its chilling requirement then it enters a stage of ecodormancy where environmental conditions, such as cold weather, prevent bud break.

Fuchigami *et al.* (1982) proposed the Degree Growth Stage (˚GS) model to describe the continuous character of bud dormancy. The ˚GS model describes a 360˚ cyclic model of woody plant growth that was not described in previous definitions. Vegetative maturity (180˚) is a stage in the ˚GS model that is defined as the point at which leaf removal no longer stimulates resting buds to resume active growth. Growth cessation and initiation of dormancy development can be correlated to the acquisition of vegetative maturity. Maximum rest (270˚) follows vegetative maturity according to the ˚GS model (Fuchigami *et al.*, 1982) and is associated with the deepest level of endodormancy. End of rest (315˚) is the point at which chilling requirements have been fulfilled (Fuchigami *et al.*, 1982). To place the ˚GS model into context with Lang *et al.* (1987): the period from the acquisition of vegetative maturity to the end of rest is endodormancy and after the end of rest is ecodormancy in which unfavourable environmental conditions prevents budbreak. The stages of dormancy proposed by Lang *et al.* (1987) and Samish (1954) are most commonly used to describe dormancy. Although the degree growth stage model allows a quantitative approach to bud dormancy, it is subjective and therefore limits this model as a measure of dormancy. Throughout this thesis, Lang's (1987) terminology will be used and endodormancy referred to as dormancy, unless otherwise specified.

Despite extensive research that has contributed to understanding dormancy (Kramer, 1936; Downs and Borthwick, 1956; Fuchigami *et al.*, 1982), the underlying mechanisms regulating dormancy have not been completely elucidated. Older studies explored morphological changes during the transition to the dormant phase and included bud set, growth cessation, and leaf senescence (van der Veen, 1951; Downs and Borthwick, 1956, Smithberg and Weiser, 1968). More recent studies focused on physiological and biochemical changes during dormancy

induction in an effort to understand the underlying mechanisms regulating dormancy. A significant proportion of this research focused on the role of plant growth regulators (see Tanino, 2004 for a review). More recently, with the development of new techniques, molecular changes and genetic expression during dormancy induction have been reported (Arora *et al.*, 1992; Rhode *et al.*, 1998; Molmann *et al.*, 2005). These advancements have contributed to knowledge of dormancy regulation and underlying mechanisms including identifying candidate genes and biochemical pathways associated with dormancy development in woody plants.

2.3.2 Environmental regulation of endodormancy induction

Photoperiod is considered to be the primary environmental stimulus regulating dormancy induction (Doorenbos, 1953; Downs and Borthwick, 1956; Wareing, 1956; Vegis, 1964). Decreasing photoperiod coincides with the onset of unfavourable conditions. Thus, dormancy has likely evolved as an adaptive response to synchronize a plant with environmental changes that coincide with decreases in photoperiod. Downs and Borthwick (1956) first proposed the term critical photoperiod for dormancy initiation. The presence of a critical photoperiod for dormancy induction is analagous to the presence of a critical photoperiod regulating flowering time in herbaceous plants. Latitudinal, altitudinal and geographical clines exist within species in which critical photoperiod is an adaptive trait that is synchronized with the natural environment. For example, photoperiodic initiation of dormancy occurs under longer days (16+ hours) at higher latitudes and shorter days at lower latitudes (12-16 hours). Examples of ecotypes with differing critical photoperiods include *Betula pendula* (Junttila, 1980), *Populus trichocarpa* (Howe *et al.*, 1995) and *Cornus sericea* (Smithberg and Weiser, 1968; Fuchigami *et al.*, 1971; Stevenson, 1994). Photoperiod is not the only factor regulating dormancy initiation. Other environmental stimuli may impact dormancy development such as temperature and stress conditions.

Temperature regulates many aspects of plant growth and development. Although photoperiod is the primary environmental stimulus of bud dormancy induction, temperature can also affect rate and depth of dormancy development. Van der Veen (1951) reported that terminal bud set occurred earlier in warmer temperatures than cooler temperatures in *Populus* under short photoperiod. Downs and Borthwick (1956) reported similar results where warm temperatures

were reported to advance the timing of bud set in red maple (*Acer rubrum* L.). Fuchigami *et al.* (1982) stated that warmer temperatures stimulate a higher level of dormancy induction than cooler temperatures in *Cornus sericea* L. Junttila *et al.* (2003) demonstrated that higher mean temperatures (15ºC compared to 9ºC and 12ºC) resulted in earlier induction of dormancy in birch (*Betula pendula* L.). Although warm temperatures resulted in earlier initiation of dormancy, warmer temperatures, as high as 21ºC, inhibit dormancy induction in birch (*Betula pendula* L.). Furthermore, Heide (2003) observed that a warm autumn temperature resulted in earlier and deeper dormancy development in forest species in a common garden planting.

In contrast, cool temperatures (<10ºC) have also been reported to override the effect of photoperiod on species and ecotypes of woody plants from northern populations. Molmann *et al.* (2005) reported that cool temperatures (5ºC) induced bud set and cold acclimation in hybrid aspen (*Populus tremula* L. × *tremuloides* Michx.). However, endodormancy induction did not occur under the low temperature treatments. Dormancy developed in northern ecotypes of *Cornus sericea* L. under cool temperature (5ºC), long photoperiod (22 hours), which is a non-dormancy inducing photoperiod (Stevenson, 1994; Svendsen *et al.*, 2007). Van der Veen (1951) observed bud set in trees exposed to 5ºC and 9-hour photoperiods. However, trees not exposed to a short-day warm period did not attain endodormancy and resumed growth when placed under favorable conditions. Fuchigami *et al.* (1982) stated that trees must undergo a warm period under short photoperiod before dormancy is induced. It may be possible that dormancy is inhibited under cool temperatures and short photoperiods for some species or ecotypes (Heide, 2003; Junttila *et al.*, 2003).

Since Went (1944) defined thermoperiodism as the effects of differences in day/ night temperature on growth and development of plants using tomato, there has been a steady development of knowledge regarding thermoperiodism. Stevenson (1994) reported that diurnal temperature differences were important for dormancy induction in *Cornus sericea* L. Plants were subjected to either 22-hour or 8-hour photoperiods with 8-hour day/ 16-hour night temperature regimes of 5/5ºC, 10/5ºC and 20/5ºC. Even under the long- photoperiod, cool night temperatures stimulated dormancy development in northern ecotypes but required a diurnal temperature difference since the 5/5ºC treatment was ineffective. It was observed that northern ecotypes show a higher degree of pigmentation under low temperature than southern ecotypes. Light exposure to plants at low temperatures may affect sensing of light quality and photoperiod. This

may subsequently affect dormancy development in northern ecotypes. In addition, stress responses can confound temperature effects. Although these two effects can occur in concert, thermoregulation of dormancy may be independent of stress.

Abiotic stresses can also affect the timing and rate of dormancy acquisition (Chen and Li, 1978; Stevenson, 1994). In addition to the ability to tolerate cold conditions associated with the onset of winter in temperate areas, trees have evolved to tolerate periodic drought events by entering a dormant state. Evolution of angiosperms began in the tropics where temperature and photoperiod were relatively stable, but cyclic annual dry seasons occurred. Plants evolved to tolerate this cyclic precipitation by losing their leaves and entering a dormant state prior to the dry season (Frankie *et al.*, 1974). Dormancy is induced by moisture stress in combination with short-day photoperiod. Decreases in water potentials have been shown to initiate leaf abscission in both tropical (Frankie *et al.*, 1974) and temperate tree species such as *Betula pubescens* (Rinne *et al.*, 1994; Welling *et al.*, 1997). An increase in abscisic acid (ABA), a stress-signaling hormone, was reported in buds during drought stress. In temperate climates, periods of severe drought or frost periods may increase the rate of dormancy induction (Rinne *et al.*, 1994).

Abiotic stresses can increase the rate of bud set to provide protection against unfavourable conditions in some species. Van der Veen (1951) observed rapid bud set of *Populus* plants placed in 5ºC constant temperature but as per Stevenson (1994), endodormancy development did not occur. Plants removed from chambers and placed in warm temperatures/long day conditions resumed growth immediately (van der Veen, 1951). Multiple pathways likely exist that regulate bud set: a stress-induced pathway, which may not necessarily lead to bud dormancy and a short-photoperiod induced pathway, which classically results in bud dormancy. Stress-induced growth cessation may be independent of photoperiod-induced growth cessation. However, under field conditions, the two processes may combine to promote rapid cessation of growth and/or induction of dormancy in late summer or autumn and therefore overlapping effects may be present at this time.

2.3.3 Environmental perception and gene expression during dormancy induction

Advances in molecular techniques have made it possible to examine gene expression associated with dormancy development in woody plants, especially in *Populus*. Recent mapping

of the genome of *Populus trichocarpa* (Brunner *et al.*, 2004) has enabled identification of candidate genes associated with dormancy in poplar. Changes in gene expression are associated with the transition from active growth to a dormant state (Coleman and Chen, 1993). Genes that regulate growth cessation, terminal bud formation, nutrient mobilization and storage, cold hardiness, desiccation tolerance and leaf senescence may all be related to dormancy induction, maintenance and release. Genetic control of dormancy development in woody plants is likely quantitative (Perry and Pauley, 1954). Identification of candidate genes and QTLs (Quantitative Trait Loci) associated with dormancy is necessary to aid in understanding the genetic control of dormancy. However, different plant systems may have differential gene expression associated with dormancy induction (Chen *et al.*, 2000). Genera such as *Acer*, *Quercus*, *Thuja*, and *Malus*, which are phenologically different than *Populus* may not share similar patterns of gene expression observed in the *Populus* model plant system.

Induction of dormancy in woody plants is primarily regulated by changes in photoperiod (Downs and Borthwick, 1956; Wareing, 1956). Changes in light are sensed in photosynthetic organs of plants, most often in leaves. Plants have evolved complex and dynamic systems for sensing changes in light duration. Changes in duration and quality of light stimulate physiological and phenological changes within the plant. Butler *et al.* (1959) demonstrated evidence of a red/far-red light activated, photoreversible pigment present in the leaves of plants. Shortly after, this pigment was named phytochrome (Butler, 1964). Phytochrome is the primary mechanism for the sensing of changes in duration of light. Currently five phytochromes (A, B, C, D and E) have been identified in plants (Briggs and Olney, 2001). All phytochromes are similar in chemical structure and amino acid sequence and have a common function in the ability to sense red and far-red light. Phytochrome is present in the cytoplasm, nucleus, mitochondria and chloroplasts (Taiz and Zeiger, 2004). Exposure to red light changes the conformation of the phytochrome protein from the inactive P_r form to the active P_{fr} form. The balance between P_{fr} and P_r has been shown to regulate growth and development in plants by activation of signal transduction pathways. These pathways regulate gene expression, which alter biochemical and physiological processes that affect development in plants (Barnes *et al.*, 1997).

Howe *et al.* (1998) suggested that phytochrome controls bud set and growth cessation, which subsequently controls dormancy and cold hardiness levels in woody plants. Candidate genes possibly connected to dormancy have been identified in *Populus* (Chen *et al.*, 2000).

Three phytochrome genes (PHYA, PHYB1 and PHYB2) are likely responsible for controlling perception of photoperiod that stimulate growth cessation and the induction of endodormancy in woody plants (Howe *et al.*, 1998). Phytochrome is one of the primary regulators of endodormancy induction in woody plant buds. Endodormancy is similar to, but distinct from bud set and growth cessation. While the mechanism by which phytochrome affects gene expression has not been completely elucidated, both phytochrome A and B have been shown to migrate into the nucleus when exposed to red-light treatments (Kircher *et al.*, 1999). This differential accumulation of phytochromes within localized areas of the cell and migration of phytochrome into the nucleus emphasizes the regulatory role of phytochrome in plant growth. Phytochrome B has been linked to the control of initiation of flowering in *Arabidopsis* (Halliday *et al.*, 2003). Far-red light inhibited phyB-green fluorescent protein (GFP) import into the nucleus but not phyA-GFP (Kircher *et al.*, 1999). Ni *et al.* (1999) reported that interactions between PhyB and PIF3 (phytochrome interacting factor 3), a gene associated with dormancy, only occurs when PhyB is in the P_{fr} form. Merkle (2001) reported that the Pfr form of PhyB in the cytoplasm activates transcription factors, which induce gene expression. Activation of phytochrome from the inactive P_r form to the P_{fr} form, promotes activation of transcription factors in the nucleus and mitochondria, subsequently inducing changes that affect plant growth and development.

While activation of phytochromes have been determined to be highly light-dependent, temperature during the dark period affects degradation and reversion of P_{fr} back to the inactive P_r form (Mumford, 1966). In addition, Bunnings (1967; cited in Anderson *et al.*, 1969) also suggested that temperature affected this reversion. Anderson *et al.* (1969) looked at the effect of temperature on reversion of P_{fr} to Pr during the dark and found that increases in temperature increase the reversion rate of P_{fr} to P_r. Applying these kinetic studies to whole-plant responses, Halliday *et al.* (2003) demonstrated the control of the FT (flowering time gene) in *Arabidopsis* by the interaction between PhyB and temperature. Temperature was shown to alter flowering time in *Arabidopsis*, which is controlled by the P_r: P_{fr} ratio of phyB. Bohlenius *et al.* (2006) reported that the poplar ortholog of the FT gene in *Arabidopsis*, PtFT1, controlled growth cessation and dormancy in *Populus tremula* x *tremuloides*. Since temperature affects the expression of the FT gene in *Arabidopsis*, it is not unreasonable to suggest that temperature could also modify the expression of PtFT1 in woody plants. Furthermore, Molmann *et al.* (2005) reported that endodormancy is not induced in poplar that over-expresses PhyA. Olsen *et al.*

(2003) investigated the expression of PhyA in hybrid aspen and its impact on critical photoperiod. It was reported that modified expression of the PhyA gene modified both critical photoperiod and growth cessation in hybrid aspen. Expression of PhyA can modify the critical photoperiod for dormancy development, which can be affected by both temperature and photoperiod. Activation of transcription factors regulating growth cessation and dormancy development depends on conformation of PhyA and other phytochromes. This suggests that temperature may contribute to variations in the critical photoperiod of woody plants through the phytochrome pathway.

2.3.4 Physiological changes related to dormancy

2.3.4.1 Hormones

The role of hormone signaling has been the focus of studies on dormancy induction, maintenance, and release of bud dormancy since Hemberg (1949). Tanino (2004) suggested that a hormone-centered approach to studying dormancy may not be successful because there are unrelated and parallel physiological processes not associated with dormancy that occur simultaneously that can impact hormone levels during dormancy development. Although hormonal levels may not completely explain dormancy, the balance of hormonal levels within plants indicates changes in gene expression, metabolite movement and overall metabolic activity. The interaction between temperature and photoperiod and their impact on hormonal levels within plants during dormancy development is of particular interest.

Abscisic acid (ABA) is commonly referred to as the stress hormone associated with inhibition of growth. Seeley and Powell (1981) observed highest concentrations of ABA in the shoot tip of apple during terminal bud formation in autumn. ABA appears to be more associated with stress tolerance from either drought or cold hardiness. Dumbroff ***et al.*** (1979) looked at ABA concentration throughout the summer and the fall in *Acer saccharum* and observed that ABA increased in July and August and decreased until leaf abscission in October. Accumulation of ABA may be dependent on temperature during dormancy induction but may be unrelated to endodormancy development in woody plants. Although cool temperatures have also been associated with an increase in bud, shoot and xylem ABA content, cool temperatures also inhibit dormancy induction in some woody plants (van der Veen, 1951; Palonen, 2006; Junttila ***et al.***, 2003). This could suggest that ABA does not have a regulatory role in dormancy development.

In addition to absolute endogenous levels of ABA, the ratio of ABA, a growth-inhibiting hormone, to growth promoting hormones such as indole-3-acetic acid (IAA) and gibberellic acid (GA) has also been explored. Rodriguez *et al.* (1991) found that the ratio of ABA: IAA was greater than one during the fall and less than one during the spring during bud break in *Corylus* buds. The ratio of ABA to growth stimulants, such as GA, may regulate dormancy development in plants. This linear approach may give the impression that these two types of hormones work antagonistically. Growth stimulants and inhibitors may contribute to a dynamic, changing network of biochemical and physiological processes as a result of environmental stimuli.

Gibberellins (GA) are associated with active growth in plants. ABA and GA may have an antagonistic relationship where ABA inhibits the activity of GA. Endogenous GA concentration correlates with stem elongation and growth cessation (Jansen *et al.*, 1986; Juntilla *et al.* 1991; Olsen *et al.*, 1995; Molmann *et al.*, 2003) and bud break (Wurzberger, 1976) in woody plants. Olsen *et al.* (1997b) showed that GA, when applied to developed terminal buds of *Salix pentandra*, initiated cell division at the apical meristem. This suggests that GA concentrations may have a regulatory effect on bud set and bud break in woody plants. GA is present in many different forms in plants. Chen (1994; cited in Tanino, 2004) showed that GA_1 and GA_8 were associated with bud break in *Euphorbia longana* while others (GA_9 and GA_{32}) increased during cessation of growth and flowering initiation.

Temperature has been shown to influence GA concentration in plants. Pinthus *et al.* (1989) reported that endogenous GA_1 concentration increased in isogenic lines of *Triticum* with increasing temperature. A thermoperiodic effect has also been observed on the synthesis of GA in plants. Went (1944) first showed the thermoperiodic effect of enhanced growth under positive day-night temperature differences in tomato. Moe (1990) suggested that observed differences in stem elongation and growth under high day/night differences was a result of changes in the sensitivity and metabolism of GA. More specifically, daily temperature alterations affect the endogenous level of bioactive GA_1 in the stems. A positive day-night temperature difference has been shown to increase GA_1, GA_{12}, GA_{19} and GA_{20} concentrations in *Begonia* x *hiemalis* Fotsch compared to no difference or negative differences (Myster *et al.*, 1997). Grindal *et al.* (1998) showed that positive day-night temperature differences resulted in less 2β-hydroxylation of bioactive GA_1 to inactive GA_8 than negative day-night temperature difference in *Pisum sativum*.

In contrast, Thingnaes *et al.* (2003) showed endogenous GA content was not affected by thermoperiod in *Arabidopsis thaliana*. The one GA compound that was significantly altered by thermoperiod was inactive GA_{29}. However, it should be noted that they did not use apical buds, which may have affected results. While the literature is not concrete, the thermoperiodic effect on GA might be another factor influencing dormancy development in vegetative buds. As demonstrated, both absolute temperature and day/night temperature differences have been shown to affect concentration of inactive and active GA compounds resulting in changes in stem elongation, commonly associated with growth. Temperature may also affect the role of GA in bud endodormancy but has yet to be investigated in woody plants.

Menhennett and Wareing (1977) compared the effect of temperature and photoperiod on growth and cytokinin levels in 'Norwegian' and 'Portugese' ecotypes of *Dactylis glomerata*. They found that both photoperiod and temperature affected cytokinin levels in the leaves. The observed effect was reduced with increasing leaf number away from the apical meristem. Interestingly, an increase in temperature under short photoperiod resulted in a decrease in cytokinin content in leaves, which may be associated with a decrease in leaf length observed with the increase in temperature. Changes in cytokinin content and activity may be correlated to changes in photoperiod and temperature but may be related to other factors related to changes in growth.

It is apparent that changes in temperature affect hormonal levels in plants, including ABA, GA and cytokinins. Further work would be useful in identifying the relationships between hormonal levels in tissues associated with dormancy and temperature during photoperiodic induction of dormancy in woody plants.

2.3.4.2 Proteins

While early studies focused on hormonal regulation of dormancy, advances in biochemistry in the late 1960's allowed for analysis of protein and RNA changes associated with dormancy. Zimmerman and Faust (1969) showed an increase in RNA and protein synthesis during dormancy induction in pear buds. The use of controlled growth chamber facilities allowed for separation of some physiological processes, such as cold acclimation and dormancy induction. Nevertheless, field studies were mostly commonly used at that time, which increased

the complexity of analyzing protein changes during dormancy induction since dormancy development and cold acclimation occur simultaneously. Plant systems containing mutants (Arora *et al.*, 1992) and F_1 hybrids (Wake and Fennell, 2000) of ecotypes and species were also used as contrasting systems to isolate changes in proteins from dormancy-related processes. Using deficient mutants or F_1 crosses allowed for quantitative and qualitative analysis of protein changes during dormancy induction. It also helped identify changes in proteins that were correlated but unrelated to dormancy. These types of systems are more advantageous because once these specific changes have been attributed to specific processes, field studies can be conducted to confirm results observed under controlled conditions. Protein changes during dormancy induction within the apical meristem, xylem and phloem of woody plants have been associated with endodormancy. Bark-storage proteins and LEA-type dehydrins have been shown to accumulate during induction of endodormancy (Arora *et al.*, 1992; Rowland and Arora, 1997; Karlson *et al.*, 2003).

Others proteins function as storage proteins for nutrients that can be remobilized once growth resumes in spring. Bark storage proteins (BSP) have been shown to accumulate during the fall during dormancy induction and degrade during bud break in spring (Sauter *et al.*, 1994; cited in Rowland and Arora, 1997; Wetzel *et al.*, 1989). Short photoperiods induce accumulation of BSP in *Populus deltoides* (Coleman *et al.*, 1991; Coleman *et al.*, 1992). BSPs of 32 kD (Wetzel *et al.*, 1989), 36 kD and 38 kD (Stepien and Martin, 1992) accumulate in protein storage vacuoles in the inner bark parenchyma (Wetzel *et al.*, 1989). Coleman *et al.* (1993) showed that chilling is required for the degradation of BSPs in *Populus deltoides*. Endodormant plants exposed to long days did not show a decline in BSP concentration but BSPs rapidly declined in ecodormant trees exposed to long days. While BSPs may not be directly related to endodormancy, research shows that accumulation and degradation are closely correlated to induction and release from a dormant state. It has been speculated that these proteins aid in seasonal nitrogen storage in the bark to be used when growth resumes the following spring (Vince-Prue, 1984).

Dehydrins, a group of LEA-type proteins, were originally discovered to accumulate in tissues under increasing drought-like conditions in plants (Robertson and Chandler, 1994; Nylander *et al.* 2001). Common characteristics of these proteins include heat stability, they are highly hydrophilic and contain at least one lysine-rich sequence comparable to

EKKGIMDKIKEKLPG (Karlson *et al.*, 2003). Accumulation of these proteins is induced by changes in temperature, water stress and photoperiod (Rowland and Arora, 1997). Salzman *et al.* (1996) looked at protein induction in *Vitis labruscana* var. 'Concord' under short photoperiods with and without low temperature and found two proteins, a 27 kD and a 47 kD protein, that accumulated under short photoperiods and warm temperature. This 47 kD protein was later found to be a dehydrin-type protein. Wake and Fennell (1996) examined protein changes during dormancy induction of *Vitis riparia* and *V. vinifera* and their F_1 hybrids. *V. riparia* entered dormancy earlier than *V. vinifera*. In both species and the hybrids, there was accumulation of 18-22 kD and 16-19 kD proteins under short-day exposure regardless of dormancy status. However, in *V. riparia* there was accumulation of a 17-20 kD protein that may be attributed to endodormancy induction. Karlson *et al.* (2003) examined photoperiod-induced accumulation of a 24 kD dehydrin protein in varying ecotypes of *Cornus sericea* L. They reported differential accumulation of the 24 kD protein that was closely associated with freezing tolerance and water content.

While some dehydrins are induced by short photoperiods and accumulate during the fall in woody tissue, it is more likely that these proteins are associated with cold acclimation than dormancy development. Arora *et al.* (1992) used a mutant evergreen peach that did not enter a dormant state and a wild-type peach to compare differential accumulation of proteins associated with endodormancy. It was found that a 16 kD protein was only present in small amounts in the non-dormant evergreen genotype but was present in higher levels in the wild-type peach. Increased expression of a 60 kD protein was highly correlated with increased cold hardiness in two varieties of blueberry (Rowland *et al.* 1996), and an evergreen and deciduous peach (Arora *et al.*, 1996). Since the 60 kD protein accumulated in the non-dormant evergreen peach, it is likely that this protein is not associated with endodormancy. Arora *et al.* (1992) suggested this 16 kD protein was not linked to cold acclimation unlike other proteins which were reported to accumulate during dormancy induction. While dehydrins appear to be more related to freezing tolerance, their relationship to dormancy cannot be discounted. However, the underlying connection between photoperiod-induced dormancy development, increased freezing tolerance and dehydrin protein accumulation may be water status and free water content within the plant. Decreased free water leads to an increase in freezing tolerance and may also regulate metabolic

processes related to endodormancy. This may be caused, in part, by the increase in accumulation of hydrophilic dehydrin proteins.

2.3.5 Relating biophysical properties of water with bud dormancy

Water is essential for life and the medium in which many metabolic and physiological processes occur. In many earlier dormancy studies, water content was shown to decrease during dormancy induction in woody plants (Jeknic and Chen, 1999; Wake and Fennell, 2000; Welling *et al.*, 2002; Li *et al.*, 2003). This decrease in water content has been correlated with both cold hardiness (Gusta *et al.*, 1979) and dormancy (Faust *et al.*, 1991; Gardea *et al.*, 1994; Faust *et al.*, 1995; Erez *et al.*, 1998). Faust *et al.* (1995) suggested that dormancy levels might impact water status in woody plants. Water status is a general term that refers to changes in water content and biophysical interactions between water and other molecules. Decreases in water content or a decrease in available water may reduce metabolic activity in meristematic tissue of formed buds. The proportion of 'free' to 'bound' water, which is the amount of water available for metabolic activity, is thought to be important to both dormancy and cold hardiness levels. Bound water can be described as water that is attached to hydrophilic molecules within plant tissue. Free water is water not bound within the cell and is available for active metabolism (Faust *et al.*, 1991).

NMR imaging has been utilized as a non-destructive technique to measure changes in biophysical characteristics of water during dormancy induction, maintenance and release. Inhibited by low resolution and high imaging times, the use of NMR was initially limited to non-tissue-specific spectroscopy measurements of water in plant tissue (Gusta *et al.*, 1979). Advances of NMR technology enabled higher resolution imaging and low imaging times to measure changes in water status in plant tissue (Ishida, 2000). Interest in how water status may contribute to dormancy regulation was renewed when Faust *et al.* (1991) investigated changes in water status in apple buds during chilling using NMR imaging. Chilling can be described as exposure to cool, non-freezing conditions that when fulfilled, allows growth of a previously endodormant bud. Chilling is critical for the transition from endodormancy to ecodormancy in woody plants. NMR imaging could be used to identify localized changes in water status within plant tissue. High T_1 and T_2 relaxation times, indicators of water content and biophysical interactions within plant tissue were more closely associated with free water while lower

relaxation times were closely associated with bound water. Faust *et al.* (1991) and Gardea *et al.* (1994) measured relaxation times at varying points during chilling and reported that T_1 relaxation times increased with increased chilling duration. Chilling may contribute to physiological responses that allow for increases in free water within stem and bud tissue of apple buds. However, prior to imaging, buds were saturated in water at room temperature for 24 hours. Changes in water may have been a result of increases in water uptake from saturation of the bud rather than a direct effect of chilling itself. Since the endodormant bud would not have the capacity to deacclimate (Tanino *et al.*, 1989), it may have resulted in the presence of less free water compared to the ecodormant bud. Instead of the chilling treatments inducing a decrease in binding of water, it conceivably increased the ability of the tissue to import water from outside during the transition from endo- to ecodormancy.

Nevertheless, the connection between dormancy and water status has been widely reported during dormancy induction. Erez *et al.* (1998) showed peach shoots exposed to long photoperiod and warm temperatures had more free water than buds that were exposed to short photoperiod-warm temperature or long photoperiod-cool temperatures during dormancy induction. Binding and movement of water out of plant tissue may be two of the processes that regulate water status in the cell. Accumulation of hydrophilic molecules can bind water within plant tissue (Faust *et al.*, 1995). In addition to changing the biophysical interactions between water and hydrophilic molecules, restriction of movement both in and out of plant tissue may regulate the state of dormancy in vegetative buds. Ashworth *et al.* (1982) identified a blockage at the base of dormany peach buds that inhibited water movement between the bud and stem. Furthermore, changes in plasmodesmata that regulate movement of water may also be responsible for the regulation of dormancy in woody plants (van der Schoot, 1996).

Water is mainly transported through the plasmodesmata and aquaporin water channels of plant cells. Intercellular communication is thought to be restricted during endodormancy by blockage of the plasmodesmata by 1-3-β-glucan (Rinne *et al.*, 2001). Blocking of the plasmodesmata would restrict access to water, essential for resumption of growth. Higher accumulation of 1-3-β-glucan has been reported in bean (*Phaseolus vulgaris*) under warmer temperatures (Abeles and Forrence, 1970). Therefore, it can be speculated that more rapid accumulation of 1-3-β-glucan, reported during dormancy induction (Rinne *et al.*, 2001), could occur under warmer temperatures. Chilling has been shown to increase activity of 1,3-β-

glucanase at the plasmodesmata showing the breakdown of 1-3-β-glucan in association with chilling (Rinne *et al.*, 2001). 1-3-β-glucanase is the enzyme responsible for degradation of 1-3-β-glucan that is present in plasmodesmata of endodormant woody plants (Rinne et al., 2001, Rinne and Van der Schoot, 2003). Opening of the plasmodesmata would allow increases in cell-cell communication within the plant.

Although a high correlation between dormancy levels and water status has been established, decreases in water are also thought to be associated with cold hardiness during the dormant period. Low water potential is considered to be critical for attaining a high degree of cold hardiness (Karlson *et al.*, 2003). During the dormancy cycle, maximum cold hardiness is normally attained after the chilling requirement has been satisfied. There are distinct differences between poplar clones in the ability to either maintain hardiness or prevent deacclimation during mid-winter thaws (Silim, 2005; pers. comm.), even when ecodormant. Maintaining that level of cold hardiness may depend upon the ability to maintain a dormant state (Tanino *et al.*, 1989) and maintain water in the relatively bound state. Mid-winter deacclimation and subsequent loss in freezing tolerance may be a result of an increase in bulk water in plant tissue. Free water is susceptible to freezing, increasing the likelihood of injury resulting from a rapid rate of ice propagation (non-equilibrium freezing) and physical injury caused by large ice crystals (Olien, 1960).

2.3.6 Morphological changes during dormancy induction

Until more advanced dormancy assays were developed, visual observation of morphological changes during dormancy development was employed to measure dormancy initiation in woody plants. In response to both short photoperiod, and in some cases, cool temperatures, growth cessation was initiated where cell elongation was inhibited and internode length decreased. The rate of new leaf emergence from the terminal bud was also reduced (Goffinet and Larson, 1981). Leaf primordia that had developed prior exposure to short photoperiod, developed into complete leaves (Larson and Pizzolato, 1977; Rhode *et al.*, 2002). Internode elongation gradually decreased between nodes (Rhode *et al.*, 2002). Once internode elongation had ceased, compaction of nodes occurred at the terminal bud occurs forming a

compact stem within the bud. Although cellular elongation has ceased, cell division remains active resulting in the formation of a compact stem that forms the terminal bud of a woody plant.

Shortly after, two or more pairs of bud scales enclosed the terminal bud (Goffinet and Larson, 1981). Under short photoperiod, leaves continue to emerge but in continuously smaller sizes until bud scales that enclose the bud form from leaf primordia. It appears as though reduction in cell elongation and reduction in leaf size signals the formation of bud scales from leaf primordia. Leaf primordia continue to develop behind the bud scales throughout the dormant period until continued growth the following season (Goffinet and Larson, 1981).

2.4 Development of Cold Hardiness during Dormancy Induction

Cold hardiness is a general term used for freezing tolerance of whole plants or specific plant tissues. Cold hardiness is an adaptation of plants and animals to survive freezing conditions. Plants have the capacity to acclimate to avoid freezing damage in winter. Plant cold hardiness will fluctuate throughout the year. Cold hardiness in woody plants during the active growth stage is normally between –1°C and –5°C (Burke *et al*., 1976; Sakai, 1965), depending on time of year (Fuchigami *et al.*, 1982; Kramer, 1936) and exposure to stress (Chen and Li, 1978). A combination of biochemical, physiological and morphological changes allows plants to increase cold hardiness to survive freezing conditions.

Fall phenological changes correlate with increases in cold hardiness in woody plants. Weiser (1970) outlined three steps of cold acclimation. The first step of cold acclimation is the initiation of dormancy induction during the autumn under short photoperiods. Once dormancy has been induced, exposure to cool but non-freezing conditions results in further increases in freezing tolerance. The last step occurs when woody plants are exposed to freezing conditions that results in cellular dehydration. While freezing tolerance increases during exposure to freezing conditions, exposure to non-freezing, cool conditions contributes the most to increases in cold hardiness (Fuchigami *et al.*, 1982). Once fully acclimated, the cold hardiness of some woody plants can exceed -196°C (Sakai, 1965).

The ability to survive freezing is generally founded through two strategies, avoidance and tolerance to ice formation (Levitt, 1972). Olien (1967) described freezing as the change in state of water molecules as a result of changes in hydrogen properties. Freezing damage occurs when

ice forms within the plant cell, bursting the cell membrane. Plant organs show different levels of cold hardiness depending on the developmental stage and location on the plant. During the transition from active to non-growth, increases in cold hardiness coincide with increases in bud dormancy levels. Once dormancy has been initiated, there is a further increase in cold hardiness in woody plants in response to specific environmental stimuli.

Among the physiological mechanisms that increase the levels of cold hardiness in woody plants, two steps, accumulation of sugars and proteins and the reduction in water content, are of particular interest. Sugars and proteins in acclimating tissue have been widely reported to increase under non-freezing cool temperatures (<10ºC) in early autumn (Cox and Stushnoff, 2003; Jeknic and Chen, 1999; Marquat *et al*., 1998; Palonen, 1999). In addition to accumulation of hydrophilic molecules, dehydration of the cell by a concentration gradient caused by intercellular ice formation under freezing conditions occurs later in autumn and during winter (Olien, 1967). Both of these steps are important for maximizing cold tolerance in woody plants and both of these steps involve changes in biophysical water characteristics in plant tissues.

Accumulation of carbohydrates may reduce freezing injury and minimize cellular damage when exposed to low temperature conditions. Soluble sugar content in buds positively correlates with cold hardiness (Palonen, 1999). Stachyose and raffinose are only detectable in significant amounts during endo- and ecodormancy in *Lonicera caerula* (Imanishi *et al.*, 1998). They accumulate in response to cold temperatures and decrease during deacclimation in the spring. Imanishi *et al.* (1998) speculated that cold acclimation to survive extreme winter conditions occurs in September and October, roughly coinciding with the observed increases in stachyose and raffinose. There is also a decrease in stachyose and raffinose content in the bud during deacclimation (Cox and Stushnoff, 2003; Imanishi *et al.*, 1998). These solutes may bind water, minimizing the amount of free water in the cell, which may increase viscosity and subsequently, lower the freezing point within the cell (Marquat *et al.*, 1998).

Additionally, cool temperatures and short photoperiod stimulates the production of LEA-type proteins called dehydrins (Dhanaraj *et al.*, 2004). These proteins are similar or identical to other stress proteins present under drought or other stress conditions. Dehydrins are considered to contribute towards the transition of free water to bound water in plants (Ingram and Bartels, 1996), which occurs during dormancy induction. Furthermore, these proteins may have other functions that may contribute to increases in cold hardiness. These dehydrin-type proteins are

abundant in the bud throughout the dormancy cycle. Faust *et al.* (1997) speculated that dehydrins are one of the binding components of free water during dormancy. Dharanaj *et al.* (2004) reported that conditions that induce dormancy also stimulate the production of these proteins in blueberry. It is thought that cool temperatures (<10ºC) stimulate the formation of these water-binding proteins. Karlson *et al*. (2003) showed that *Cornus*, which supercooled, lacked a 24 kDa dehydrin protein. Additionally, stem water content also did not decrease with exposure to short day conditions. However, it has been shown that photoperiod induces only a small portion of dehydrin proteins (Arora and Rowland, 1992; Karlson *et al.*, 2003) and the specific interactions between dehydrin proteins and water has not been completely elucidated.

Reduced available water content is thought to play a dominant role in regulating cold hardiness during the dormant period, and a low water potential is thought to be central to attaining cold hardiness (Karlson *et al.*, 2003). Although Gusta *et al.* (1979) suggested biophysical water characteristics do not correlate well with cold hardiness in winter wheat, localized changes in water within plant tissues may be more closely related to cold hardiness than could be distinguished through previous techniques. Recent advances in NMR technology may be able to identify tissue-specific changes in biophysical water characteristics that are more closely related to cold hardiness in plants.

While much progress has been made in many areas of cold hardiness research, research linking these disciplines together has been minimal. Cold acclimation is a complex process involving interacting factors that result in increases in cold hardiness. Comprehensive approaches involving changes in the biophysical interactions within plant tissues may yield new insight into how plants attain high levels of cold hardiness. It is apparent that there are high levels of genetic variation within woody plant populations that result in differential cold hardiness (Howe *et al.*, 2003). Identifying the underlying mechanisms and limitations that produce this variation would be valuable.

3.0 THE IMPACTS OF AUTUMN TEMPERATURES ON DEVELOPMENT OF DORMANCY AND COLD HARDINESS IN HYBRID POPLAR

3.1 Introduction

Over the next 75 years, annual global mean temperature is forecast to increase between 1.1ºC and 6.4ºC (IPCC, 2007). Temperature increases will be more pronounced at northern latitudes, including the Great Plains region of North America. Temperate woody plants undergo a cyclic pattern of active growth and non-growth, which is commonly referred to as dormancy. In early autumn, the transition from active growth to the dormant phase occurs. This transition is a necessary step for the winter survival of woody plants from these regions. In autumn, when dormancy and cold acclimation are initiated, temperatures are forecast to increase by 3-5˚C (Wheaton, 2001), thereby extending the growing season (Motha and Baier, 2005). The impacts of this temperature increase on phenological and physiological processes associated with the induction of dormancy and cold hardiness in temperate woody plants are relatively unknown.

Woody plant species native to temperate regions develop the ability to survive cold temperatures in mid-winter, including temperatures as cold as –196˚C (Sakai, 1965). However, actively growing plants will be killed or damaged by temperatures as high as -1˚C to –3˚C (Burke *et al.*, 1976). The process of cold acclimation will not commence until growth has stopped. Dormancy acquisition is, therefore, a response by woody plants in temperate regions to synchronize growth with the environment and subsequently survive the cold winters. Seasonal growth must be balanced with the ability to survive cold conditions. The effect of autumn temperature on the trade-off between attaining maximum growth and winter survival is not known. It is possible that genotypic variation in dormancy development and induction of cold acclimation may exist in response to increases in temperature. Identification of these responses may indicate whether certain plant species or genotypes have a greater capacity to adapt to increases in temperature than others.

Photoperiod is accepted as the primary factor regulating dormancy induction (Wareing, 1956; Downs and Borthwick, 1956). The responses of different species and ecotypes, including the physiological and biochemical processes involved in sensing photoperiod have been extensively studied (Kramer, 1936; Downs and Borthwick, 1956; Nitsch, 1957; Junttila, 1980;

Wake and Fennell, 2000; Olsen *et al.*, 2003; Molmann *et al.*, 2005). However, temperature can also contribute to changes during dormancy induction. Cool temperatures have been shown to induce dormancy in northern ecotypes in birch (Junttila, 1980) and dogwood (Stevenson, 1994; Svendsen *et al.*, 2007), even under a 22 to 24 hour photoperiod. Conversely, it has also been reported that warmer temperatures can increase the rate of dormancy development in woody plants (Fuchigami *et al.*, 1982; Heide, 2003; Juntilla *et al.*; 2003). These contrasting dormancy responses of woody plants to temperature during dormancy induction underline the importance of identifying how dormancy and related processes are affected by temperature.

In this study, we examine how temperature influences dormancy development and cold acclimation initiation in hybrid poplar. We were particularly interested in determining how phenological changes associated with dormancy and freezing tolerance will be impacted by predicted temperature increases. Poplar is an established model system for studying woody plant responses (Brunner *et al.*, 2004), including dormancy and related processes. Compared to natural ecotypes, variability exists in hybrid poplar resulting from interspecific recombination that yields contrasting responses useful for studying physiological processes. The clones used in this study were selected from a group of cultivars currently grown on the Canadian Prairies. Selected clones demonstrated high variability in dormancy and cold acclimation patterns (Silim *et al.*, 2005; unpublished data).

'WP-69' ('Okanese') enters dormancy earlier than other clones under natural Saskatchewan autumn conditions and is considered to be among the hardiest clones tested (Silim *et al.*, 2005, unpublished data). 'Walker' and 'Katepwa' show intermediate dormancy and cold acclimation initiation characteristics. 'Walker' is also the most widely planted hybrid poplar on the Canadian prairies. 'Prairie Sky' acclimates later in the fall and is susceptible to freezing damage in some parts of the Canadian Prairies. From these observations, we expected 'WP-69' to enter dormancy first and be less impacted by temperature than 'Prairie Sky'. Clones such as 'Walker' and 'Katepwa' are more commonly planted on the Canadian Prairies. Both clones are distributed as shelterbelt and forest plantation trees. Since there is increasing interest in using hybrid poplar for short-rotation woody crop plantations, identifying how these trees respond to increases in temperature is important in order to determine whether these clones will be suitable for continued distribution on the Canadian Prairies under a climate change scenario.

The synchronization of phenology with dormancy development and cold acclimation initiation is essential for cold acclimation and prevention of untimely deacclimation during the winter. Weiser (1970) outlined the three steps of cold acclimation and suggested cold acclimation is dependent on initiation of dormancy. In contrast, Fuchigami *et al.* (1971) suggested that growth cessation is required for cold acclimation to occur. There are examples of woody plants that only require growth cessation before cold acclimation can occur (Silim and Lavender, 1993). This experiment examined the effect of day/night temperature and the underlying relationships between growth cessation, endodormancy and cold acclimation induction in hybrid poplar.

Stevenson (1994) reported that day/night temperature differences affected dormancy development in *Cornus sericea* L. ecotypes. Dormancy development occurred earlier when exposed to higher day/night temperature differences using cooler night temperatures compared to lower day/night temperature differences using warmer night temperatures under a short photoperiod. However, a constant low temperature 5/5^{o}C under short photoperiod inhibited dormancy development (Stevenson, 1994; Van der Veen, 1951). There may be night or day temperature effects that contribute to the overall temperature response to short photoperiod in hybrid poplar. Therefore, in addition to exploring the overall impact of temperature on dormancy and related processes, this study also looked at day or night temperature effects on dormancy development and cold acclimation initiation.

3.2 Materials and Methods

3.2.1 Plant Material and Growth Conditions

Hybrid poplar (*Populus x sp.*) clones used in this study developed by Agriculture and Agri-Food Canada breeding stations at Indian Head, Saskatchewan (50°N 30.705') and Morden, Manitoba (49° 11' N), which were interspecific hybrids between various native and exotic species. The combination of interspecific genotypes results in high variation among clonal selections. Four different clones were selected for variation in cold acclimation and dormancy

induction responses observed from seasonal cold hardiness evaluation of hybrid poplar grown on the Canadian prairies by Silim *et al.* (2005, unpublished data). The clones used were: 'Walker', 'WP-69' (recently released as cultivar 'Okanese'), 'Katepwa' and 'Prairie Sky'. 'Walker' is a cross of *P. deltoides* and *P.* x *petrowskyana*. 'WP-69' and 'Katepwa' are progenies of 'Walker'. While 'Katepwa' was open-polinated, the male parent of 'WP-69' was *P.* x *petrowskyana*. 'Prairie Sky' is a cross between *P. deltoides* and *P. nigra*. 'WP-69' enters dormancy earlier than other clones and is considered to be among the hardiest clones tested (Silim *et al.*, 2005, unpublished data). 'Walker' and 'Katepwa' show intermediate dormancy and cold acclimation initiation characteristics. 'Walker' is also the most widely planted hybrid poplar on the Canadian Prairies. 'Prairie Sky' acclimates later in the fall and is susceptible to winter injury in some parts of the Canadian Prairies.

Clonal material for the four clones was obtained from the PFRA Shelterbelt Centre in February 2006, after chilling requirement had been fulfilled. Plants were propagated from hardwood cuttings (approximately 6-8 cm) in Spencer-Lemaire (Spencer-Lemaire Industries, Edmonton, Alberta) root trainers filled with soil-less media (Sunshine No. 4, Sungro Hort Inc, Bellevue, WA) and grown in a greenhouse (20 ± 5 °C) under natural light supplemented with 400 W high-pressure sodium lights (18 h photoperiod, 600 μmol m^{-2} s^{-1}). The rooting medium was kept constantly moist during the rooting and establishment period (approximately 2 weeks). Cuttings were watered when required, usually every one or two days. Plants were fertilized with 100 ppm 20-20-20 water-soluble fertilizer + micronutrients once every week for the first 2 weeks, and twice per week after 2 weeks. After 5 to 6 weeks of growth in the greenhouse (plants 30-45 cm tall), uniform sized plants were selected and alocated to experimental treatments in growth chambers and grown for 60 days.

Controlled environment chambers (Conviron model PGR15) used for this study were located in the Phytotron facility in the College of Agriculture and Bioresources at the University of Saskatchewan. Light levels were 300 μmol·m^{-2}·s^{-1} produced by an even ratio of incandescent and fluorescent lights. The relative humidity was 40-60%. Plants were exposed to a 12-hour photoperiod, which is similar to the average photoperiod present in September in Saskatoon, Saskatchewan (52°7'N) for 30 days. After 30 days, the photoperiod was reduced to 10 hours to account for reduction in photoperiod in the fall and plants grown for another 30 days. The plants were elevated with plastic pots about 10 cm above the aluminum floor, and watered when

required, normally once every one or two days. Plants were fertilized with 100 ppm 20-20-20 (N-P-K) water-soluble fertilizer + micronutrients twice per week for the first 3 weeks and once per week from that point to the end of the 60 day induction period. The plants were not allowed to dry out at any time.

3.2.2 Induction Temperature Regimes

Four day/night temperature regimes were used during the induction period (Table 3.1). These temperatures were selected to reflect current and predicted future annual variation in autumn temperature. The four temperature regimes were produced from a 2 x 2 factorial design with factors of mean temperature and day/night temperature difference. Two mean temperatures, the 30-year mean temperature (1961-1991) and the forecasted future mean temperature for September in Saskatchewan (2080), were used. Two day/night temperature differences (5°C and 15°C) accounted for diurnal variations of temperature that exist during the autumn.

Table 3.1 Induction temperature regimes within controlled environment chambers

	Day/Night Temperature Difference	
Mean Temperature (˚C)	5°C	15°C
11.0*	**13.5 ˚C /8.5 °C** Cool Day/ Intermediate Night (**CD/IN**)	**18.5˚C/ 3.5°C*** Intermediate Day/ Cool Night (**ID/CN**)
16.0	**18.5˚C/ 13.5°C** Intermediate Day/ Warm Night (**ID/WN**)	**23.5˚C/ 8.5°C** Warm Day/ Intermediate Night (**WD/IN**)

*Represents the mean temperatures for Saskatoon, Saskatchewan for the month of September based on 30 year means (1961-1991) (Environment Canada, 2005)

3.2.3 Measurements

3.2.3.1 Height

Eight plants from each treatment were randomly tagged when plants were placed in the chambers. These plants were measured weekly for height (cm) from the top of the cutting to the apical bud. Growth increment was calculated for each week. Using TableCurve2D (SPSS, Inc., Chicago, IL) software, regression curves, using the best-fit equation, were fit for each subject (plant) and the x-intercept point (x = 0) was considered to be the date of growth cessation.

Equation 3.1. Determination of time to growth cessation during dormancy induction derived from weekly growth increment.

$$y = a + bx^3$$

y = days

x = growth increment (cm)

a and b are constants

3.1

3.2.3.2 Dormancy

Dormancy development was measured using the bud-break method modified from Rinne *et al.* (1998). Five plants were sampled from each treatment every 10 days. Plant stems were defoliated and cut into two-node cuttings (approximately 6 cm) using the middle section, about 10 cm below the terminal bud. Plant stem sections were randomized within each treatment and put into 50 mL test tubes with 5 mL distilled water and placed into an E8H model controlled environment chamber (Controlled Environments Ltd., Winnipeg, Manitoba, Canada) under 18 hour photoperiod and 23 °C constant temperature. Light intensity was approximately 300 $\mu mol \cdot m^{-2} \cdot s^{-1}$ produced by an even ratio of incandescent and fluorescent lights. These environmental conditions promoted bud break in stem sections. Bud-break was determined as the point at which the first leaves started to emerge and unfold from the dormant bud (Figure 3.1). Longer times to bud-break indicate a higher level of dormancy.

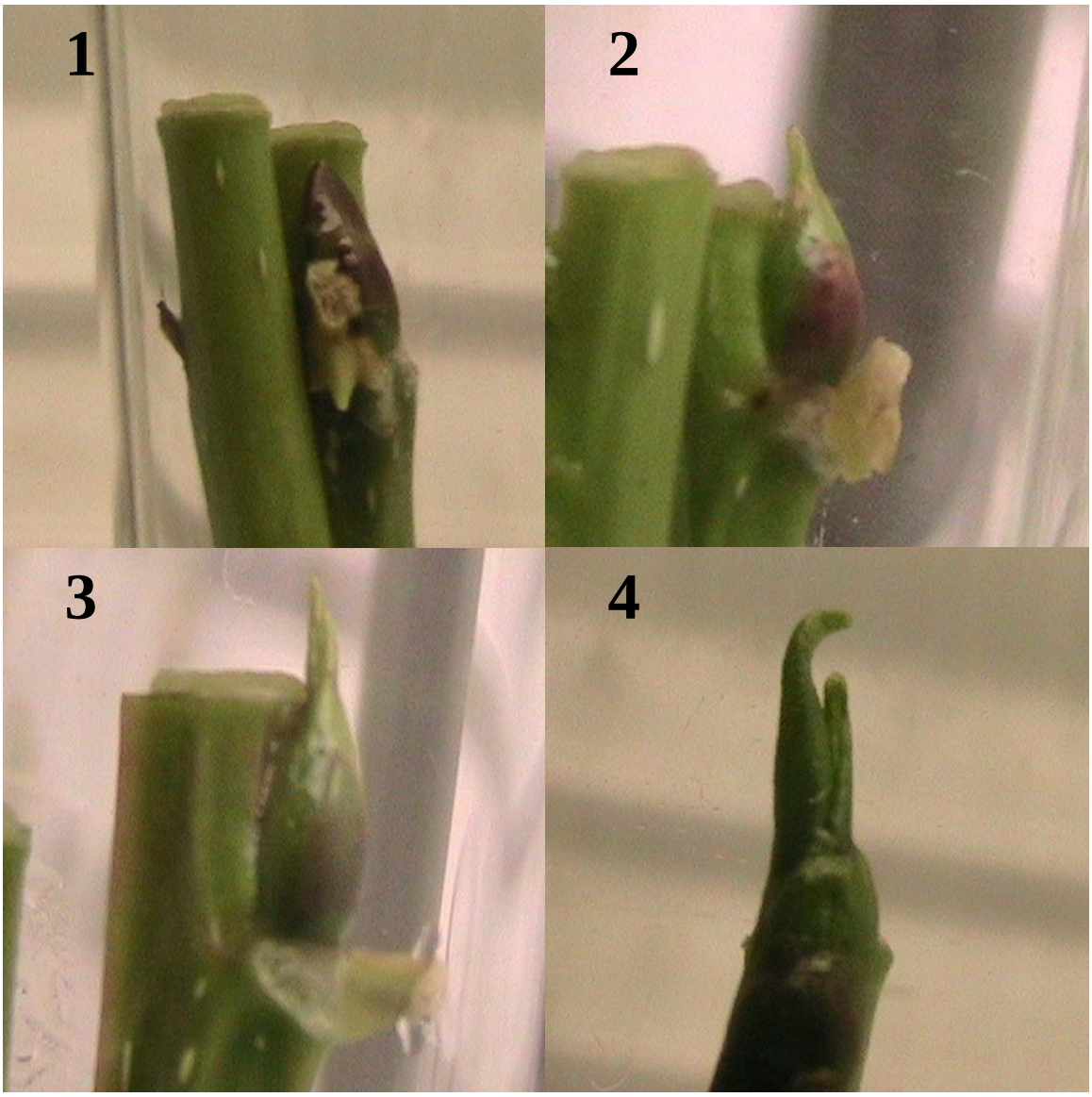

Figure 3.1 Progression of bud-break in hybrid poplar (Stages 1-4). Bud break is considered to be the point at which the first leaf starts to unfold after emerging from the dormant bud (Stage 4).

Incipient vegetative maturity (i-VM), a quantitative measure of the rate of dormancy acquisition, was calculated from dormancy levels in each treatment during the induction period. Mean Days-to Bud-Break (DBB), derived from the mean of 8 replicates within the treatment sample, was plotted against days of induction treatment. The date of i-VM (Svendsen *et al.*, 2007) was considered to be the date (x) when the first derivative (slope) was equal to 0.5 (y' = 0.5). Pair-wise t-tests were used to determine differences in i-VM dates between treatments.

Equation 3.2. Determination of timing of i-VM during dormancy induction derived from DBB data

$$y = a+be^{cx}$$

y = days to bud break,

x = days of dormancy induction,

a, b and c are constants derived through iteration until their values and correlation coefficients no longer change.

3.2

3.2.3.3 Cold Hardiness

Cold hardiness was determined using the electrolyte leakage method described by Flint *et al.* (1967). This methodology is closely correlated with actual field hardiness of poplar clones (Silim, pers. comm.). Five plants were sampled every 20 days and the top 10 cm of the stem tissue was used for testing. Stem segments were defoliated, double rinsed with distilled water and patted dry. Stem segments were cut into approximately one-millimetre slices, pooled and randomly distributed into scintillation vials. One mL of distilled water and a light dusting of silver iodide was added to the samples to stimulate ice nucleation. Scintillation vials (N = 3) were then assigned into four groups: an unfrozen control and three samples to be frozen at pre-determined temperatures. The unfrozen control and the other three treatments were placed in a Conviron, open-air chamber at 2°C (Controlled Environments Ltd., Winnipeg, Manitoba, Canada).

The three freezing temperatures were predetermined estimates from previous weeks cold hardiness values. Samples were run through a pre-programmed cycle in which the chamber cooled at 2°C/ hour to -2°C. Samples were held at this temperature for 6 hours, until freezing

equilibrium was attained. The chamber continued to cool to the first target temperature. When the temperature was reached, samples were held at the target temperature for 30 minutes and the set of samples for that temperature were removed and placed in a chamber at 2°C to thaw (normally overnight). This was repeated for the other two sample temperatures.

Once all samples were thawed, 10 mL of distilled water was added and samples were placed on a shaker at 90 rpm for 8 hours. After shaking, samples were measured for electrical conductivity (EC) using a CON 100 Series 'Oakton' Conductivity Meter (Eutech Instruments, Singapore). Samples were then placed in a water bath at 80 ºC for 15 minutes to kill the tissues. Samples were removed and left to cool to room temperature. Once samples cooled (normally overnight), EC was measured again. Injury index (Equation 3.3) was determined for each sample temperature in each treatment. Using regression curves, injury index was plotted against sample freezing temperature and the point, determined by best-fit curve, at which 50% injury occurred, was considered to be LT_{50}.

Equation 3.3. Determination of injury index from electrolyte leakage measurements after freezing tests

$$I_j \text{ (Injury Index)} = RC_{Temperature(X)} - RC_{Control} / 1 - RC_{Control}$$

$$RC_{Temperature\ (X)} = EC_{Temperature\ (X)} / EC_{Max\ -\ Temperature\ (X)} * 100\%$$

$$RC_{Control} = EC_{Control} / EC_{Max\ -\ Control} * 100\%$$

EC = Measured Electrical Conductivity (μmol)

X = Target Temperature (°C)

3.3

A completely randomized 4 x 4 factorial design was used. The treatments consisted of four clones, two mean temperatures and 2 day/night temperature amplitudes (4 temperature treatments). The entire experiment was repeated to confirm results. Growth cessation dates (N = 8) were analyzed using a two-way ANOVA using Tukey's LSD ($\alpha = 0.05$) for mean separation. DBB (N = 16) at 60 days of induction were analyzed using a two-way ANOVA, with the factors of temperature and poplar clone. Tukey's LSD ($\alpha = 0.05$) was used for mean separation. Differences in cold hardiness (N = 8) between treatments were analyzed at 20, 40 and 60 days of

induction using a two-way ANOVA with Tukey's LSD ($\alpha = 0.05$) used for mean separation. Pearson correlation coefficients were calculated between dormancy and cold hardiness for each clone. Correlation coefficients were also calculated, using Pearson correlation test, between day and night temperatures with growth cessation date, dormancy development and cold hardiness after 60 days of dormancy induction.

3.3 Results

3.3.1 Growth Cessation

Growth cessation was the first observed response of the plants to short-day photoperiod, preceding dormancy development and cold acclimation. Growth cessation occurred in all treatments before 60 days under the short photoperiod. Timing of growth cessation corresponded with terminal bud set (data not included). Depending on clone and induction temperature, the mean time to growth cessation ranged from 27.4 to 57.5 days (Table 3.2).

Induction temperature affected timing of growth cessation of each clone differently (Table 3.2). Time to growth cessation under a constant, short-day photoperiod in 'WP-69' (early acclimation) was not influenced by temperature ($P>0.05$). The range in mean days to growth cessation in 'WP-69' between temperature treatments was 3.2 days. However, temperature affected time to growth cessation in 'Walker' (intermediate acclimation), 'Katepwa' (intermediate acclimation) and 'Prairie Sky' (late acclimation) ($P<0.05$). Range between maximum and minimum days to growth cessation between temperature treatments for 'Walker', 'Katepwa' and 'Prairie Sky' were 29.3, 21.8 and 15 days, respectively.

Higher night temperature treatments reduced days to growth cessation in 'Katepwa', 'Prairie Sky' and 'Walker' but not 'WP-69'. Furthermore, as night temperature increased, the range in mean days to growth cessation between clones decreased. It appeared that cooler night temperatures resulted in a wider range in time to growth cessation between clones. Under the Intermediate Day/Cool Night (ID/ CN) temperature regime, the clonal difference between the maximum and minimum days to growth cessation was 20.3 days. Under the Intermediate Day/Warm Night ID/WN temperature treatment, the clonal difference between the maximum ('Katepwa') and minimum ('WP-69') days to growth cessation was 1.7 days. Night temperature was negatively correlated with days to growth cessation. Pearson correlation coefficients for

growth cessation and night temperature were -0.72 ($P<0.01$), -0.73, -0.82 ($P<0.001$) and -0.99 ($P<0.01$) for 'Walker', 'WP-69', 'Katepwa' and 'Prairie Sky', respectively (Table 3.3). Since there were minimal differences between temperature treatments for 'WP-69', correlations between night temperature and days to growth cessation was not significant.

Day temperature did not significantly affect growth cessation in any of the clones. There were no significant differences between the Warm Day/ Intermediate Night (WD/IN) (23.5^{o}C/ 8.5^{o}C day/night temperature) treatment and the Cool day/ Intermediate night (CD/IN) (13.5^{o}C/ 8.5^{o}C day/night temperature) treatment in any of the clones ($P=0.05$). Furthermore, day temperature did not significantly correlate with growth cessation in any of the clones (Table 3.3).

Although growth cessation occurred earlier under warmer night temperature, initial growth increment was higher once placed in inducing conditions. Growth increment was highest in the ID/WN temperature treatment in all clones (*Appendix A*). However, growth increment decreased faster and growth cessation was earlier in the ID/WN induction treatment than the ID/CN treatment. Overall growth was reduced under the warmer temperature treatments than cool temperature treatments as a result of earlier growth cessation (data not included). Plants in the ID/CN induction treatment were visibly taller than plants in the ID/WN induction treatment after 60 days of induction.

Table 3.2 Days to growth cessation during dormancy induction in four hybrid poplar clones (Walker, WP-69, Katepwa, Prairie Sky) under four different induction temperature regimes (13.5°C /8.5°C, 18.5°C /3.5°C, 18.5°C /13.5°C, 23.5°C /8.5°C).

Clone	Temperature	Days to Growth Cessation*
'Katepwa' (Intermediate Acclimation)	18.5°C / 3.5°C	50.9 a
	13.5°C / 8.5°C	33.4 b
	23.5°C / 8.5°C	29.7 bc
	18.5°C /13.5°C	29.1 c
'Prairie Sky' (Late Acclimation)	18.5°C / 3.5°C	42.4 a
	13.5°C / 8.5°C	35.2 b
	23.5°C / 8.5°C	33.6 b
	18.5°C /13.5°C	27.4 c
'Walker' (Standard Cultivar)	18.5°C / 3.5°C	57.5 a
	13.5°C / 8.5°C	34.5 b
	23.5°C / 8.5°C	33.8 b
	18.5°C /13.5°C	28.2 c
'WP-69' (Early Acclimation)	18.5°C / 3.5°C	30.6 a
	13.5°C / 8.5°C	29.7 ab
	23.5°C / 8.5°C	26.8 b
	18.5°C /13.5°C	27.4 ab

*Values determined by fitting weekly growth increments to the curve equation Y=a+bx3 and finding the point in which the weekly growth increment (cm/week) was equivalent to 0). Tukey's LSD ($\alpha = 0.05$) used for mean separation of temperature treatments within clones. N = 8

Table 3.3 Pearson correlation coefficients between night and day temperature treatment and mean growth cessation, mean dormancy development and mean cold hardiness of four poplar clones ('Walker', 'WP-69', 'Katepwa' and 'Prairie Sky'), * P≤0.05, ** P≤0.01, *** P≤0.001

Clone	Temperature Factor	Growth Cessation N = 4	Dormancy Development N = 8	Cold Hardiness N = 8
'Katepwa'	Day	-0.14	-0.44	0.44
	Night	-0.82***	0.81**	-0.87**
'Prairie Sky'	Day	-0.11	0.09	0.39
	Night	-0.99**	0.70	-0.87**
'Walker'	Day	-0.09	-0.23	0.22
	Night	-0.72***	0.44**	-0.61***
'WP-69'	Day	-0.64	-0.02	0.03
	Night	-0.73	0.26	-0.78*

3.3.2 Dormancy Development

Induction temperatures affected dormancy levels within axillary buds of each clone during 60 days of induction. In all actively growing clones, days to bud-break, a measure of bud dormancy, was approximately 5 days. Once growth cessation occurred, dormancy levels began to increase. DBB subsequently increased in all treatments during the induction period. Dormancy levels were highest in treatments where growth cessation occurred the earliest. Therefore, timing of growth cessation appeared to affect depth of dormancy development. There was an increasing range in mean days to bud-break of 10.4, 15.3 and 26.8 DBB between treatments at 20, 40 and 60 days of induction treatment, respectively (Figure 3.2, 3.3, 3.4, and 3.5). The interaction between clone and temperature resulted in the increasing range in dormancy levels at each measurement time.

Dormancy levels between temperature treatments were significantly different in 'Walker', 'Katepwa' and 'WP-69' (P=0.05) by 60 days of induction (Figure 3.2, 3.4, and 3.5). However, temperature affected dormancy levels in 'Walker' and 'Katepwa' to a greater extent than in 'WP-69'. 'Prairie Sky' was the exception where dormancy acquisition did not occur (Figure 3.3). For all clones except 'Prairie Sky', dormancy levels increased earlier under the ID/WN treatment than the ID/CN treatment. Dormancy development was significantly delayed under ID/CN induction temperatures (P=0.05). Similar to growth cessation, cool nights appeared to inhibit dormancy development in 'Walker', 'Katepwa' and 'WP-69'. Pearson correlation coefficients between night temperature and dormancy development were 0.44 (P<0.01), 0.26, 0.81 (P<0.01) and 0.70 for 'Walker', 'WP-69', 'Katepwa' and 'Prairie Sky', respectively (Table 3.3). Since night temperature was more closely correlated with dormancy development in 'Walker' and 'Katepwa', night temperature had a greater influence on dormancy development in these clones than 'WP-69' and 'Prairie Sky'. Day temperature did not significantly correlate with dormancy development in any of the clones (Table 3.3).

While DBB provides an accurate reflection of increases in dormancy over time, it cannot measure rate of dormancy acquisition. Incipient Vegetative Maturity (i-VM) allows for the quantification of change in rate of dormancy development during the induction period. Since DBB did not increase in 'Prairie Sky', it did not reach i-VM under any of the temperature conditions (Table 3.4). Conversely, in 'WP-69', all induction temperatures reached i-VM. i-VM was not attained under the ID/CN induction treatment for 'Walker' and 'Katepwa' but reached i-VM in all other induction treatments. For those induction treatments where i-VM was achieved, differences in dates to i-VM acquisition between induction temperatures were greater in some clones ('Walker' and 'Katepwa') than others ('WP-69').

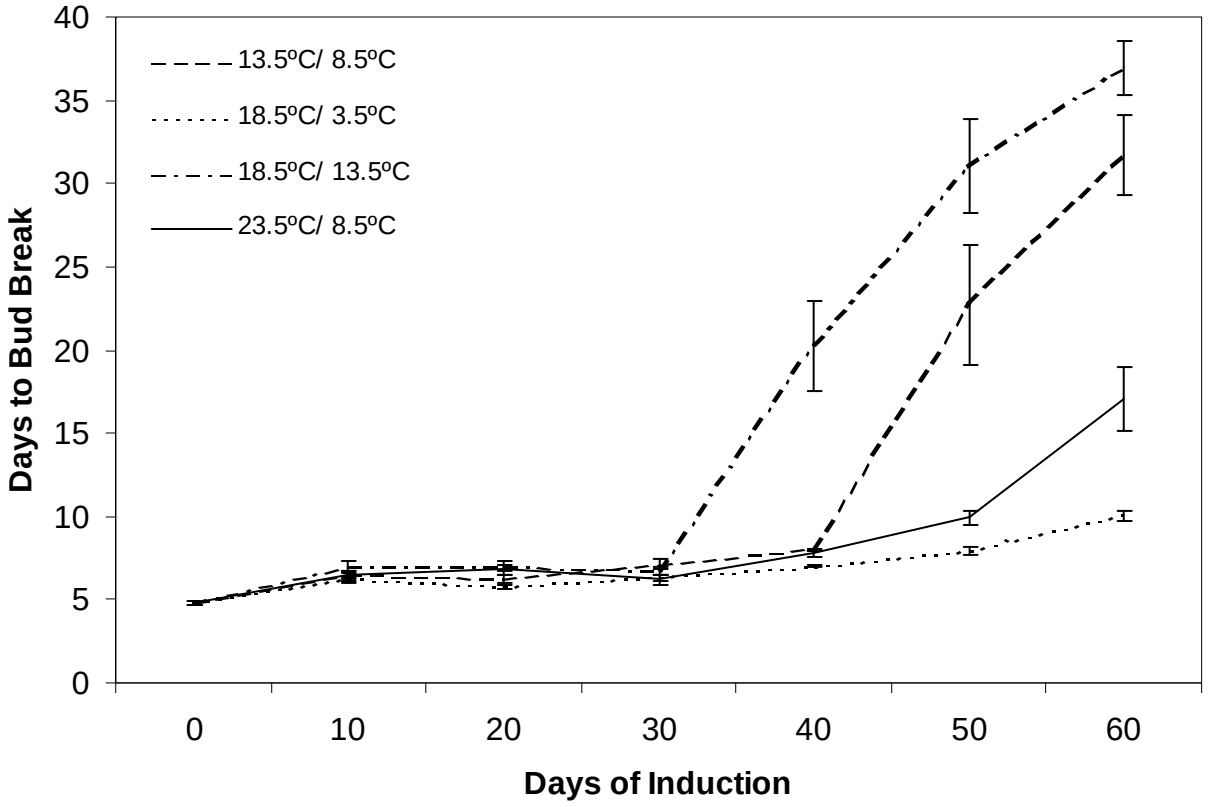

Figure 3.2 Dormancy development (days to bud-break) of 'Katepwa' hybrid poplar clones under short photoperiods in four controlled environment temperature conditions (13.5ºC/8.5ºC, 18.5ºC/3.5ºC, 18.5ºC/13.5ºC and 23.5ºC/8.5ºC day/night temperatures). Error bars denote ± SE. N = 16.

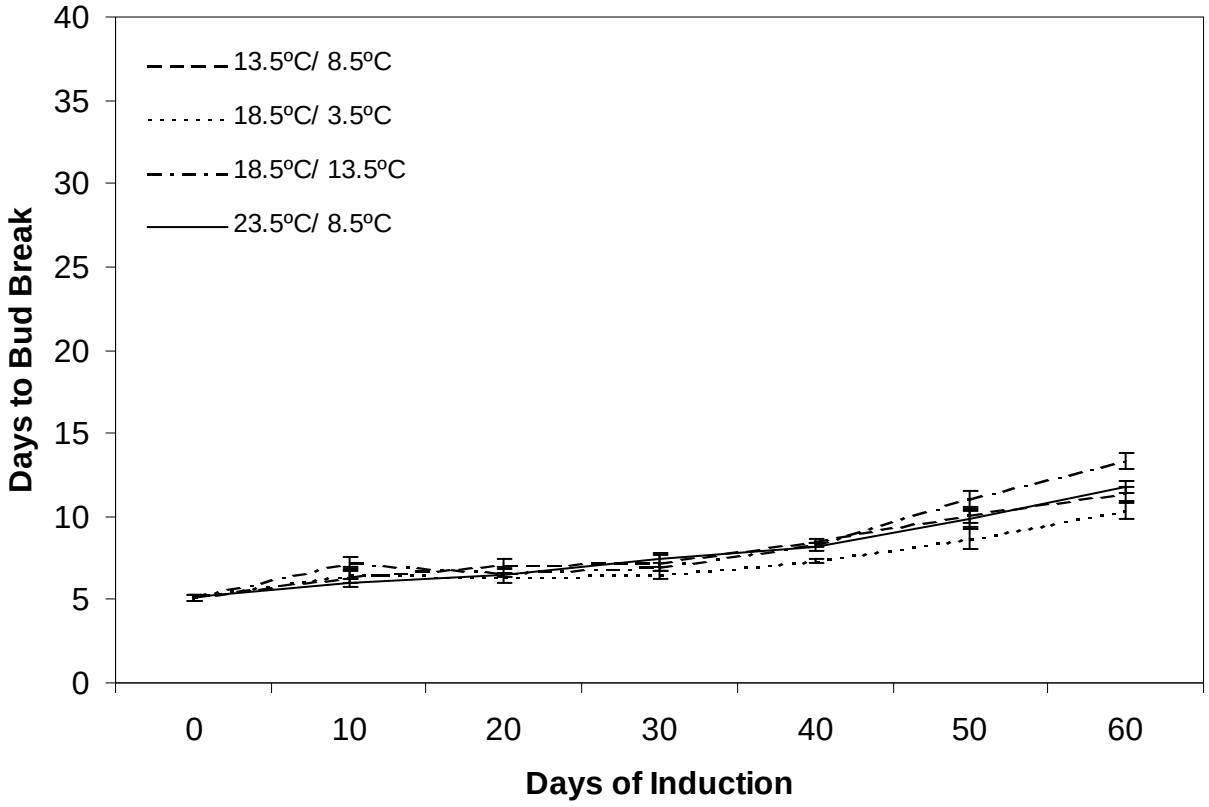

Figure 3.3 Dormancy development (days to bud-break) of 'Prairie Sky' hybrid poplar clones under short photoperiods in four controlled environment temperature conditions (13.5ºC/8.5ºC, 18.5ºC/3.5ºC, 18.5ºC/13.5ºC and 23.5ºC/8.5ºC day/night temperatures). Error bars denote ± SE. N = 16.

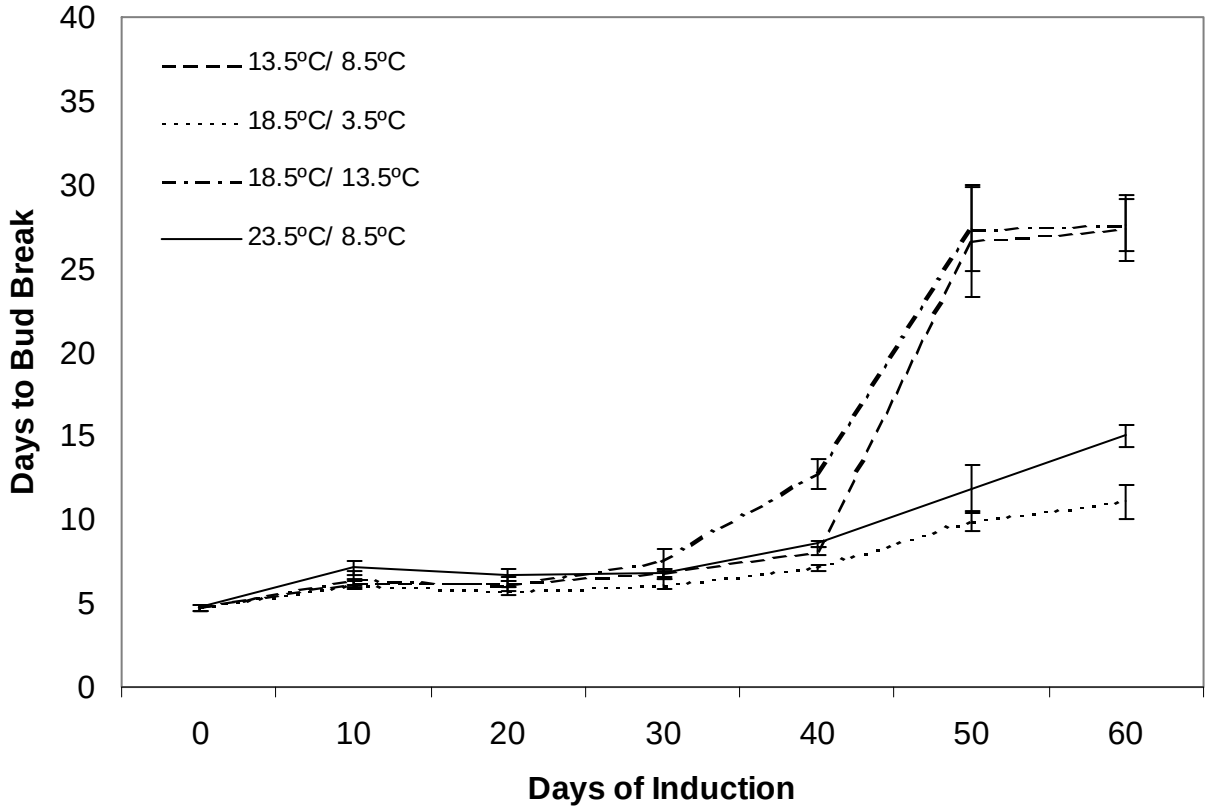

Figure 3.4 Dormancy development (days to bud-break) of 'Walker' hybrid poplar clones under short photoperiods in four controlled environment temperature conditions (13.5ºC/8.5ºC, 18.5ºC/3.5ºC, 18.5ºC/13.5ºC and 23.5ºC/8.5ºC day/night temperatures). Error bars denote ± SE. N = 16.

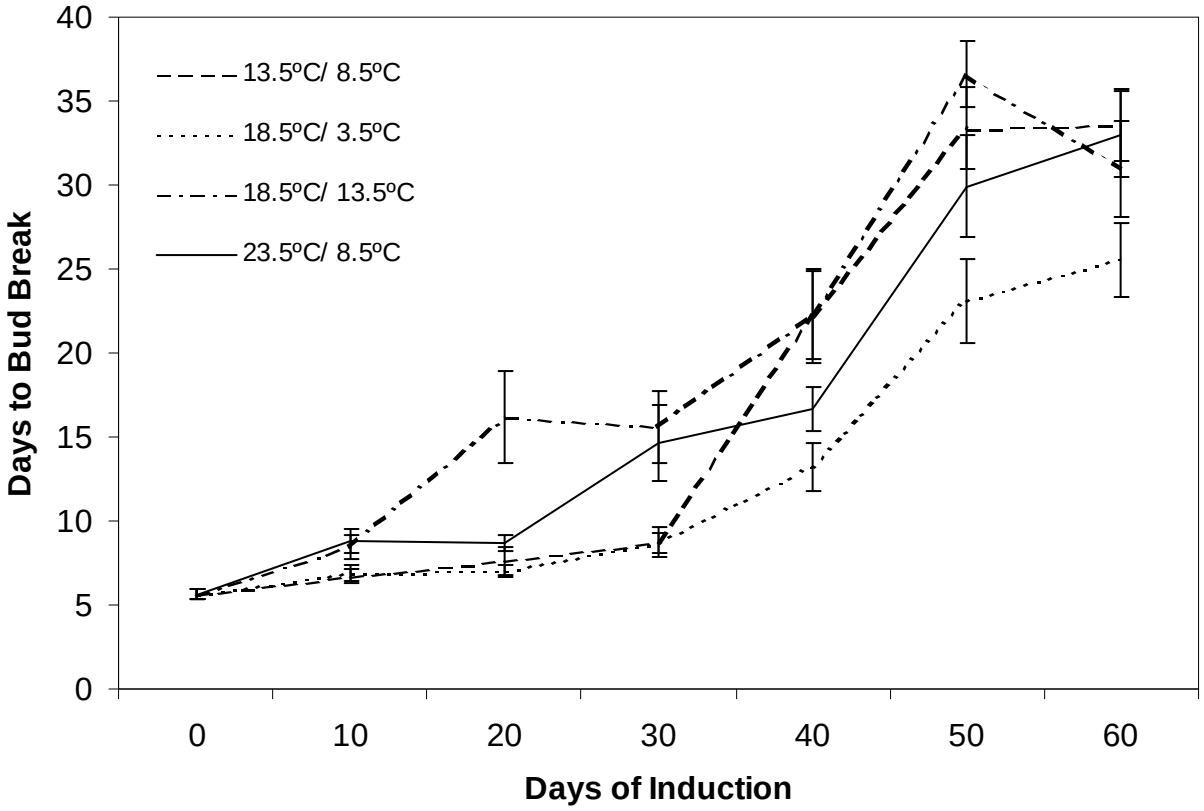

Figure 3.5 Dormancy development (days to bud-break) of 'WP-69' hybrid poplar clones under short photoperiods in four controlled environment temperature conditions (13.5ºC/8.5ºC, 18.5ºC/3.5ºC, 18.5ºC/13.5ºC and 23.5ºC/8.5ºC day/night temperatures). Error bars denote ± SE. N = 16.

Unlike growth cessation, day temperature also affected i-VM dates for 'Katepwa' and 'Walker' but not 'WP-69' with higher day temperatures delaying i-VM acquisition (Table 3.4). i-VM attainment under the WD/IN treatment was delayed an average of 11.1 and 7.9 days compared to the CD/IN induction temperatures in 'Walker' and 'Katepwa', respectively. While there were differences between day temperature treatments, the differences between night temperature (ID/CN and ID/WN) induction treatments were greater (Table 3.4). In 'WP-69', in which i-VM was attained under all induction treatments, on average, i-VM was reached 12.6 days later under the ID/CN induction treatment compared to the ID/WN induction treatment. In 'Walker' and 'Katepwa', the difference between the ID/CN and ID/WN induction treatments was greater than 23.2 and 24.2 days, respectively, since i-VM attainment did not occur under the ID/CN induction treatment for either clone.

Table 3.4 Incipient Vegetative Maturity (i-VM) dates for four hybrid poplar clones under short photoperiods under four different temperature conditions (13.5°C/8.5°C, 18.5°C /3.5°C, 18.5°C /13.5°C and 23.5°C /8.5°C day/night temperatures)

Clone	Temperature	% Reached i-VM	Days to i-VM[1]
'Katepwa'	13.3/8.5	100	44.3 b
	18.5/3.5	0	no i-VM
	18.5/13.5	100	35.8 a
	23.5/8.5	88	52.4 c
'Prairie Sky'	13.3/8.5	0	no i-VM
	18.5/3.5	0	no i-VM
	18.5/13.5	19	no i-VM
	23.5/8.5	13	no i-VM
'Walker'	13.3/8.5	100	43.0 b
	18.5/3.5	13	no i-VM
	18.5/13.5	100	36.8 a
	23.5/8.5	100	54.1 c
'WP-69'	13.3/8.5	100	33.2 b
	18.5/3.5	100	40.8 c
	18.5/13.5	94	27.4 a
	23.5/8.5	100	34.3 b

[1] Letters indicate significant differences separated using Tukey's mean separation (P=0.05)

3.3.3 Cold Hardiness under Dormancy-inducing treatments

Under conditions promoting active growth (long photoperiod), cold hardiness of each clone was between –2 to –3°C. Mean cold hardiness between induction treatments at 20 days of induction ranged from –2.5 to –3.7°C. Significant differences in cold hardiness between clones and temperature treatments were observed only at 40 and 60 days of induction. At 40 days of induction, cold hardiness ranged from –4.2 to –10.7°C while after 60 days, cold hardiness ranged from -5.0°C to -22.5°C depending upon clone and temperature treatment. At 60 days of induction, the range in mean cold hardiness between temperature treatments was 11.2°C, 4.1°C, 11.4°C and 3.7°C for 'Walker', 'WP-69', 'Katepwa' and 'Prairie Sky', respectively (Figures 3.6, 3.7, 3.8, and 3.9).

A high range in cold hardiness between induction temperatures was a reflection of timing of growth cessation, which is reinforced by a close negative correlation between growth cessation and cold hardiness in all clones. Pearson correlation coefficients for timing of growth cessation and cold hardiness after 60 days of induction for 'Walker', 'WP-69', 'Katepwa' and 'Prairie Sky' were –0.94, -0.84, -0.97 and –0.89, respectively. Cold hardiness, after 60 days of induction, was higher under treatments, which produced earlier growth cessation. Delayed growth cessation resulted in delayed cold acclimation.

In 'Walker', there were minimal increases in cold hardiness under all induction treatments over the first 40 days of induction. Increases in cold hardiness were observed at 40 days of induction for all induction temperatures, except under the ID/CN induction treatment (Figure 3.8). When early growth cessation occurred (ID/WN treatment), increases in cold hardiness were observed at 40 days. Cool night temperatures (ID/CN) reduced cold hardiness of 'Walker' at 60 days of induction while maximum cold hardiness was observed for each clone at 60 days of induction under the ID/WN induction treatment. Mean cold hardiness of 'Walker' was –6.3°C and -17.5°C at 60 days of induction in the ID/CN and ID/WN induction treatment, respectively. Dormancy induction is distinct from growth cessation since day temperature also influenced dormancy response. Correspondingly, cooler day temperature appeared to result in increased cold hardiness in 'Walker'. Cold hardiness was greater in the CD/ IN temperature treatment at 60 days (-14.3°C) compared to the WD/IN temperature treatment (-9.9°C).

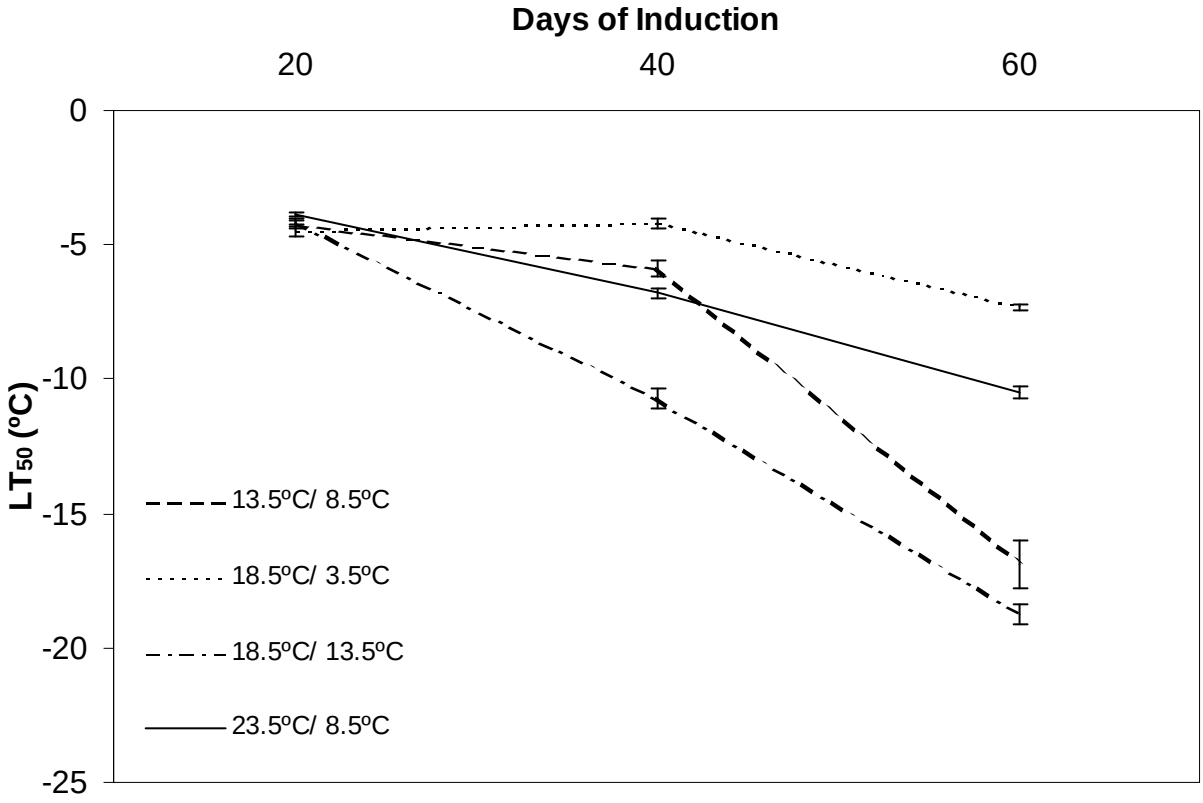

Figure 3.6 Cold hardiness of 'Katepwa' hybrid poplar (Populus sp.) clone under short photoperiods in four controlled environment temperature conditions (13.5ºC/8.5ºC, 18.5ºC/3.5ºC, 18.5ºC/13.5ºC and 23.5ºC/8.5ºC day/night temperatures). Error bars denote ± SE. N = 8.

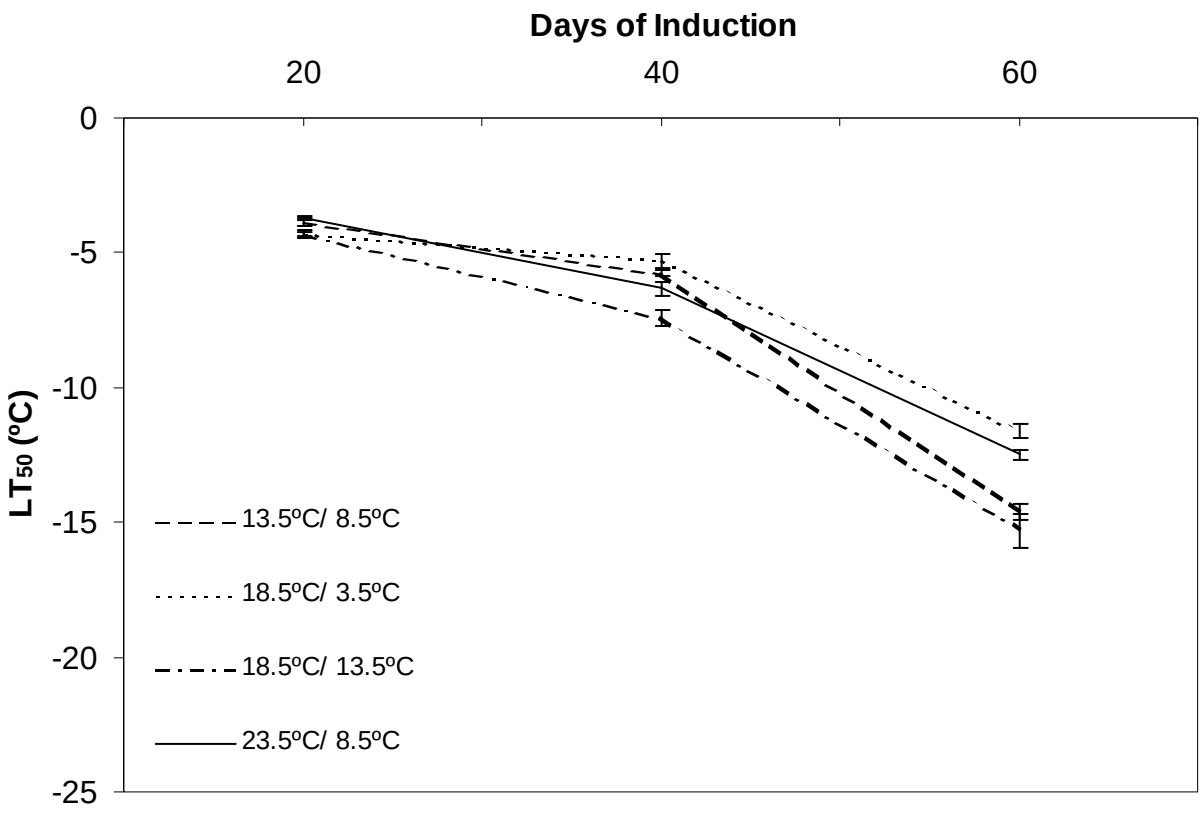

Figure 3.7 Cold hardiness of 'Prairie Sky' hybrid poplar (Populus sp.) clone under short photoperiods in four controlled environment temperature conditions (13.5ºC/8.5ºC, 18.5ºC/3.5ºC, 18.5ºC/13.5ºC and 23.5ºC/8.5ºC day/night temperatures). Error bars denote ± SE. N = 8.

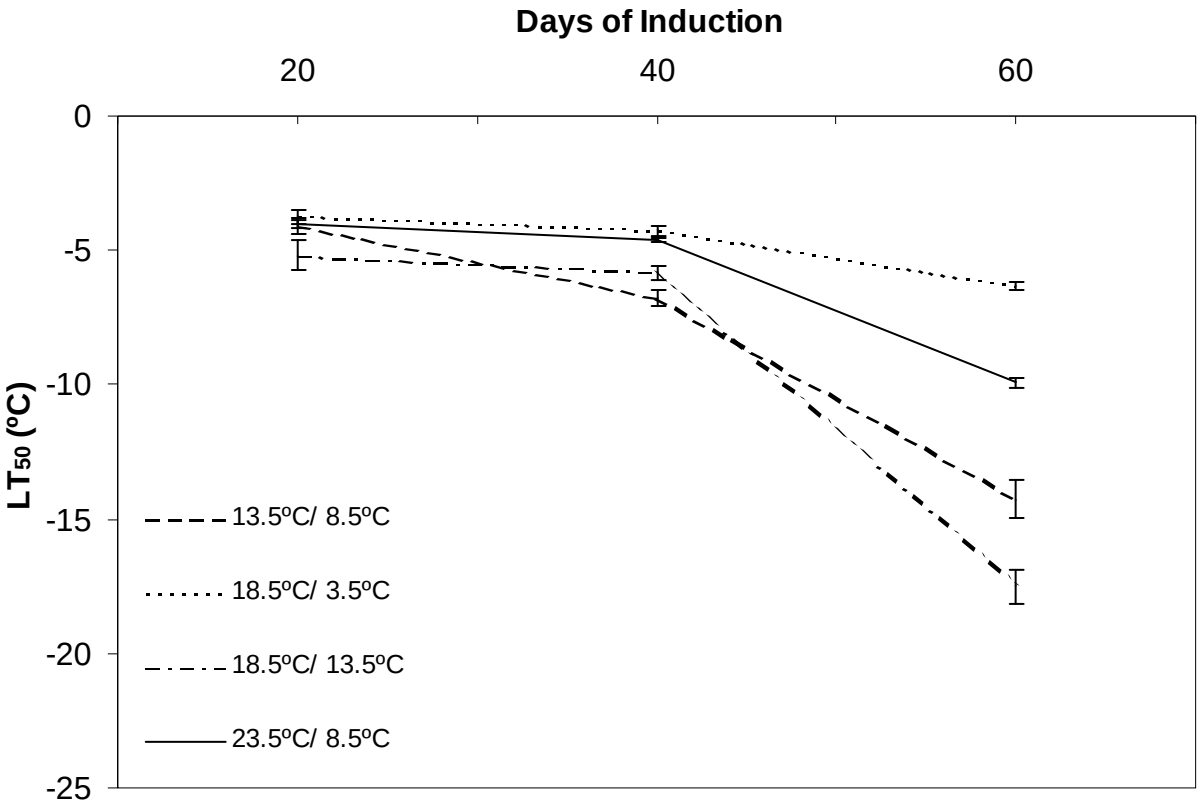

Figure 3.8 Cold hardiness of 'Walker' hybrid poplar (*Populus sp.*) clone under short photoperiods in four controlled environment temperature conditions (13.5ºC/8.5ºC, 18.5ºC/3.5ºC, 18.5ºC/13.5ºC and 23.5ºC/8.5ºC day/night temperatures). Error bars denote ± SE. N = 8.

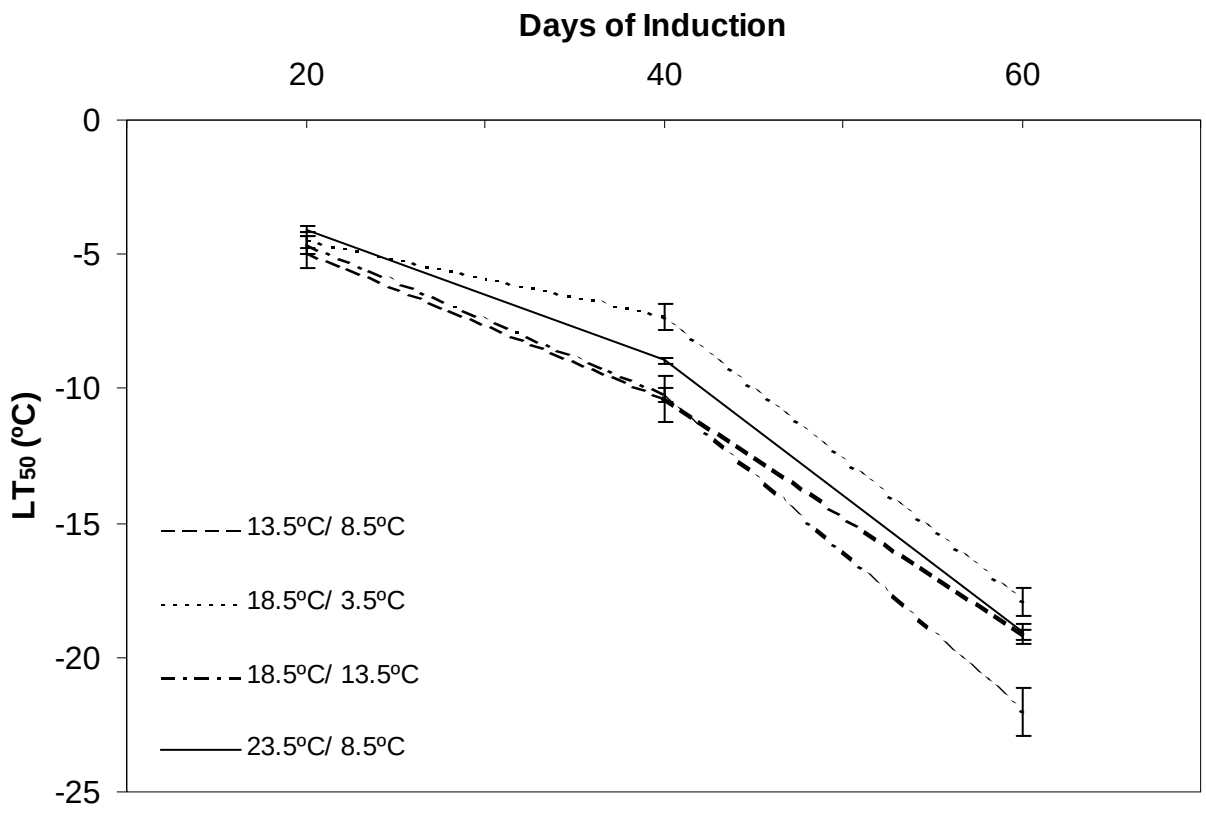

Figure 3.9 Cold hardiness of 'WP-69' hybrid poplar (*Populus sp.*) clone under short photoperiods in four controlled environment temperature conditions (13.5ºC/8.5ºC, 18.5ºC/3.5ºC, 18.5ºC/13.5ºC and 23.5ºC/8.5ºC day/night temperatures). Error bars denote ± SE. N = 8.

Similar to ‘Walker’, increases in cold hardiness in ‘Katepwa’ were observed at 40 days of induction (Figure 3.6). Cold acclimation in ‘Katepwa’ was reduced under cool night temperature treatments compared to warm night temperature treatments. At 40 and 60 days, cold hardiness was greatest for ‘Katepwa’ in the ID/WN induction treatments. In the ID/WN temperature treatment, ‘Katepwa’ had a mean cold hardiness of –10.7^{o}C and -18.7^{o}C at 40 and 60 days of induction treatment, respectively. In contrast, cold hardiness in ‘Katepwa’ was –7.3^{o}C at 60 days in the ID/CN induction treatment. Similar to ‘Walker’, cooler day temperatures resulted in increases in cold hardiness in ‘Katepwa’ at 60 days of induction. Cold hardiness was greater under the CD/IN (-16.9^{o}C) induction treatment compared to the WD/IN (-10.5^{o}C) treatment.

Distinct from ‘Walker’ and ‘Katepwa’, ‘WP-69’ showed earlier increases in cold hardiness (Fig 3.8). This is consistent with growth cessation where timing of growth cessation for ‘WP-69’ was earlier than other clones. Similar to other clones, cold hardiness for ‘WP-69’ was lower in the ID/CN induction temperature compared to the ID/WN induction treatment at 60 days of treatment. However, the range in cold hardiness between induction treatments was not as pronounced in ‘WP-69’. The difference in mean cold hardiness at 60 days between the ID/CN and ID/WN induction treatments was 3^{o}C for ‘WP-69’ compared to 11.2^{o}C and 11.4^{o}C for ‘Walker’ and ‘Katepwa’, respectively.

‘Prairie Sky’ was able to acclimate under growth cessation and in the absence of dormancy development. At 60 days of induction, there was no significant difference in cold hardiness for ‘Prairie Sky’ between induction temperatures. The range in cold hardiness between the maximum (ID/WN) and minimum (ID/CN) cold hardiness between induction treatments was 3.7^{o}C. While induction temperature minimally affected cold acclimation, overall cold hardiness was reduced in ‘Prairie Sky’ compared to other clones. The maximum cold hardiness under the ID/WN induction treatment for ‘Prairie Sky’ was –15.3^{o}C compared to –18.7, -17.5 and –22.0^{o}C for ‘Katepwa’, ‘Walker’ and ‘WP-69’, respectively.

3.4 Discussion

Low night temperature appeared to inhibit growth cessation, which subsequently delayed dormancy development and cold acclimation in hybrid poplar. The exception was ‘Prairie Sky’,

in which growth cessation occurred but an endodormant state was not attained. Conversely, under long photoperiod and low temperatures, the short photoperiod requirement for growth cessation and dormancy induction was bypassed in northern ecotypes of *Cornus sericea* L. and dormancy levels were higher under a cooler temperature treatment (Svendsen *et al*., 2007). Under short photoperiods, cool temperatures have been shown to inhibit dormancy development in *Cornus sericea* L. (Fuchigami *et al*., 1971) and *Betula pubescens* (Heide, 2003). Furthermore, earlier studies examining photoperiod effects on woody plants reported cool temperatures inhibit dormancy development under short photoperiod according to visual bud-set (Van der Veen, 1951; Downs and Borthwick; 1954). Molmann *et al.*, 2005 also reported that bud set occurs in plants placed into cold conditions (approximately 5°C), however endodormancy was not attained.

While this study used day/night temperature differences in combination with mean temperature treatments, these factors were not analyzed individually due to an interaction between day/night temperature difference and mean temperature. This indicates each factor was not individually contributing toward dormancy induction or cold acclimation, but these two factors contributed together to the overall response. Therefore, temperature treatments were analyzed as night and day temperature contributions rather than day/night and mean temperature effects.

Another factor that may explain some of the discrepancies between the results and previous studies may be the temperature treatments applied during induction. Paton and Willing (1968) showed that temperatures above 20ºC inhibited dormancy induction, suggesting an optimum temperature for dormancy induction in woody plants. This optimum range in temperature may be greater for some species of ecotypes than others. Temperature treatments were not much greater than 20ºC for either day or night temperature. Night temperature treatments were never greater than 13.5°C in this study. This lies within the middle of the treatment range for temperatures applied during dormancy induction by Junttila *et al.* (2003) using *Betula pendula.* Higher temperatures in that study were also used, which resulted in a decrease in dormancy development. This suggests an optimum temperature between 15 and 18°C exists. It is possible there are optimum temperatures for dormancy development and genetic variation exists for this response. Temperature treatments chosen for induction treatments were selected from environmental data at a time when the trees are going dormant from the region

where these poplar clones grow. Temperature treatments, particularly day temperature, may not have been low enough to show responses observed in Stevenson (1994), Junttila *et al.* (2003), and Svendsen *et al.* (2007).

One hypothesis that may explain some of the growth cessation and dormancy responses to night temperature under short photoperiod is the effect of temperature on the phytochrome pathway. Phytochrome conversion to the active P_{fr} form occurs instantaneously when exposed to red light. However, during the night P_{fr} reverts back to the inactive P_r form in addition to breakdown of the P_{fr} form of phytochrome. This reaction is a function of both time and temperature (Anderson, 1969; Mumford, 1966). Phytochrome reversion and breakdown may be inhibited by cooler night conditions, which may subsequently delay photoperiod-induced bud-set. Changes in expression of PhyA can alter critical photoperiod for growth cessation in hybrid aspen (*Populus tremula* x *tremuloides*) (Olsen *et al*., 1997). Mollman *et al*. (2005) observed a delay in bud-set and growth cessation in cooler temperatures under short photoperiods. Halliday and Whitelam (2003) and Thingnaes *et al*. (2003) observed decreasing temperature altered flowering in *Arabidopsis thaliana*, similar to responses observed by increasing photoperiod. Cooler night temperature could reduce the rate of P_r reversion, essentially maintaining a higher ratio of P_r: P_{fr}, which may counteract or inhibit the effect of short photoperiods.

Although using hybrid clones with differential acclimation patterns in the fall was useful in studying how temperature affects dormancy, it is difficult to identify why each clone responded so differently. Using hybrid poplar made it difficult to identify whether latitudinal or other geographical origins accounted for the differential response to temperature. 'Walker' is a *P. deltoides* x *P.* x *petrowskyana* selection released in 1963. *P.* x *petrowskyana* is a cross between *P. nigra* and *P. laurifolia.*. 'WP-69', a seedling from 'Walker' did not show the same high response to temperature.Since 'WP-69' is a backcross and showed different dormancy traits than 'Walker', conceivably, the observed low temperature response came from the other parent, not 'Walker'. This is consistent with the observation that 'Katepwa', an open-pollinated seedling of 'Walker', was similarly affected by temperature as 'Walker'. However, without knowing the geographic origin of the parents of the selections, it is difficult to establish whether latitudinal origin affects response to temperature. Junttila *et al.* (2003) and Heide (2003) both observed that northern ecotypes were less responsive to temperature than southern ecotypes in birch (*Betula pendula* L. and *Betula pubescens* L.). While using latitudinal or altitudinal ecotypes may be

more ecologically applicable, high variation in dormancy acquisition and cold acclimation patterns observed in hybrid poplar clones during the fall make them useful in studying the impact of temperature on dormancy-related processes.

'Prairie Sky' would also be a useful control in dormancy studies since dormancy acquisition did not occur under any of the induction treatments. 'Prairie Sky' is a *P. deltoides* x *P. nigra* selection that may be a clonal anomaly where growth cessation occurs but does not reach an endodormant state. There are other species of trees that do not achieve true endodormancy such as *Thuja plicata and Chameocyparis nootkantensis,* which only attains ecodormancy and new growth occurs upon return to environmental conditions favourable for growth (Silims and Lavender, 1993). These trees can be susceptible to winter damage on the Canadian prairies where deacclimation can occur under winter warming periods (Colombo and Raitanen, 1991). 'Prairie Sky' can be also used to separate dormancy from cold hardiness processes. This enables investigation of other physiological responses corresponding to dormancy induction in woody plants.

Since dormancy development was not necessary for cold acclimation initiation to occur in 'Prairie Sky' and increases in cold hardiness directly followed growth cessation, it can be suggested that growth cessation, not dormancy initiation, is the prerequisite for cold acclimation. Cold acclimation and dormancy development occurred simultaneously in other clones. However in all cases, increases in cold hardiness and dormancy levels followed growth cessation. Furthermore, when growth had ceased in less than 50 days and plants were placed in a growth chamber at 5°C, all plants attained a high level of cold hardiness (>-40°C) (data not shown). Treatments where growth cessation occurred between 50 and 60 days were not able to acclimate to the same extent. Weiser (1970) suggested cold acclimation is dependent on dormancy induction. Moreover, Fuchigami *et al.* (1982) in the description of the 360^{o} degree growth stage cycle, suggested that cold acclimation is dependent on attainment of the 180^{o} stage of dormancy, vegetative maturity. Vegetative maturity can be defined as the point in which a bud, when exposed to favourable environmental conditions, does not grow. It is more likely that growth cessation is a prerequisite for both dormancy development and cold acclimation in woody plants, which is consistent with Fuchigami *et al*. (1971).

The effect of temperature on growth cessation and dormancy development does not only affect processes during the autumn. Dormancy development is essential to prevent deacclimation

during the winter. Tanino *et al.* (1989) reported that *Cornus sericea* L. did not deacclimate if the chilling requirement was not satisfied. Since 'Prairie Sky' does not enter a dormant state, it may be susceptible to damage during the winter, particularly under mid-winter warming periods. Moreover, Silim *et al.* (2005, unpublished data) observed that 'Prairie Sky' would deacclimate or stop acclimating in response to warm temperatures late into the autumn. Clones that maintain a higher level of dormancy (higher chilling requirement) may be less susceptible to mid-winter warming (Heide, 2003). The true value of dormancy is to be able to maintain that cold hardy state even under warm, mid-winter conditions (Tanino, pers. comm., 2007). Dormancy prevents deacclimation (Irving and Lanphear, 1967; Litzow and Pellett, 1980; Tanino *et al.*, 1989) but is not the prerequisite for acclimation to occur.

In conclusion, photoperiod interacted with temperature during dormancy induction. Increases in temperatures, particularly night temperature impacted dormancy development and cold acclimation induction in hybrid poplar. Changes in temperature can significantly alter timing of growth cessation, which subsequently may affect dormancy development and cold acclimation. Cool night temperatures inhibit growth cessation under short photoperiod. Conceivably, warmer temperatures during the autumn would result in higher dormancy levels in plants, possibly resulting in earlier cold acclimation and an increased chilling requirement. Delays of growth cessation, resulting from cooler night temperatures, were greater in 'Katepwa' and 'Walker' than 'Prairie Sky' and even less in 'WP-69'. It appears that dormancy development and cold acclimation is more variable in response to temperature in some genotypes than others. Under future warmer temperatures, genotypes like 'WP-69' that are less sensitive to temperature may be able to better adapt to changing climates than clones like 'Walker' and 'Katepwa'. Applying these results to ecotypes and different species with different dormancy acquisition characteristics would be useful in determining whether these results obtained in hybrids can be applied to natural plant species.

4.0 USING MAGNETIC RESONANCE MICRO-IMAGING (MRMI) TO MEASURE LOCALIZED CHANGES IN BIOPHYSICAL CHARACTARISTICS OF WATER DURING DORMANCY INDUCTION IN HYBRID POPLAR

4.1 Introduction

Dormancy induction is associated with a reduction in water content of woody plant tissue (Rinne *et al.*, 1994; Jeknic and Chen, 1999) including bud water content (Li *et al.*, 2003). A decrease in water content corresponds with growth cessation, which occurs during autumn in woody plants. Since water is an essential component of life and the catalyst in which many metabolic processes occur, biophysical interaction of water with the cellular environment may contribute to maintenance of dormancy in woody plants (Faust *et al.*, 1995). Changes in concentration of hydrophilic molecules, such as sugars and dehydrin proteins contribute to changes in water binding in plant tissues that can affect water and its biophysical interactions within plant tissue during dormancy induction and cold acclimation. Changes in osmotic balance and membrane permeability can also impact movement of water between cellular and extracellular spaces. Studying biophysical changes in water during dormancy induction will therefore yield information on how changes in water mobility and biophysical properties are related to dormancy development and regulation of the dormant state in woody plants.

While water content has been shown to decrease during dormancy induction, previous research examined whole plant changes in water content during dormancy induction using the gravimetric method (Rinne *et al.*, 1994; Li *et al.*, 2003). However, this method cannot non-destructively identify localized changes in water content within isolated tissues. Furthermore, water content is only one component of the interaction of water with the cellular environment. Other components include water mobility, membrane permeability and osmotic balance. Nuclear magnetic resonance (NMR) imaging is a comprehensive measurement of all factors affecting biophysical parameters of water within plant tissues. Magnetic resonance images of heterogeneous systems are derived from nuclear magnetic resonance relaxation characteristics based on bulk water distributed within a three dimensional sample (Morris, 1986; cited in Connelly *et al.*, 1987). NMR imaging was primarily developed for large sample sizes in clinical health applications limiting the need for increases in image resolution. Recent advances in NMR imaging technology has permitted the development of techniques to observe water content,

biophysical parameters of water and water mobility within plant tissues at a higher resolution (Ishida *et al.*, 2000).

NMR imaging maintains several advantages over more conventional methods of measuring water in plants. NMR imaging is non-destructive and can identify localized changes within plant tissues at an anatomical resolution. However, despite these advantages, earlier studies using NMR imaging suffered from low image resolution and long acquisition time (Connelly *et al.*, 1987), limiting the applicability of NMR imaging. Instead, NMR spectroscopy was often used to identify changes in water relaxation peaks in plants. This is not advantageous compared to NMR imaging because spectroscopy is not able to localize tissue-specific changes of water within the sample (Ishikawa *et al.*, 1998). More recently, increased image resolution resulting from higher powered magnets and better software increased the applicability of NMR imaging beyond clinical health applications (Ishida *et al.*, 2000).

Magnetic resonance micro-imaging (MRMI) represents a higher resolution NMR image. MRMI can be used to identify tissue-specific changes in both animals and plants. In plants, studies using MRMI include seed germination (Connelly *et al.*, 1987; Hou *et al.*, 1997), dormancy (Faust *et al.*, 1991; Erez *et al.*, 1998), and vascular water movement (Bottomly *et al.*, 1986). Using MRMI to study changes in water during dormancy induction in woody plants is useful because high-resolution images can detect changes in water in axillary buds and vascular tissue (Faust *et al.*, 1991). MRMI can be used to detect both static and dynamic changes in biophysical interactions of water during dormancy induction. T_1 or T_2 relaxation times are a reflection of water content, biophysical interactions of water within plant tissue and interactions with macromolecules (Belton and Ratcliffe, 1985; cited in Snaar and van As, 1992).T_1 or T_2 images are produced from a two-dimensional array of H_1-NMR spin-lattice (T_1) or spin-spin (T_2) relaxation times for each voxel in a sample.

Studies investigating water changes during dormancy have used NMR imaging during all stages of dormancy. This includes dormancy induction (Fennell and Line, 2001), chilling (Faust *et al.*, 1991; Gardea *at al.*, 1994; Erez *et al.*, 1998) and bud burst (DeFay, 2000). A significant portion of studies using NMR to investigate dormancy measured T_1 and/or T_2 relaxation times (Faust *et al.*, 1991; Gardea *et al.*, 1993; Fennell and Line, 2001 ; Erez *et al.*, 1998). While these parameters demonstrated changes in water content and biophysical characteristics within plant tissue, changes in water mobility could not be identified. Identifying changes in water mobility is

important because cell-cell communication and movement of metabolites is necessary for maintenance of metabolic activity promoting growth of the plant. Water content is a 'static' measurement of water that is not a true measure of water mobility.

Water mobility within biological tissue can be measured using diffusion-weighted imaging, is an NMR image that uses diffusion gradients to calculate a self-diffusion coefficient (Tanner, 1983). Its original application was to measure blood flow and fluid movement in animal tissue. However, this method can also be used for plant studies. Hou *et al.* (1997) used diffusion-weighted imaging to determine localized water uptake in *Avena fatua* seeds during germination. Diffusion-weighted imaging has been used to study dormancy in a handful of studies. Defay (2000) used diffusion-weighted images to show increases in water movement and activity during budburst in *Picea abies* buds after fulfillment of its chilling requirement. Diffusion-weighted images have also been used to observe increases in water mobility, reflected by higher Apparent Diffusion Coefficient (ADC) values, in tulip bulbs throughout the chilling period (van der Toorn, 2000). However, use of a low field strength magnet (1.5 Tesla) and large sample size (5 cm field of view) limited the resolution of the images produced. Increasing magnet power increases the resolution making it possible to produce diffusion-weighted images of axillary buds and stem tissue of woody plants, potentially yielding insight into localized changes in water mobility during endodormancy development.

This study addressed the question, 'do biophysical properties of water and water mobility change during endodormancy development?' T_1 and diffusion-weighted images that reflect changes in water content, biophysical interactions of water with the cellular environment and water mobility, were measured during dormancy induction using hybrid poplar clones expressing differential dormancy acquisition patterns in response to temperature. Localized changes in water within plant tissue such as axillary bud, vascular tissue, and vascular transition zones were observed. Furthermore, the extent to which T_1 and diffusion-weighted images (reflecting water status and mobility, respectively) correlate with dormancy development was also explored. This research will contribute to understanding how changes in biophysical characteristics of water and water mobility correspond with dormancy induction in woody plants.

4.2 Materials and Methods

4.2.1 Plant Material

Two hybrid poplar clones, 'Walker' and 'WP-69', were placed under two temperature conditions (18.5/3.5ºC and 18.5/13.5ºC day/night) previously shown to promote the largest differential dormancy development under short photoperiod (12 h). Days to Bud-Break (DBB), an indicator of dormancy development was significantly less in 'Walker' (11.1 days) under the cool night temperature (18.5ºC/3.5ºC day/night) compared to 27.6 DBB under warm night temperature (18.5ºC /13.5ºC day/night) at 60 days of inductive treatments (Chapter 3). DBB was also delayed in 'WP-69' under the cool night temperature but not to the same extent as in 'Walker'. DBB was 25.6 and 30.9 days for 'WP-69' under the cool night and warm night temperature treatments, respectively. Since 'WP-69' and 'Walker' show different dormancy development in response to night temperature, it provides an effective system for examining biophysical changes in water associated with endodormancy induction.

4.2.2 Sample Collection and Preparation

Plant samples were collected at the following time intervals during the induction treatment: 0, 40 and 60 days. Three plants were sampled from each treatment at each sampling date. For each plant, axillary buds, approximately 15 cm from the terminal bud were selected and used for imaging to ensure maturity of stem tissue. Stem sections were cut 2 cm below and above the selected axillary bud. Stem sections with axillary buds were placed in 10 cm NMR-sample tubes with moist cotton balls to prevent dehydration. Dormancy levels were also assessed on the same plants used for NMR at 0, 40 and 60 days, using a bud-break assay described in Chapter 3.

4.2.3 NMR Micro-imaging and Data Processing

Micro-imaging experiments were conducted using a Bruker AM (Bruker Biospin, Milton, Ontario) 8.45 Tesla, wide-bore spectrometer equipped with the standard micro-imaging accessories located at the National Research Council, Plant Biotechnology Institute (NRC-PBI)

in Saskatoon, SK, Canada. Data was collected on a 128 (read) x 128 (phase) array, using a 10.5 mm (i.d.) radio frequency (rf) Helmholtz coil. An orientation matrix was designed to orient slices to ensure slice uniformity across samples. Four image slices were produced from each bud: two slices corresponding to the vascular stem and vascular branching into the axillary meristem (slice 4 and 3, respectively in Fig. 3.1) and two slices within the axillary bud (lower and upper bud region, slice 2 and 1, respectively in Fig. 3.1). Regions Of Interest (ROIs) for the images were the vascular tissue, vascular transition region, and axillary mersitem region (Figure 3.1).

4.2.4 Diffusion-weighted experiments

Diffusion-weighted images were conducted using a spin-echo diffusion-weighted program with weighting factors of 30, 275 and 845 $s \cdot mm^{-2}$. Higher weighting factors encode less mobile water molecules such as tissue water. Increasing the weighting factor causes spins to de-phase faster. Free mobile water (extra-cellular water and water in vascular tissue) will appear at low diffusion weighting factors. If spins do not move, they will be re-phased and if they do move, they will not be re-phased and will be lost to the signal. Three weighting factors were used to plot weighting factors (x) against intensity (y) and develop a best-fit equation that identifies I_o (Y-intercept). To calculate Apparent Diffusion Coefficient (ADC), the two higher weighting factors (275 $s \cdot mm^{-2}$ and 845 $s \cdot mm^{-2}$) were used to reduce detection of highly mobile extra-cellular water that is detected at lower weighting factors. The apparent diffusion coefficient was calculated using Equation 4.1. Higher ADC values represent greater water mobility within the sample tissue. Once the ADC was calculated for each ROI in each sample, the data was analyzed using a two-way ANOVA ($\alpha = 0.05$) for each ROI, where clone and temperature regime were fixed effects. Paired t-tests ($\alpha = 0.05$) were used to determine differences between ROIs.

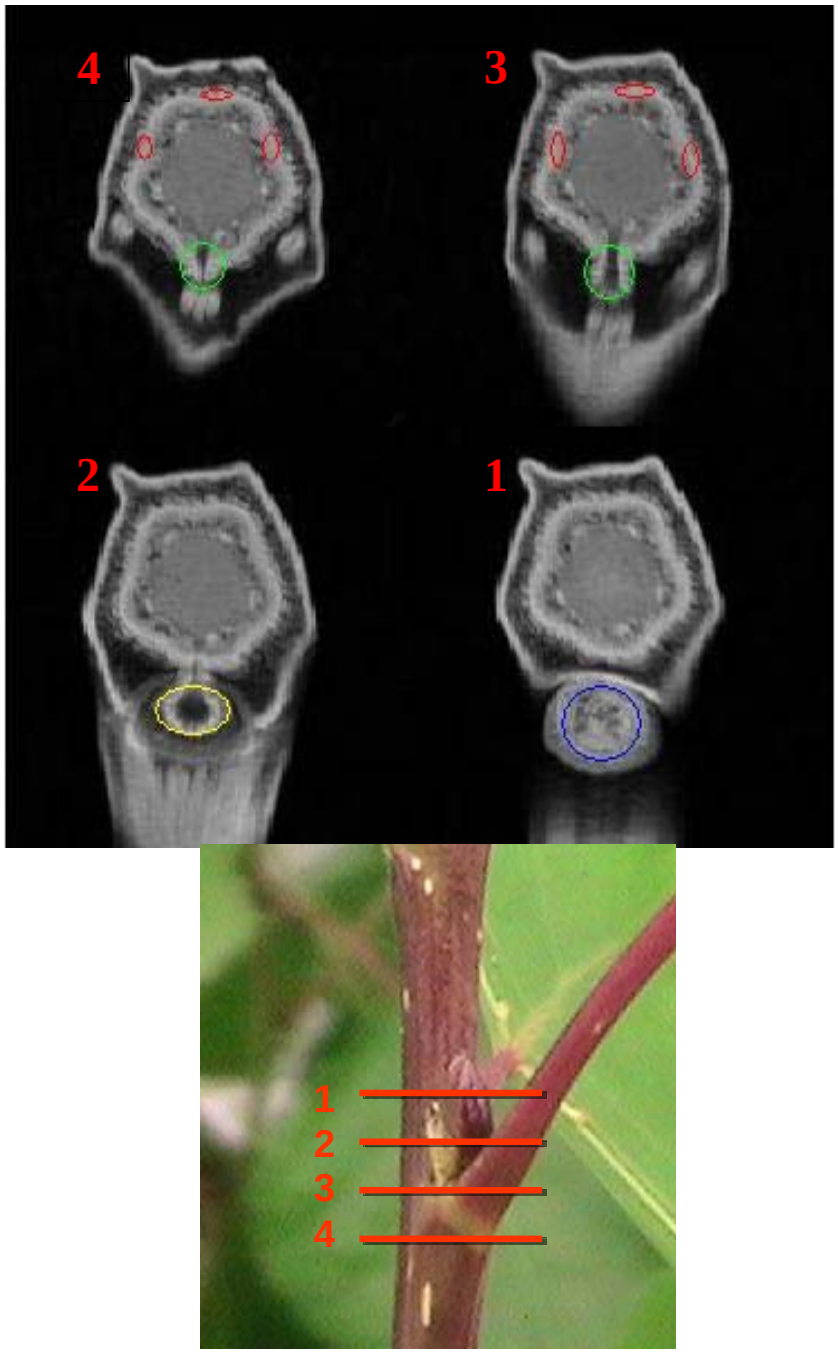

Figure 4.1 Sampling of T_1 relaxation and Apparent Diffusion Coefficient (ADC) raw data NMR images in a hybrid poplar stem and axillary bud. 1 = Upper axillary bud, 2 = Lower axillary bud, 3 = Base of axillary bud, 4 = Stem tissue. Four cross sectional slices taken through the stem and axillary bud: Red = vascular stem tissue, Green = vascular transition, Blue = upper axillary bud, Yellow = lower axillary bud.

Equation 4.1. Calculation of Apparent Diffusion Coefficient (ADC) derived from intensity values sampled from diffusion-weighted NMR images

$$\mathbf{ADC = [\ln(I_o - I_b) - \ln(I_o - I_a)]/(b-a)}$$

$a = 275\ s \cdot mm^{-2}$,

$b = 845\ s \cdot mm^{-2}$

I_a, I_b = mean intensity of ROI from images with weighting factor of

$275 s \cdot mm^{-2}$ and $845 s \cdot mm^{-2}$, respectively

4.1

4.2.5 T_1-weighted Experiments

T_1-weighted images were produced using a spin-echo T_1-weighted program. Three sets of images corresponding to three TR (repetition time) of 100, 500 and 1000 ms were produced. Mean intensity values for each ROI (y) was plotted against TR (x) and T_1 values calculated using Equation 4.2.

Equation 4.2. Calculation of T_1 relaxation times from intensity values sampled from T_1-weighted NMR images

$$I = I_0 (1 - e^{-t/T_1})$$

I = Intensity,

I_0 = maximum intensity,

t = TR (ms)

T_1 = T_1 relaxation time (ms).

4.2

Higher T_1 relaxation times correspond to higher free water content and less binding of water in the sample tissue. Once the T_1 values were calculated for each ROI in each sample, the data was analyzed using a three-way ANOVA ($\alpha = 0.05$) for each ROI where day, clone and

temperature regime were fixed effects. Paired t-tests ($\alpha = 0.05$) were used to determine differences between ROIs.

4.3 Results

4.3.1 Dormancy Development

Differential dormancy development was induced between treatments in axillary buds during the induction period (Figure 4.2). Dormancy levels, indicated by DBB, in 'Walker' were 19.4 and 8.6 days under the cool night (CN) (3.5ºC) and warm night (WN) (13.5ºC) induction regimes, respectively. Cool night temperatures delayed dormancy development in both 'Walker' and 'WP-69'. The difference in dormancy development between the cool night (CN) and warm night (13.5ºC) (WN) treatments for 'Walker' was greater than 'WP-69'. There were differences in dormancy development of 'Walker' and 'WP-69' in response to night temperature ($P<0.05$).

4.3.2 Water mobility measured by diffusion-weighted imaging.

The Apparent Diffusion Coefficient was different between the four Regions of Interest (ROI), vascular tissue region, vascular transition region, lower axillary bud and upper axillary bud regions. ADC was highest in the vascular stem tissue region (ranging from 8.77 to 13.98 $mm^2\ sec^{-1} \times 10^{-4}$), indicating greater water mobility and lowest in the upper axillary bud (ranging from 3.13 to 5.85 $mm^2\ sec^{-1} \times 10^{-4}$), even in actively growing plants (Figs. 4.3 – 4.6). The ADC significantly decreased during the transition to dormancy in the vascular stem tissue region (Figure 4.3), vascular transition region (Figure 4.4) and lower axillary bud (Figure 4.5) ($P<0.05$). However, ADC did not significantly decrease during the induction period in the upper axillary bud (Figure 4.6). The vascular transition region and lower axillary bud showed earlier decreases in the ADC compared to the vascular stem tissue region. Significant decreases in ADC were observed at 40 days of induction ($P<0.05$) in both the vascular transition region and the lower axillary bud region. However in the vascular stem tissue region, significant differences in the ADC between treatments were observed only at 60 days of induction ($P<0.05$) (Figure 4.3).

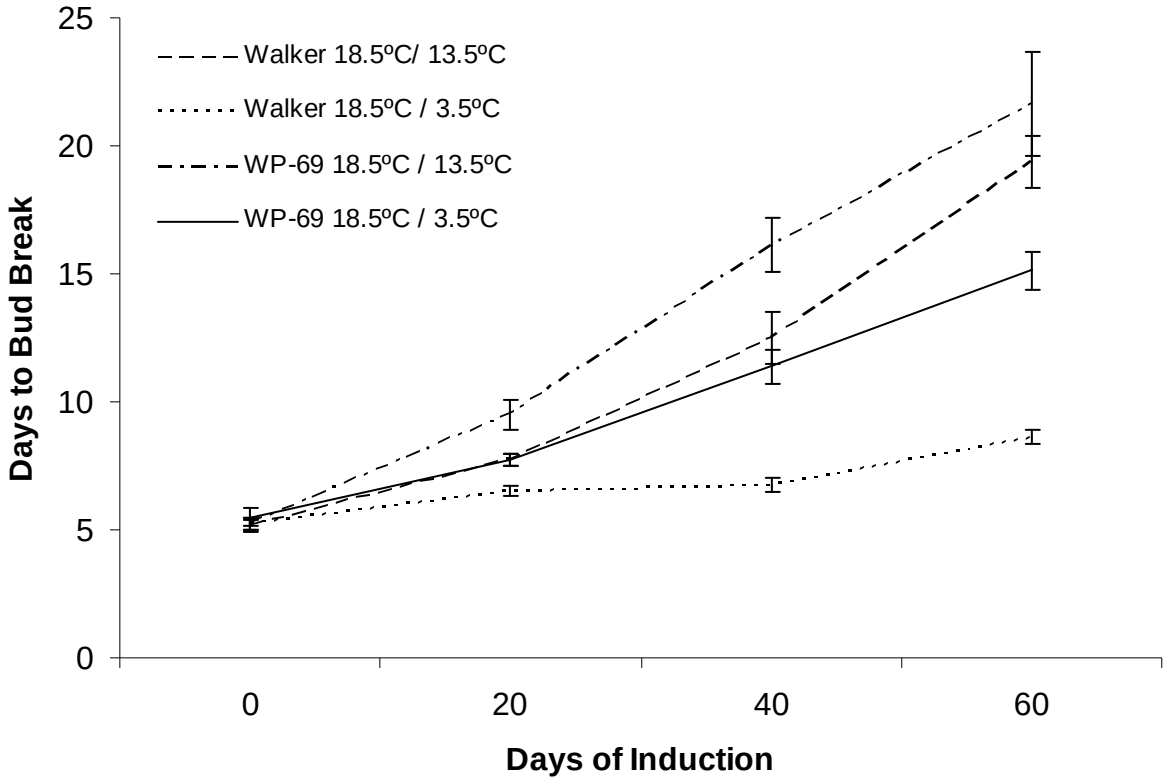

Figure 4.2 Dormancy development for two hybrid poplar (*Populus sp.*) clones, 'Walker' and 'WP-69', under controlled environment temperature conditions (18.5ºC/3.5ºC and 18.5ºC /13.5ºC day/ night temperatures) at 0, 20, 40 and 60 days. Error bars denote ±SE. N = 8.

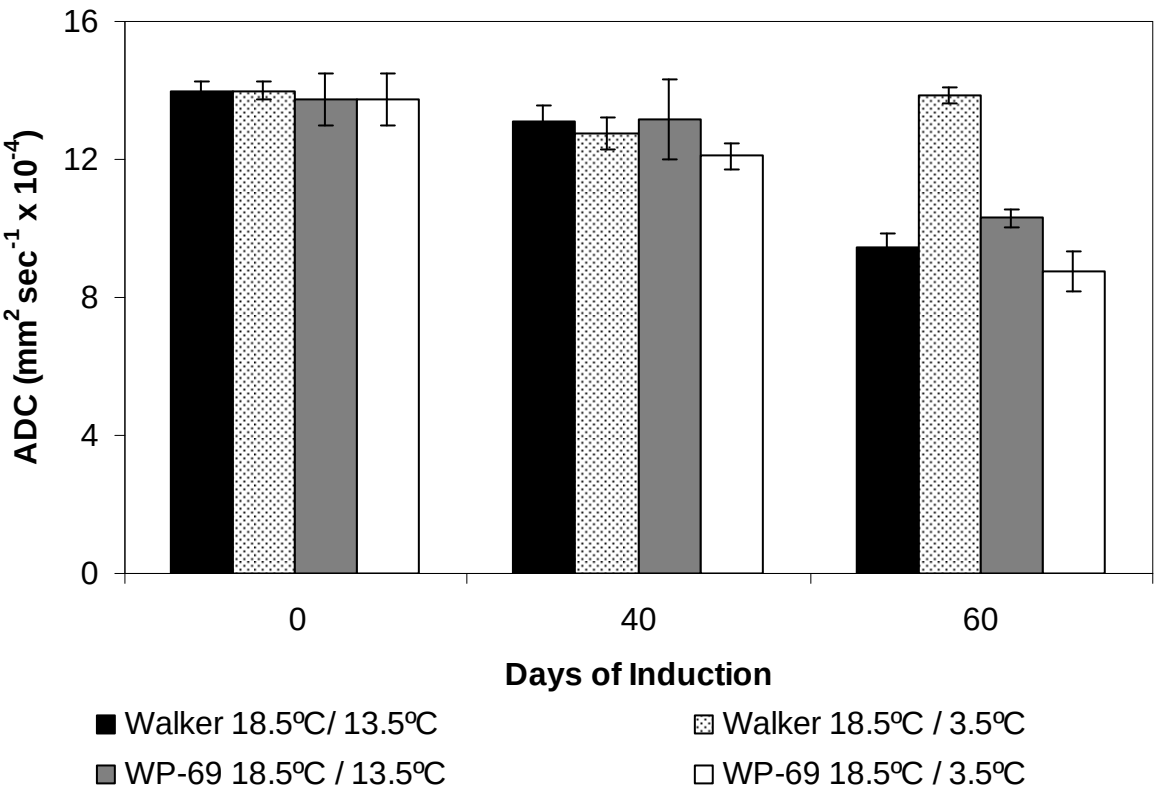

Figure 4.3 Apparent Diffusion Coefficient (ADC) for tissue within the vascular stem tissue region for two hybrid poplar (*Populus sp.*) clones ('Walker' and 'WP-69') under controlled environment temperature conditions (18.5ºC/3.5ºC and 18.5ºC /13.5ºC day/ night temperatures) after 0, 40 and 60 days of dormancy induction. Error bars denote ±SE. N = 3

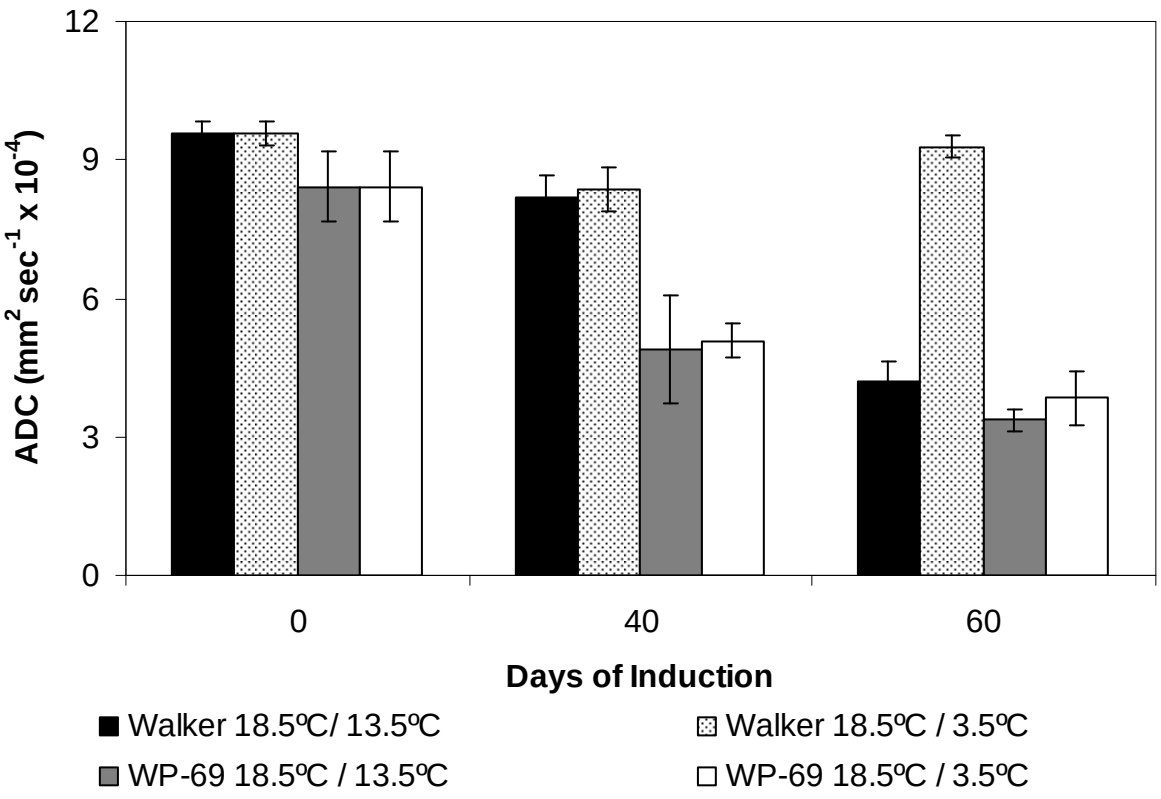

Figure 4.4 Apparent Diffusion Coefficient (ADC) for tissue within the vascular transition region for two hybrid poplar (*Populus sp.*) clones ('Walker' and 'WP-69') under controlled environment temperature conditions (18.5ºC/3.5ºC and 18.5ºC /13.5ºC day/ night temperatures) after 0, 40 and 60 days of dormancy induction. Error bars denote ±SE. N = 3

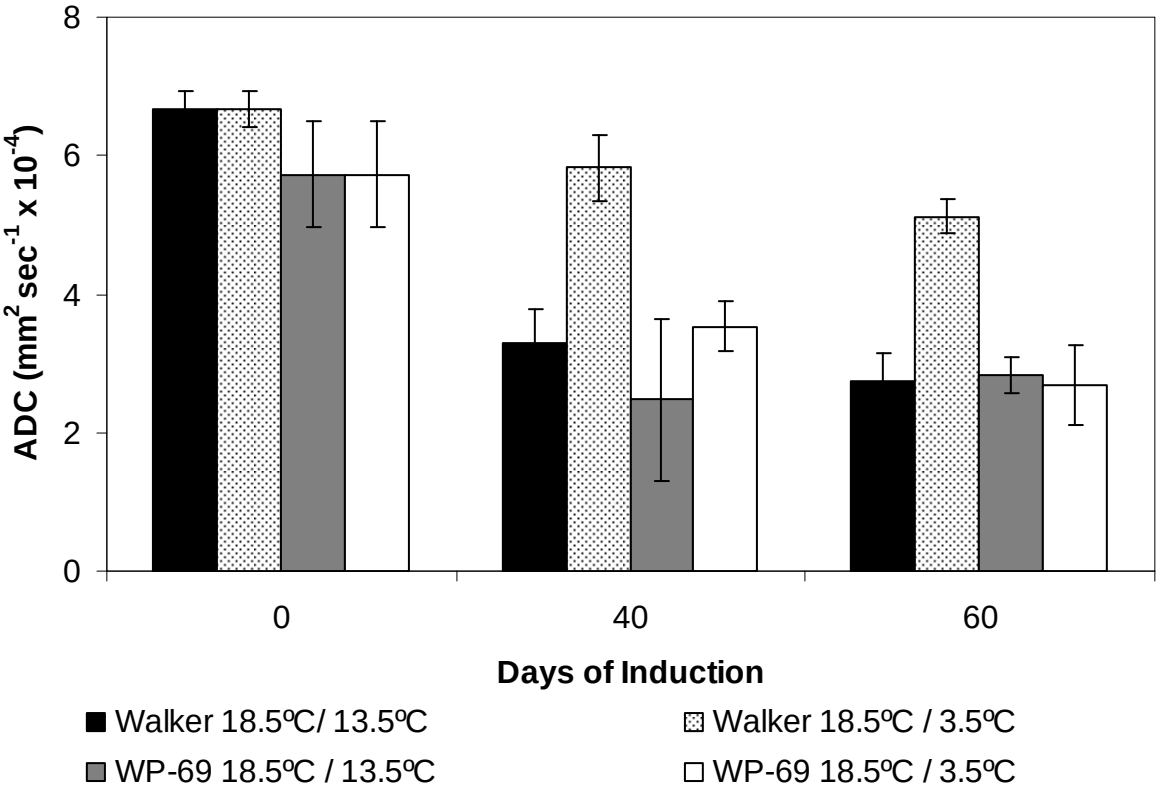

Figure 4.5 Apparent Diffusion Coefficient (ADC) for tissue within the lower axillary bud region for two hybrid poplar (*Populus sp.*) clones ('Walker' and 'WP-69') under controlled environment temperature conditions (18.5ºC/3.5ºC and 18.5ºC /13.5ºC day/ night temperatures) after 0, 40 and 60 days of dormancy induction. Error bars denote ±SE. N = 3

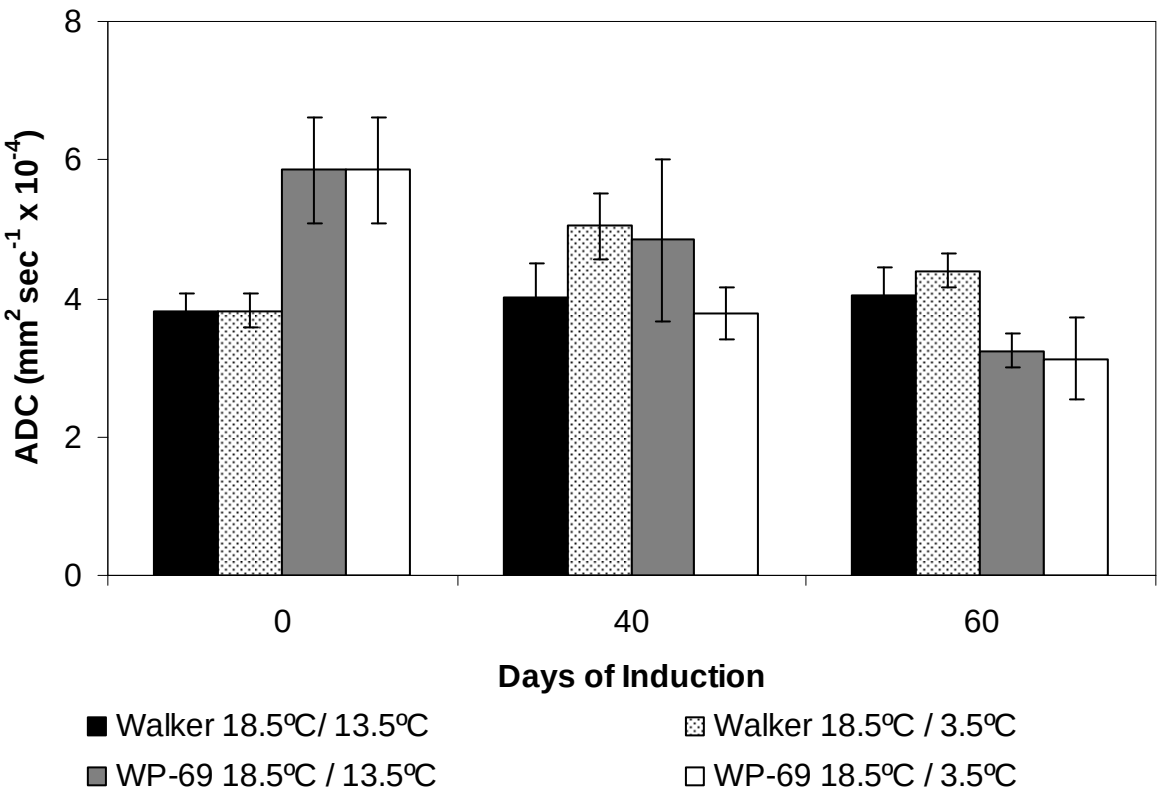

Figure 4.6 Apparent Diffusion Coefficient (ADC) for tissue within the upper axillary bud region for two hybrid poplar (*Populus sp.*) clones ('Walker' and 'WP-69') under controlled environment temperature conditions (18.5ºC/3.5ºC and 18.5ºC /13.5ºC day/ night temperatures) after 0, 40 and 60 days of dormancy induction. Error bars denote ±SE. N = 3

For the vascular stem tissue, vascular transition and lower axillary bud regions, decreases in the ADC for 'Walker' and 'WP-69' during the induction period were influenced by night temperature ($P<0.05$). This response is similar to differential dormancy development in response to night temperature for 'Walker' and 'WP-69' reported earlier. There were significant decreases in the ADC during the induction period for 'Walker' in the Warm Night (WN) induction treatment but no significant decreases in the ADC for 'Walker' in the Cold Night (CN) induction treatment ($P<0.05$). Taking a closer look at each ROI individually, decreases in ADC were influenced by induction treatment.

Corresponding to dormancy development in each treatment the ADC decreased in the vascular stem tissue region by 32% and 1% for 'Walker' and by 36% and 25% for 'WP-69', for the WN and CN induction treatments, respectively over the 60-day induction period (Figure 4.3). In 'WP-69' at 60 days of induction, the ADC in the vascular stem tissue region was 10.3×10^{-4} and 8.8×10^{-4} mm^2 sec^{-1} for the CN and WN induction treatments, respectively and was not significantly different ($P=0.05$) (Figure 4.3). In contrast, the ADC for 'Walker' at 60 days of induction from the CN treatment was significantly greater than the WN induction treatment ($P=0.05$) and was 13.9×10^{-4} and 9.5×10^{-4} mm^2 sec^{-1} for the CN and WN induction treatments, respectively.

Similar to the vascular stem tissue region, the ADC decreased in the vascular transition region into the axillary bud by 56% and 3% for 'Walker' and 60% and 54% for 'WP-69' from the WN and CN induction treatments, respectively. Relative decreases in ADC were greater in the vascular transition region than the vascular stem tissue region during the 60-day induction period. The ADC for 'Walker' was 4.2×10^{-4} and 9.3×10^{-4} mm^2 sec^{-1} for the CN and WN induction treatments, respectively at 60 days of induction. Conversely, the ADC for 'WP-69' at 60 days of induction was 3.4×10^{-4} and 3.8×10^{-4} mm^2 sec^{-1} for the CN and WN induction treatments, respectively. Temperature significantly affected changes in ADC in the vascular stem tissue region and the vascular transition region during the induction period in 'Walker' but not 'WP-69'.

Changes in the ADC were variable between the lower and upper axillary bud region during the induction period. Although the ADC in the lower axillary bud region was initially higher than the upper axillary bud at the beginning of induction, decreases were observed during the 60-day induction period. Decreases were not observed in the ADC in the upper axillary bud

during the induction period. The ADC in the lower axillary bud region was less than the upper axillary bud region (Figure 4.5 and 4.6) indicating higher water mobility in the upper axillary bud region. During the induction period in the lower axillary bud region, the ADC decreased by 59% and 23% for 'Walker' and by 51% and 53% for 'WP-69', in WN and CN induction treatments, respectively. At 60 days of induction, the ADC for 'Walker' was 2.8 x 10^{-4} and 5.1 x 10^{-4} mm^2 sec^{-1} and for "WP-69' was 2.8 x 10^{-4} and 2.7 x 10^{-4} mm^2 sec^{-1} in the WN and CN induction treatments, respectively. The differential decreases in ADC of 'Walker' and 'WP-69' in response to induction temperature was not observed in the upper axillary bud region. Decreases in the ADC in the upper axillary bud region were observed in 'WP-69' but not in 'Walker' at 60 days of induction (P=0.05). In this region, ADC decreased by 45% in 'WP-69' during the induction period.

4.3.3 T_1-Weighted Experiments

T_1 relaxation times are a reflection of water content and biophysical interactions of water within plant tissue and are the most widely measured parameter to date. Higher T_1 relaxation times suggest increased water content, decreased binding or a combination of both. In general, mean T_1 relaxation times were highest in the vascular stem tissue region (ranging from 452 to 1030 ms) (Figure 4.8), reduced in the vascular transition zone (ranging from 219 to 564 ms) (Fig. 4.9) and lowest in the axillary bud (ranging from 177 to 356 ms) (Figures 4.10 and 4.11). This indicates more free water in the vascular tissue region than buds. T_1 relaxation times in the lower axillary bud (Figure 4.10) were less than the upper axillary bud region (Figure 4.11). There was a significant decrease in T_1 times during the induction period ($P<0.05$), however, T_1 times did not decrease after 40 days.

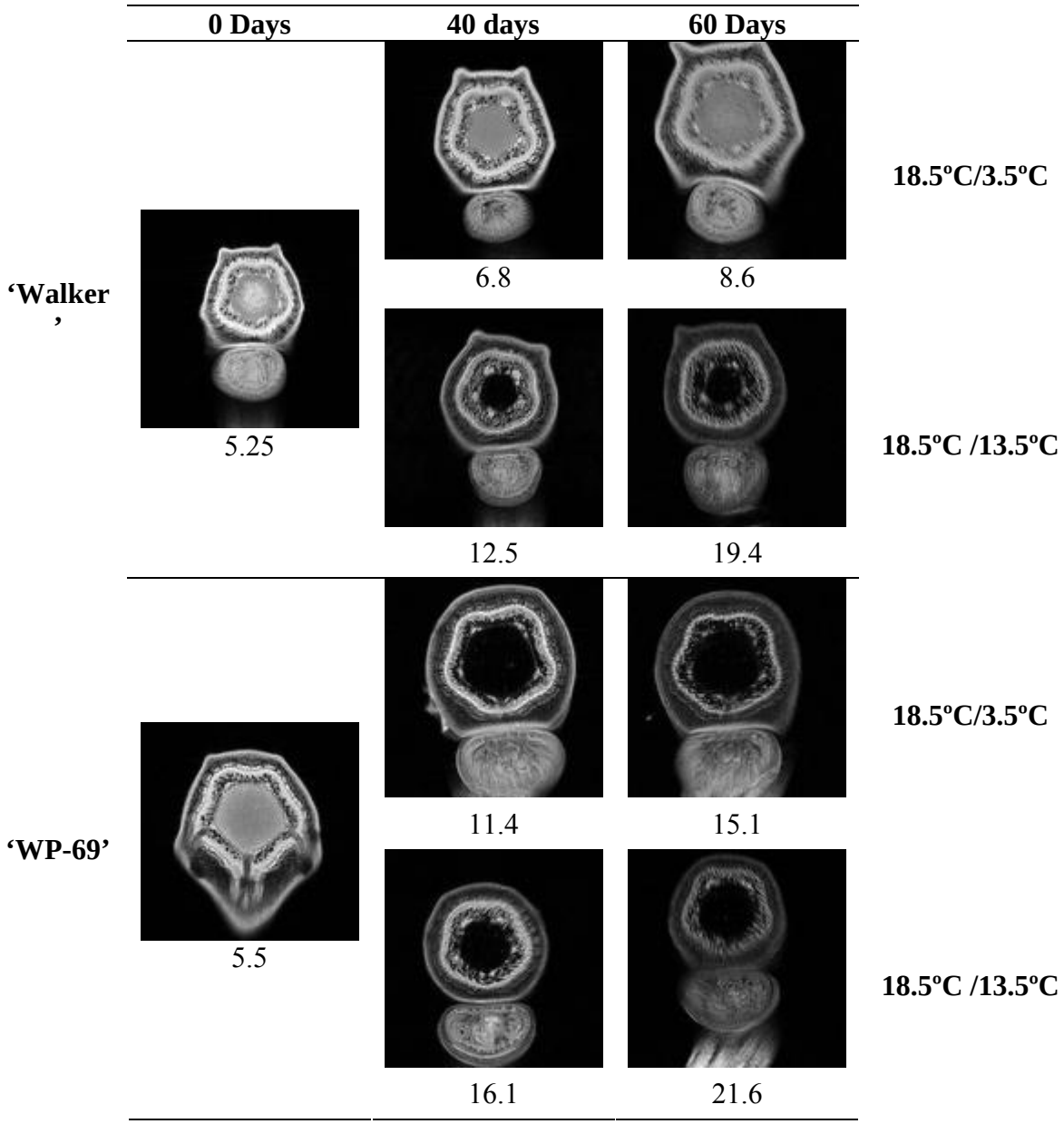

Figure 4.7 Raw data cross-sectional NMR images of 'Walker' and 'WP-69' hybrid poplar (*Populus sp.*) stem and axillary bud after 0, 40 and 60 days in controlled environment chambers at 18.3/3.5 day/night temperature and short photoperiod. Dormancy levels indicated below image (Days to Bud Break).

Interestingly, unlike the ADC diffusion response, there were no differences in T_1 times based on temperature treatments for either clone. This is also in contrast to differences in time to growth cessation, in which temperature was a significant factor for both 'Walker' and 'WP-69' (data not included). Looking at each ROI separately, T_1 times between 'Walker' and 'WP-69' were significantly different in the vascular stem tissue region and the vascular transition region but not within the axillary bud (Figures 4.10 and 4.11). T_1 times in 'Walker' appeared to decrease at a faster rate than 'WP-69'. This interaction was most pronounced in the vascular transition region and lower axillary bud (Figure 4.9 and 4.10). T_1 times significantly decreased over time in 'Walker' in the vascular stem tissue region, vascular transition and lower axillary bud regions. There were no significant decreases over time in 'WP-69' in the vascular transition zone and the lower axillary bud while there were significant decreases observed in the vascular stem tissue region (Figs 4.8, 4.9, 4.10 and 4.11). Although the vascular stem tissue region showed the highest T_1 times, it also expressed the greatest decrease in T_1 times over the induction period. There was a 36% decrease in mean T_1 relaxation time between 0 and 60 days of dormancy induction compared to 28%, 15% and 9% for the vascular transition zone, lower- and upper axillary bud region, respectively.

4.3.4 Correlation between dormancy development and ADC and T_1 relaxation times

The ADC was more highly correlated with endodormancy development compared to T_1 relaxation times (Table 4.1). The ADC for the vascular stem, vascular transition region and lower axillary bud regions were all negatively correlated with changes in bud dormancy levels during the induction period (Table 4.1). The ADC of the vascular transition region and the lower axillary bud showed the highest correlation with dormancy in which the Pearson correlation coefficients were -0.90 ($P<0.001$) and -0.92 ($P<0.001$), respectively. The ADC did not significantly correlate with dormancy development in the upper axillary bud. There was also a significant negative correlation between T_1 times in the vascular stem tissue region and dormancy development ($P<0.05$). However, changes in T_1 times in other ROIs did not significantly correlate with dormancy levels observed during the induction period.

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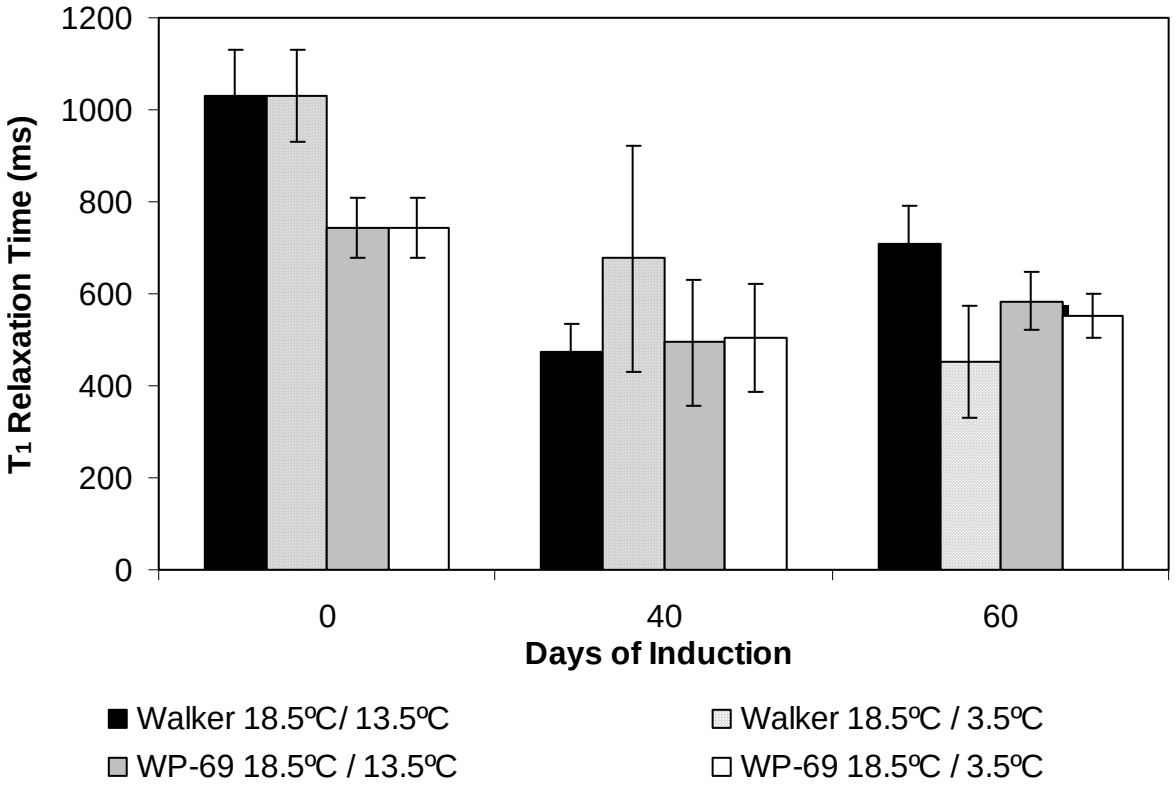

Figure 4.8 T_1 relaxation times in the vascular stem tissue region for two hybrid poplar (*Populus sp.*) clones ('Walker' and 'WP-69') under controlled environment temperature conditions (18.5ºC/3.5ºC and 18.5ºC /13.5ºC day/ night temperatures) after 0, 40 and 60 days of dormancy induction. Error bars denote ±SE. N = 3

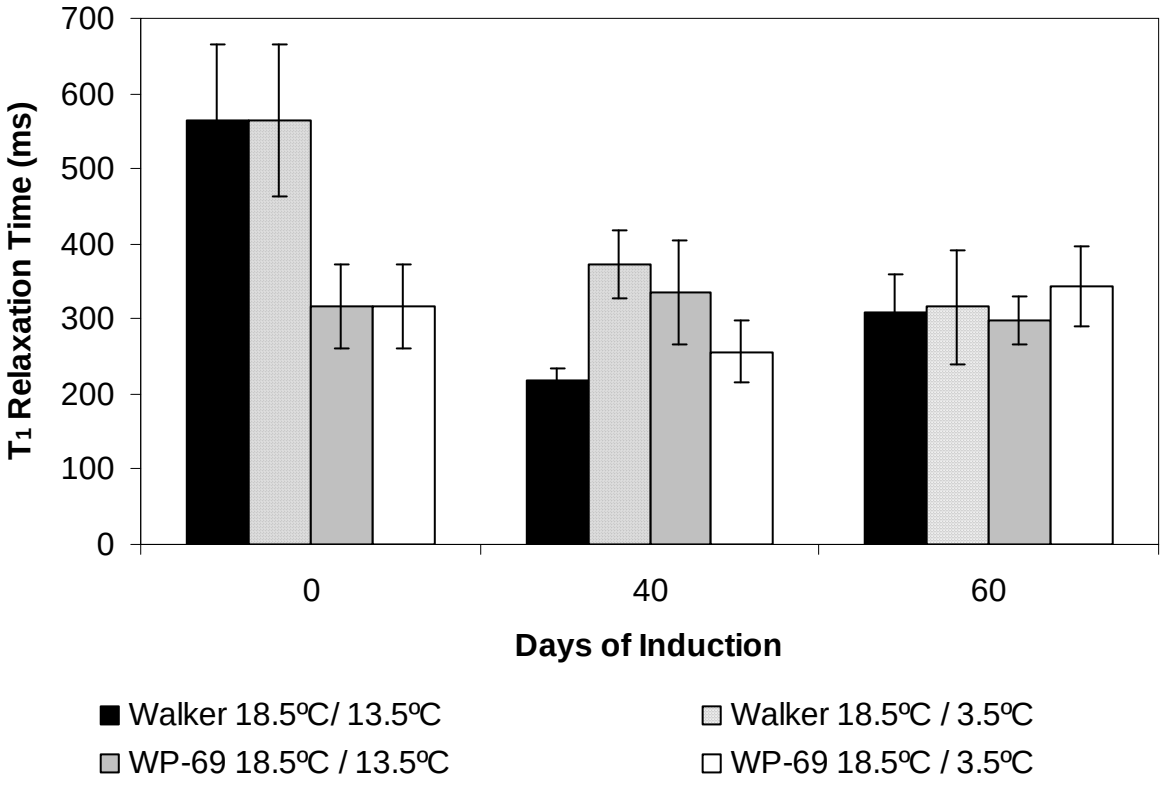

Figure 4.9 T_1 relaxation times in the vascular transition region for two hybrid poplar (*Populus sp.*) clones ('Walker' and 'WP-69') under controlled environment temperature conditions (18.5ºC/3.5ºC and 18.5ºC /13.5ºC day/ night temperatures) after 0, 40 and 60 days of dormancy induction. Error bars denote ±SE. N = 3

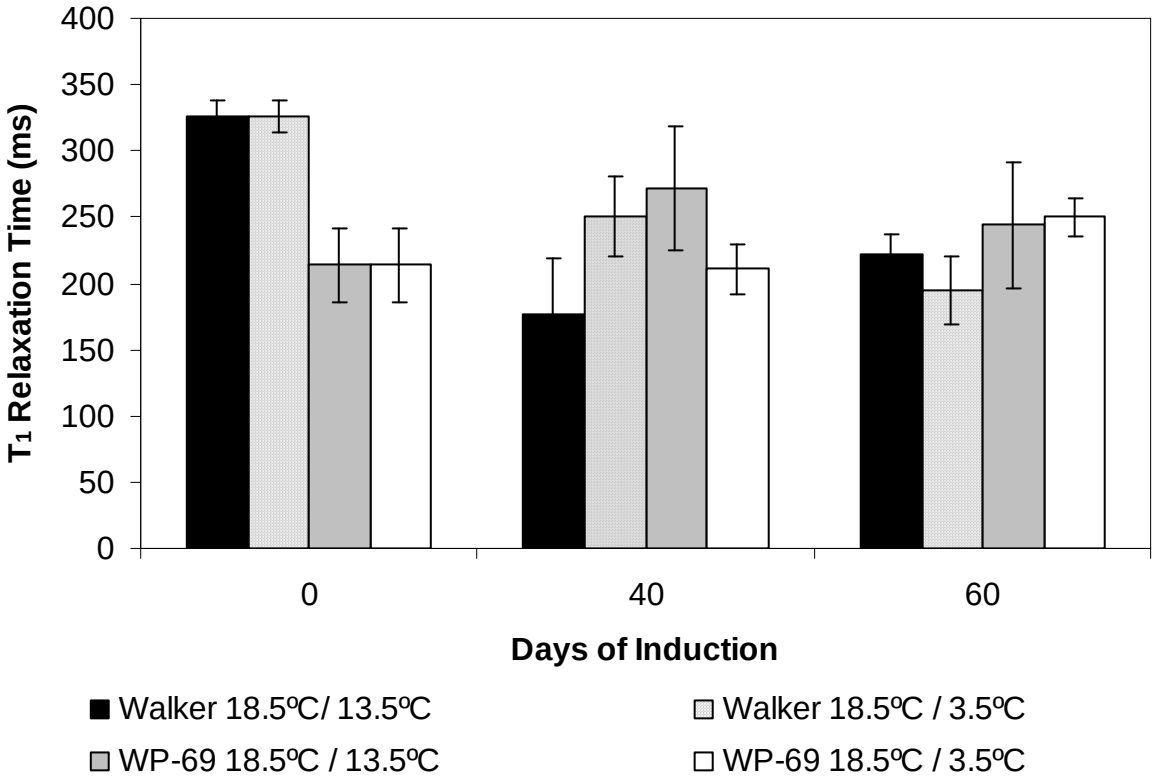

Figure 4.10 T_1 relaxation times in the lower axillary bud region for two hybrid poplar (*Populus sp.*) clones ('Walker' and 'WP-69') under controlled environment temperature conditions (18.5ºC/3.5ºC and 18.5ºC /13.5ºC day/ night temperatures) after 0, 40 and 60 days of dormancy induction. Error bars denote ±SE. N = 3.

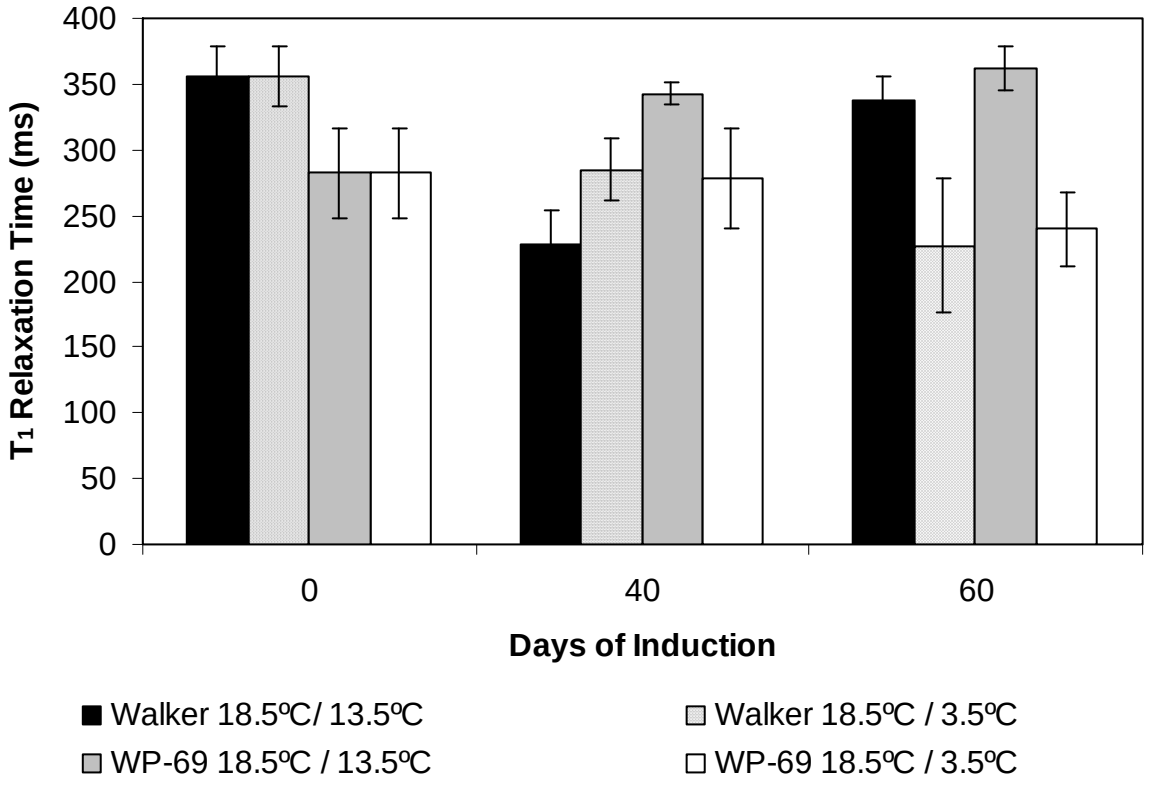

Figure 4.11 T_1 relaxation times in the upper axillary bud region for two hybrid poplar (*Populus sp.*) clones ('Walker' and 'WP-69') under controlled environment temperature conditions (18.5ºC/3.5ºC and 18.5ºC /13.5ºC day/ night temperatures) after 0, 40 and 60 days of dormancy induction. Error bars denote ±SE. N = 3.

Table 4.1 Correlation coefficients between dormancy and Apparent Diffusion Coefficient (ADC) and T_1 relaxation times within various Regions of Interest (ROI) (vascular stem, vascular transition into axillary bud, lower and upper axillary bud region). * ≤0.05, **P≤0.01, ***P≤0.001. N = 9

ROI	ADC	T1 Relaxation Times
Vascular Tissue Region	-0.79**	-0.65*
Vascular Transition Region	-0.90***	-0.36
Lower Axillary Bud	-0.92***	-0.25
Upper Axillary Bud	-0.53	-0.41

4.4 Discussion

Once plants were placed under the dormancy induction treatments, the region of the stem section that showed the earliest decrease in ADC was the lower axillary bud followed by the transitional area between the vascular transition into the axillary meristem. Ashworth (1982) described a blockage at the base of axillary buds in peach that restricts water movement between the bud and vascular stem tissue. Furthermore, Fennel and Line (2001) identified an early decrease in free water in the cortex/gap tissue between the vascular stem tissue and the axillary bud that occurs during dormancy induction in *Vitis riparia*. Water mobility may be reduced in this region limiting hormonal or physiological communication between the axillary bud and the stem. This corresponds with increases in dormancy levels in axillary buds that may result in lower susceptibility to bud break during favourable conditions, subsequently contributing to endodormancy development.

Since greater decreases in the ADC in the lower axillary bud region were observed compared to the upper axillary bud (above the apical meristem), it can be suggested that changes within meristematic tissue, between the two sampled regions, may have resulted in the differential decrease in the ADC. The control of dormancy in woody plants may be regulated within the meristem (Fuchigami *et al.*, 1982; Lang *et al.*, 1987; Rinne *et al.*, 2001). Furthermore, Rinne and van der Schoot (2003) identified the presence of 1,3-β-D-glucan that narrows or blocks plasmodesmata, controlling cell-to-cell movement of metabolites within the apical meristem. Chilling has been shown to increase activity of 1,3-β-glucanase in the plasmodesmata.

1-3-β-glucanase is the enzyme responsible for degradation of 1-3-β-glucan that is present in plasmodesmata of endodormant woody plants (Rinne et al., 2001; Rinne and Van der Schoot, 2003). Cell to cell communication and signaling is integral in regulation and stimulation of meristematic growth. Intercellular communication by blocking of the plasmodesmata by 1-3-β-glucan is thought to be restricted during endodormancy (Rinne *et al.*, 2001). Water mobility would be expected to decrease below the meristem increasing plasmodesmata blocking and reducing cell-to-cell communication possibly resulting in a decrease in metabolic activity. Until chilling induces increases in 1,3-β-glucanase activity resulting in the breakdown of 1-3-β-glucan blockages in the plasmodesmata, water mobility below the meristem will be reduced.

Decreases in ADC were not observed in the upper axillary bud region. While this may seem contradictory to progression towards dormancy, Goffinet and Larson (1981) observed continual development of leaf primordia during the dormant period in *Populus*. If growth and metabolic activity is still occurring in the upper axillary bud region, it can be expected that there would not be a decrease in the ADC, reflecting reduced water mobility. Ideally, imaging of the apical meristem would have yielded insight into changes in water status and mobility at the meristem. However, that level of accuracy was difficult to achieve because of different bud sizes present between clones. Although an orientation matrix was used to ensure uniform imaging of the bud, that level of accuracy was not possible. In future, a vertical slice through the axillary bud will be sampled.

Decreases in T_1 relaxation times did not correspond with dormancy development during the induction period. These results are different from past studies where dormancy has been attributed to changes in T_1 and T_2 relaxation times (Faust *et al.*, 1991; Liu *et al.*, 1993). T_1 and T_2 relaxation times are parameters that reflect changes in water content, binding and viscosity in a sample. This study was distinct from previous studies because samples for NMR were taken from non-acclimating, dormancy inducing conditions instead of cold hardiness inducing, chilling conditions that promote dormancy release. Measuring changes during dormancy induction allowed for separation of cold acclimation and dormancy development. Faust *et al.* (1991) observed increases in T_2 times in apple buds during chilling. However, the separation of cold hardiness and dormancy is difficult because plants will not deacclimate if endodormant but will deacclimate if ecodormant (Tanino *et al.*, 1989). While deacclimation may have occurred, it underlines the importance of changes in water movement and permeability during dormancy

release. Erez *et al.* (1998) observed a decrease in T_2 times during dormancy induction in peach shoots placed under dormancy inducing conditions. However, these decreases were not as pronounced compared to the increase in T_2 times in response to chilling observed in Faust *et al.* (1991).

Accumulation of hydrophilic molecules such as sugars (Palonen, 1999; Cox and Stushnoff, 2003) and proteins (Rowland and Arora, 1997) affect the viscosity of the cytoplasm, through binding water, contributing to a decrease in T_1 times. However, a decrease in tissue water content is also observed during dormancy induction (Welling *et al.*, 2002; Jeknic and Chen, 1999; Li *et al.*, 2003; Wake and Fennell, 2000). Furthermore, using H^1-NMR spectroscopy, Gardea *et al.* (1993) reported changes in water status in grape buds under exposure to short photoperiods. Sampling of specific anatomical regions of interest may not be representative of all changes in water content. There were other tissues, such as the pith, that revealed a decrease in water content (Figure 4.7). There was visible darkening of the pith in the samples during the induction period, indicating reduced water content. Thus, while T_1 times in specific regions of interest did not indicate a decrease in water content, decreases in water content in the pith, a region not sampled, may account for the discrepancies between this study and previous studies using non tissue-specific water measurements (Gusta *et al*., 1979). The pith region may be of significance to cold hardiness in first year growth in woody plants but due to subsequent lignification, may not be a factor in mature wood.

Previous studies suggest that there are two populations of water in plant tissue, 'bound' and 'free' water (Faust *et al.*, 1991; de Fay, 2000; Gusta *et al.*, 1979). 'Bound' water is water bound to hydrophilic molecules that is not readily available for metabolic activity. Many of these studies that identified two populations of water in plant tissue were spectroscopic measurements, which does not identify localized changes within tissues. Within specific tissue regions, there was only one population of water and the samples from each ROI were normally distributed.

In this study, T_1 relaxation times did not decrease in conjunction with an increase in dormancy levels in axillary buds. Furthermore, there were no observable increases in sugars such as raffinose and stachyose (*Appendix C*). While protein changes were not measured, few LEA-type dehydrin proteins increase in response to short-day photoperiod alone (Rowland and Arora, 1992; Karlson *et al.*, 2003). Cool, non-freezing temperatures induce both sugars, such as raffinose and stachyose, and proteins, such as dehydrins. This suggests that changes in T_1 times

relating to increases in hydrophilic molecules and/or a decrease in water content may contribute more to cold hardiness than dormancy in woody plants.

By contrast, changes in ADC corresponded to changes in dormancy more effectively than T_1 measurements. While T_1 relaxation times indicate biophysical parameters of water within a pixel, it cannot measure changes in mobility of that water within plant tissue. In addition to indicating the degree of free water in a tissue, the ADC also indicates its mobility. De Fay *et al.* (2000) measured both the ADC and T_2 relaxation times in tulip bulbs during chilling and found the ADC did not decrease during chilling. However, the resolution of the image was not as high as images in this study and measurements of the ADC may only be useful using high-resolution images. Furthermore, measurements were made during dormancy induction where as in De Fay *et al.* (2000), diffusion measurements were made when buds were ecodormant. There may be changes in diffusion that occur during the transfer from endodormancy to ecodormancy in buds.

In conclusion, water is inherently linked to dormancy development. In addition to a decrease in water content reported in previous studies, water mobility, indicated by ADC, decreases during dormancy induction in hybrid poplar. Water mobility is more closely correlated with dormancy development than T_1 relaxation times, which are common measurements in NMR experiments exploring dormancy development in woody plants. It appears that water content decreases under exposure to short photoperiods but T_1 relaxation times do not correlate well with dormancy development in hybrid poplar, based on the sampled Regions of Interest. Tissue-specific sampling of biophysical water characteristics, such as T_1 relaxation times, likely affected the dislinkage between T_1 relaxation times and dormancy. Water mobility, reflected by ADC, corresponds with increases in dormancy levels in axillary buds in hybrid poplar and reflected differential temperature treatments. Decreases in the ADC in specific anatomical regions correlate more highly with dormancy development than T_1 relaxation times. The vascular transition zone and the lower axillary bud region showed the highest decrease in ADC during dormancy development. Further work should include identifying the physiological mechanism responsible for the decrease in water mobility in this region and possibly, vertical slice sampling for microimaging NMR. These changes lend strength to theories attributing localized biochemical and physiological changes related to water within vascular and meristematic tissues with dormancy development in woody plants.

5.0 SUMMARY AND CONCLUSIONS

Temperature is an often-overlooked factor contributing to dormancy induction in woody plants. Temperature appears to interact with photoperiod to regulate endodormancy induction and related processes. Although both day and night temperatures affected dormancy development, night temperature impacted dormancy more. Cool night temperature significantly delayed growth cessation, which subsequently delayed dormancy development and cold acclimation initiation. Overall growth was reduced in poplar exposed to warmer night temperatures, which was a result of earlier growth cessation. Growth cessation is a prerequisite for both dormancy development and cold acclimation initiation.

Changes in biophysical properties of water, which can affect metabolic activity, are connected with dormancy development in hybrid poplar. One of the underlying physiological cheanges related to dormancy, which is affected by temperature, is water mobility and free water. Cool night temperature treatments that delayed growth cessation and inhibited dormancy development also resulted in a delay in reduction in water mobility and free water when measured using NMR micro-imaging. Changes in dormancy development are closely correlated with changes in water mobility, reflected by changes in the apparent diffusion coefficient. Tissue water mobility decreased during dormancy induction, which could be a result of a decrease in water content or anatomical changes that occur during dormancy induction. Strong decreases in water mobility in the vascular transition region and lower axillary meristem region, which were closely correlated with dormancy, indicate that dormancy may be controlled from these two regions. In contrast, T_1 relaxation times, a reflection of water content and biophysical interactions of molecules with water, did not correlate with dormancy development. T_1 times may be closer related to accumulation of hydrophilic solutes such as soluble sugars and proteins that accumulate under cool, non-freezing conditions.

Using hybrid poplar as a system to study dormancy is useful because of its wide geographic range and adaptations to different environmental conditions ranging from subtropical to arctic environments. Further reinforcing the value of poplar as a system, easily developed inter-specific hybrids allow for selection of differential responses that would not have occurred naturally. Furthermore, using this established system of differential clonal-temperature responses

allowed us to explore physiological questions related to dormancy and postulate as to their role in dormancy development in woody plants. Overall, genetic variability exists in how dormancy and cold acclimation induction are affected by temperature. Identifying responses to temperature change during dormancy induction contributed towards answering the question "How do plants respond to temperature change in the autumn?" This has implications in many areas including but not limited to plant physiology, ecology, forestry and even agricultural production systems. Woody plants that are less affected by temperature may be more likely to survive under changing temperatures in the future. Warmer temperatures during autumn will not likely result in increased susceptibility to freezing damage during the fall in hybrid poplar. Clones such as 'WP-69' may be better able to tolerate temperature change than other clones.

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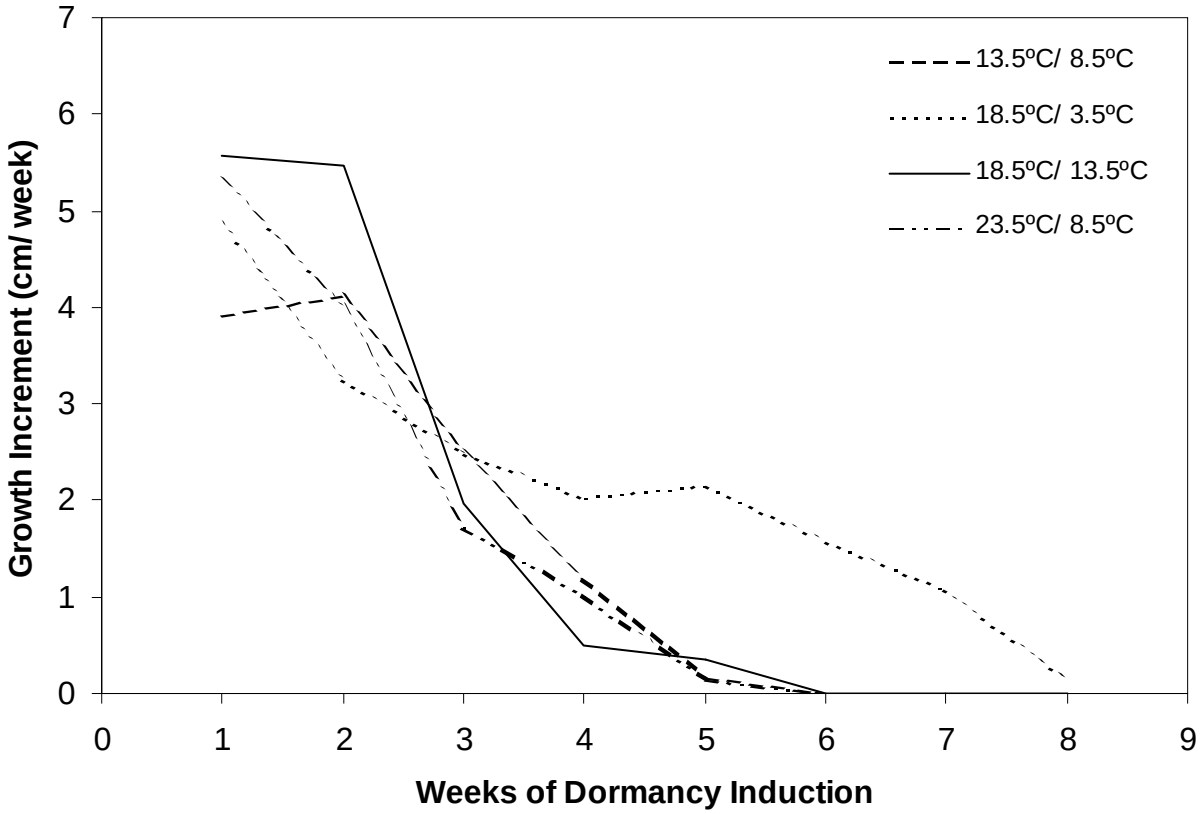
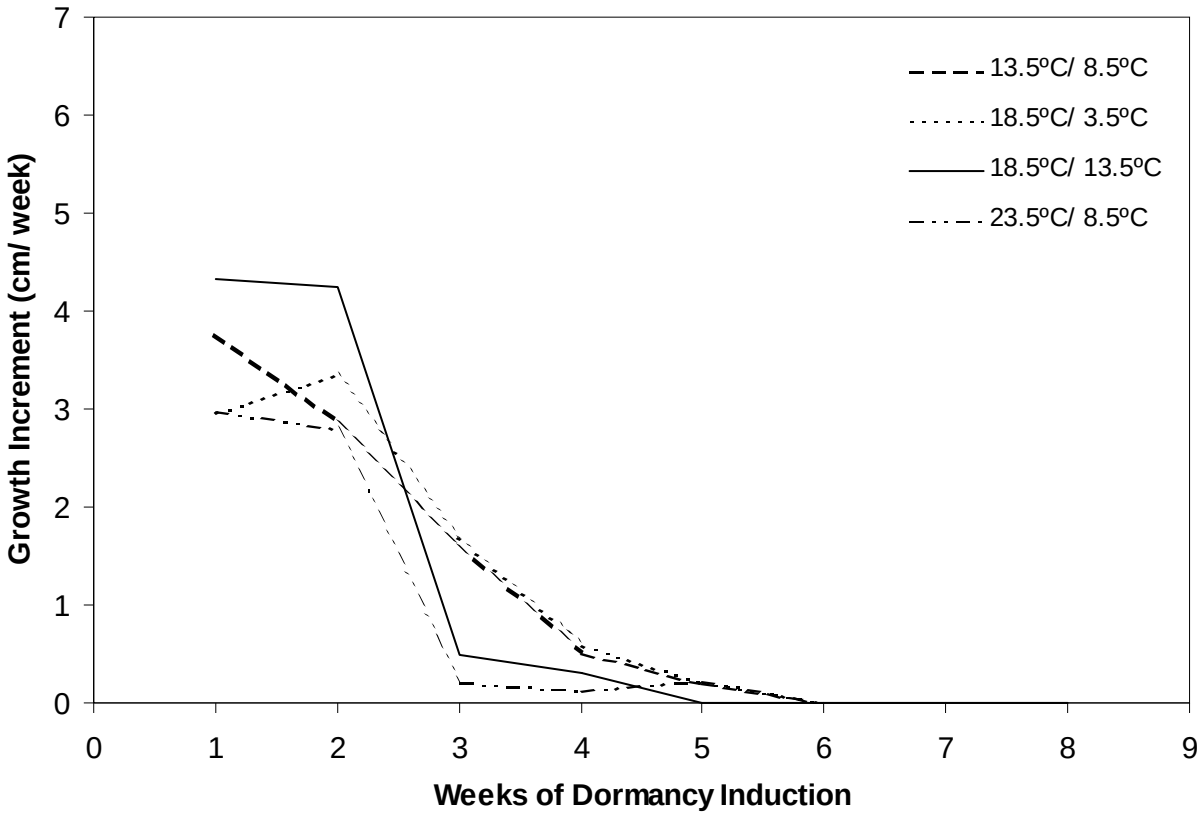
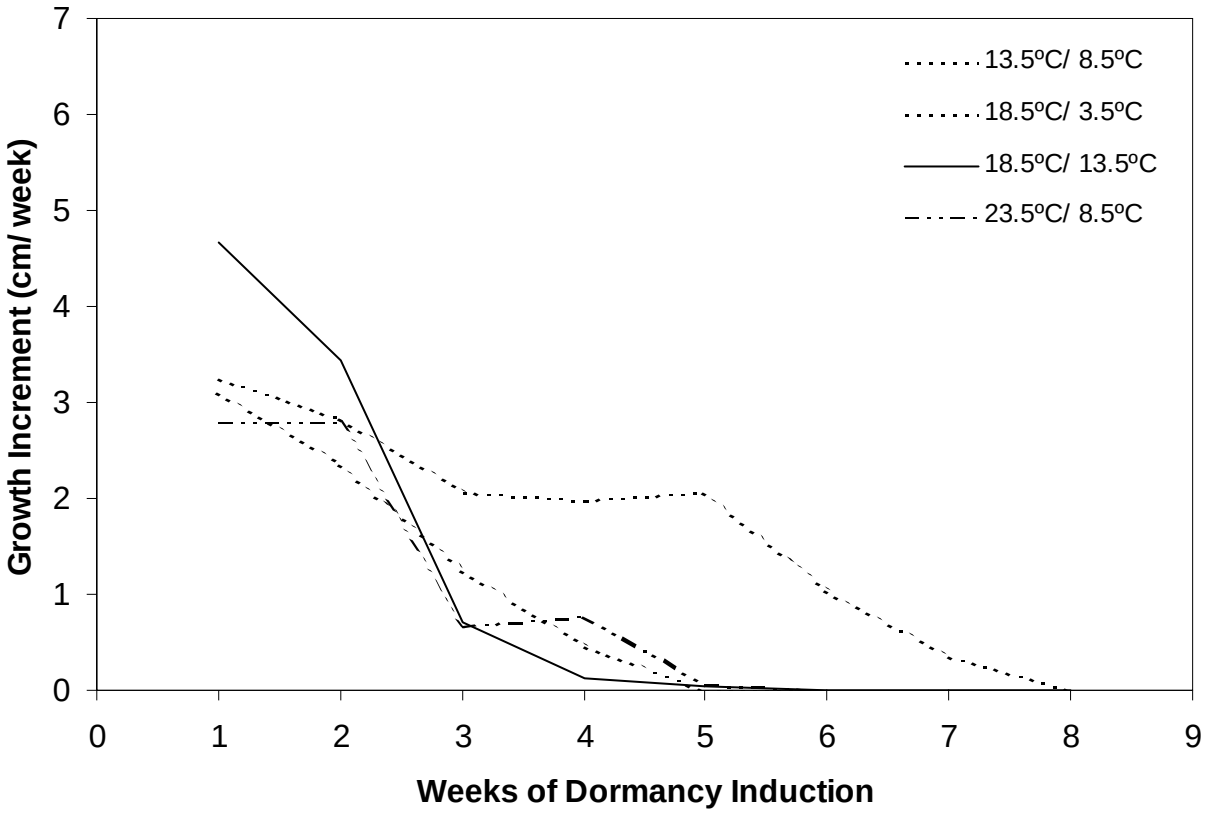
APPENDIX A. GROWTH INCREMENT DATA



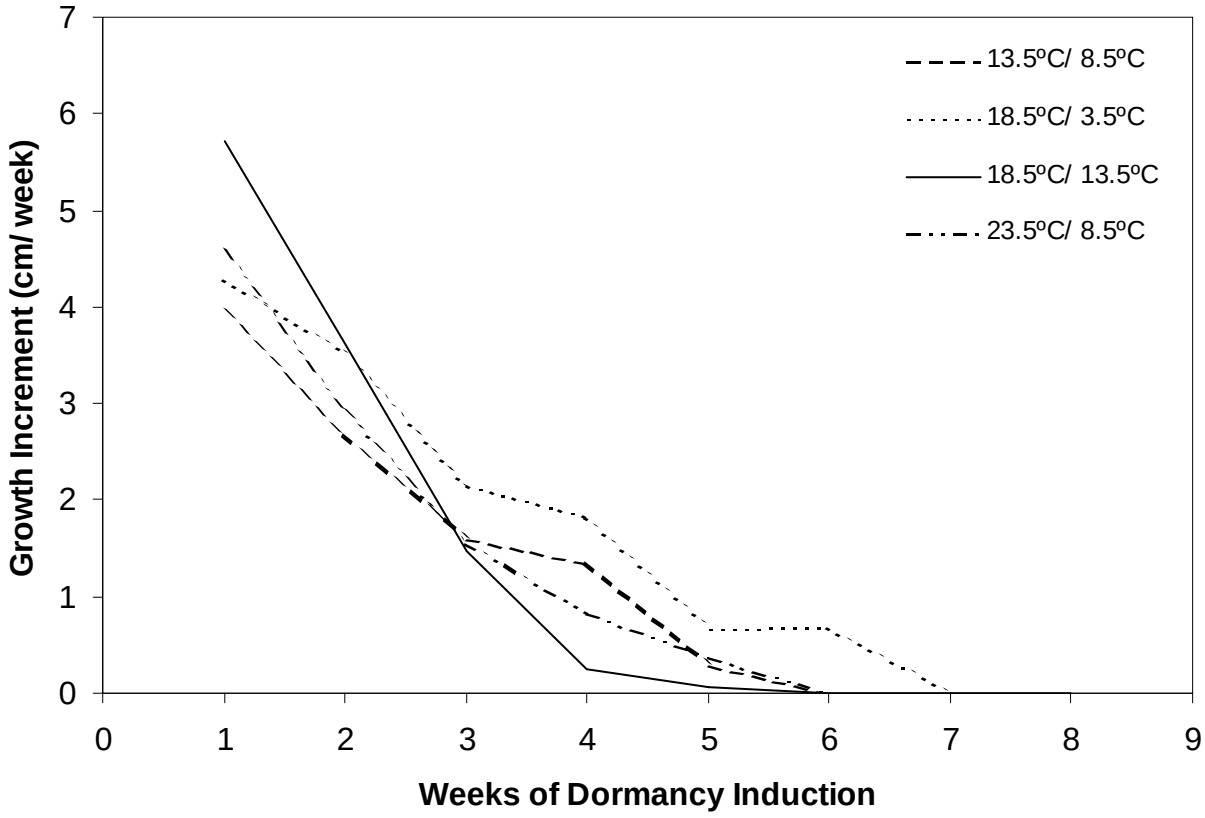
Growth increment of 'Walker' hybrid poplar (*Populus sp.*) clones under 4 controlled environment temperature regimes, 13.5°C /8.5°C, 18.5°C /3.5°C, 18.5°C/13.5°C and 23.5°C /8.5°C, over the period of 9 weeks.



Growth increment of 'WP-69' hybrid poplar (*Populus sp.*) clones under 4 controlled environment temperature regimes, 13.5°C /8.5°C, 18.5°C /3.5°C, 18.5°C/13.5°C and 23.5°C /8.5°C, over the period of 9 weeks.



Growth increment of 'Katepwa' hybrid poplar (*Populus sp.*) clones under 4 controlled environment temperature regimes, 13.5°C /8.5°C, 18.5°C /3.5°C, 18.5°C/13.5°C and 23.5°C /8.5°C, over the period of 9 weeks.



Growth increment of 'Prairie Sky' hybrid poplar (*Populus sp.*) clones under 4 controlled environment temperature regimes, 13.5°C /8.5°C, 18.5°C /3.5°C, 18.5°C/13.5°C and 23.5°C/8.5°C, over the period of 9 weeks.

APPENDIX B. PHOTOGRAPHS OF BUD SET DURING DORMANCY INDUCTION

‘WALKER’

18.5ºC/ 3.5ºC

18.5ºC/ 13.5ºC

‘WP-69’

18.5ºC/ 3.5ºC

18.5ºC/ 13.5ºC

Terminal bud development in ‘Walker’ (left) and ‘WP-69’ (right) poplar after 40 days of dormancy induction at 18.5ºC/ 3.5ºC and 18.5ºC/ 13.5ºC

‘WALKER’

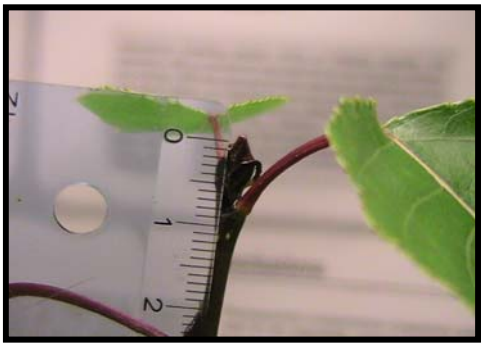
18.5ºC/ 3.5ºC

18.5ºC/ 13.5ºC

‘WP-69’

18.5ºC/ 3.5ºC

18.5ºC/ 13.5ºC



Terminal bud development in ‘Walker’ (left) and ‘WP-69’ (right) poplar after 60 days of dormancy induction at 18.5ºC/ 3.5ºC and 18.5ºC/ 13.5ºC

APPENDIX C. CORRELATION BETWEEN SOLUBLE SUGAR CHANGES DURING DORMANCY INDUCTION AND DORMANCY DEVELOPMENT AND COLD HARDINESS

C.1 Introduction

Sugars, derived from photosynthetic activity, are an essential part of growth, energy storage and transport within the plant. Additionally, sucrose has recently been reported to contribute towards plant growth and development, essentially acting as a plant hormone (Sheen *et al.*, 1999). Source-sink interactions at a cellular and whole plant level regulate, through feedback loops, developmental processes within the plant. These may include phenological changes such as bud-set and flowering. Additionally, sugars, such as raffinose, stachyose and sucrose have been attributed to increases and decreases in cold hardiness in woody plants. They my function by acting as stabilizers because of hydrophilic properties that bind water and decrease susceptibility to cellular dehydration resulting from abiotic stresses such as drought and cold temperatures.

Sugar changes during dormancy induction and cold acclimation have been previously reported (Jeknic and Chen, 1999; Palonen, 1999; Cox and Stushnoff, 2003). Sugars increase the viscosity of water within the cell to increase the capacity for a plant to super-cool and avoid freezing. Specific sugars shown to increase during the dormancy/ cold acclimation period are sucrose, raffinose and stachyose. Cox and Stushnoff (2003) reported increases in stachyose and raffinose in stem tissue from *Populus tremuloides* during acclimation. Palonen (1999) also reported increases in sugars in Raspberry canes during dormancy induction and cold acclimation however, raffinose and stachyose were either undetectable or found in minimal concentrations. This was attributed to a lack of acclimation period. Separation of cold hardiness and dormancy development in natural environments is difficult because the two processes occur simultaneously during autumn. Understanding the relationship between dormancy, cold hardiness and sugars will contribute to understanding the complex mechanisms associated with dormancy.

This study addressed the question of 'whether sugar profiles change over time during dormancy induction and how are the observed changes related to dormancy development?' Using HPLC, sugar content was determined in hybrid polar stems during dormancy induction to determine changes in sucrose, glucose, fructose, raffinose and stachyose. Furthermore, it was

determined whether changes in sugar content correlate with dormancy development in hybrid poplar with differential dormancy induction trends.

C.2 Materials and Methods

C.2.1 Experimental Design and Sample Collection

Temperature treatments and clones were the same as chapter 1. Samples were taken every 20 days from day 0, 20, 40 and 60 days of dormancy induction. Samples were 0.5 to 1 cm stem segments sampled from directly below the terminal bud. Immediately after samples were taken from the stem segment after removal from the chamber, they were immersed in liquid nitrogen. Samples were stored at –80ºC until sample preparation at the Faculty of Forestry at the University of British Columbia.

C.2.2 Sample Preparation

Samples were transported in a cryo-flask at temperatures of approximately –196ºC from the University of Saskatchewan to the University of British Columbia in Vancouver, British Columbia, Canada. Individual samples were fresh-ground in liquid nitrogen and placed in 1.5mL cryo-tubes for freeze-drying. Samples were freeze dried at –50ºC for 24 hours and placed in a dehydrator to avoid rehydration of the ground plant tissue.

Samples were weighed and placed into 15mL falcon tubes. Sample masses ranged from approximately 5 mg to 20 mg dry weight. In addition to freeze-dried samples, 100µL of a 10-mg/ml standard fucose solution was added to the falcon tubes and weighed again. 4mL of a 12:5:3 methanol:chloroform:water solution was added to the falcon tubes and samples were stored at –20ºC overnight to allow for soluble sugars to enter solution.

Soluble sugar extraction was done the following day. Falcon tubes were placed into a centrifuge and spun at 4000 rpm for 10 minutes at 4ºC. Supernatant was pipetted into 50mL falcon tubes and stored at 4ºC. 4mL of methanol:chloroform:water solution was added again to the sample and the centrifuge/ extraction was repeated. This whole process was repeated once more producing about 12mL of supernatant from each sample. 5mL of water was added to each

falcon tube and flipped upside down to allow for adequate mixing of the supernatant with the water. Samples were placed at 4ºC and allowed for phase separation for approximately two hours. Samples were then centrifuges at 4000 rpm for 5 minutes at 4ºC to remove debris from the upper phase. The upper phase (approximately 10mL) was pipetted into scintillation vials and stored at 4ºC until HPLC analysis.

C.2.3 HPLC Analysis

1.5mL of the solution from the scintillation vials was placed in 2mL microtubes and placed in a speed-vac and allowed to dry down on medium heat (approximately 40ºC). This step was repeated two more times until there was 4.5mL of the solution dried down in the microtubes. Samples were resuspended in 0.5mL of deionized water and filtered into an HPLC vial through a 0.45 nm HPLC filter using a 1mL syringe.

Samples were analyzed using a PA-1 column (DIONEX) in a DIONEX600 HPLC (Dionex Corporation, Sunnyvale, CA) using the method 'rfo4' that resolved raffinose family oligosaccarides in addition to sucrose, fructose and glucose. Sample time was 70 minutes. Peaks for sucrose, glucose, fructose, raffinose and stachyose were identified using standard solutions. Peaks areas were selected and normalized for fucose and concentrations determined using regression equations from standard concentration solutions of 25, 50 and 100µg/mL run in HPLC.

C.2.4 Statistical Analysis

A two-way ANOVA was performed to determine sugar concentration changes between temperature and clones. For significant interactions, Tukey's LSD ($\alpha = 0.05$) was used for mean separation. Sugar concentrations (individual and total simple sugars content) were correlated to both dormancy development and cold hardiness during dormancy induction.

C.3 Results and Discussion

Soluble sugars did not increase during non-acclimating, dormancy-inducing conditions. This is in contrast to previous studies examining sugar accumulation during dormancy induction.

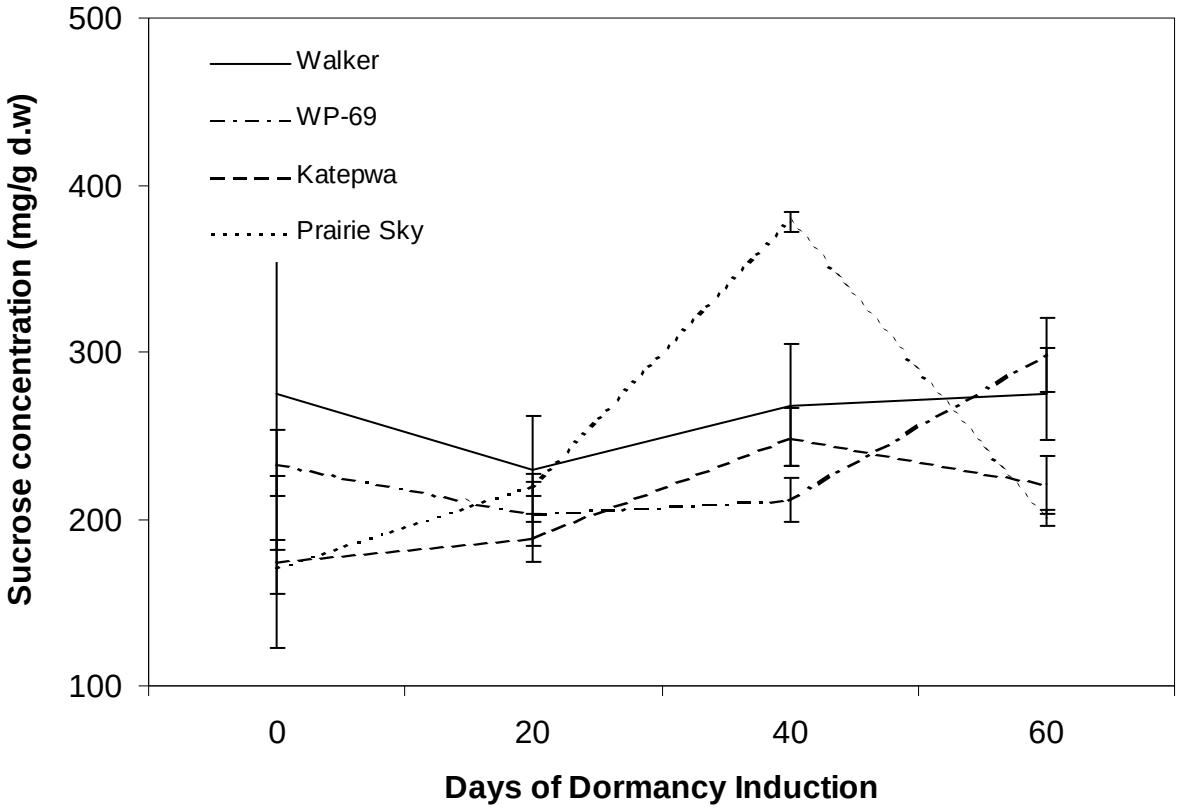
Cox and Stushnoff (2003) observed high accumulation of sugars, specifically raffinose and stachyose in aspen during the fall. However, dormancy induction was not separate from cold acclimation since plants were grown under field conditions. During dormancy induction, there were no measurable increases in raffinose and stachyose in stem tissue. For the majority of the samples, raffinose and stachyose were not detectable in any significant amounts (data not shown).

Temperature treatments did not promote cold acclimation, even under the cool night treatment (18.5ºC /3.5ºC). This was a result of late growth cessation in cool night treatments. Increases in raffinose and stachyose occur once terminal bud-set has occurred (Palonen, 1999). However, even in clones where bud set occurred early, soluble sugar accumulation was not significant. Accumulation in soluble sugars can be attributed to cold acclimation rather than dormancy induction, specifically exposure to cool, non-freezing conditions.

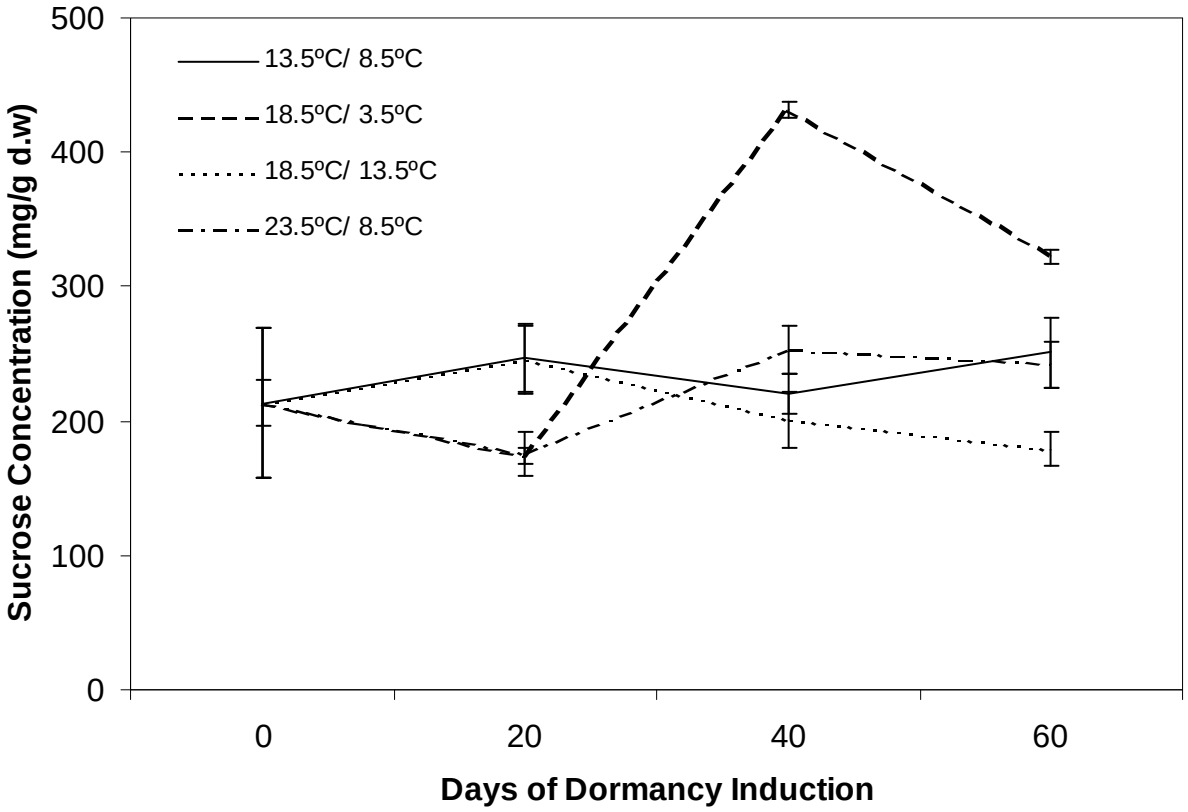
Fructose was also not detectable in any significant amounts (data not shown). Glucose and sucrose were detectable consistently in all samples. Sucrose and glucose did not significantly increase over time in either clones or temperatures (Figure 5.1, 5.2, 5.3, and 5.4). Although insignificant, there were observed increases in sucrose over time. It is possible that once growth cessation occurred, source-sink relationships changed resulting in accumulation of sugars, produced from photosynthetically active leaves, in stem tissue.

There were no significant interactions effects between clone and temperature (data not shown)($P<0.05$). Soluble sugar content did not significantly correlate with dormancy development. Other factors are involved during dormancy development. Growth cessation may be a prerequisite for soluble sugar accumulation, subsequently resulting in increases in freezing tolerance. However, increases in soluble sugars are still dependent on exposure to cool, non-freezing temperatures.

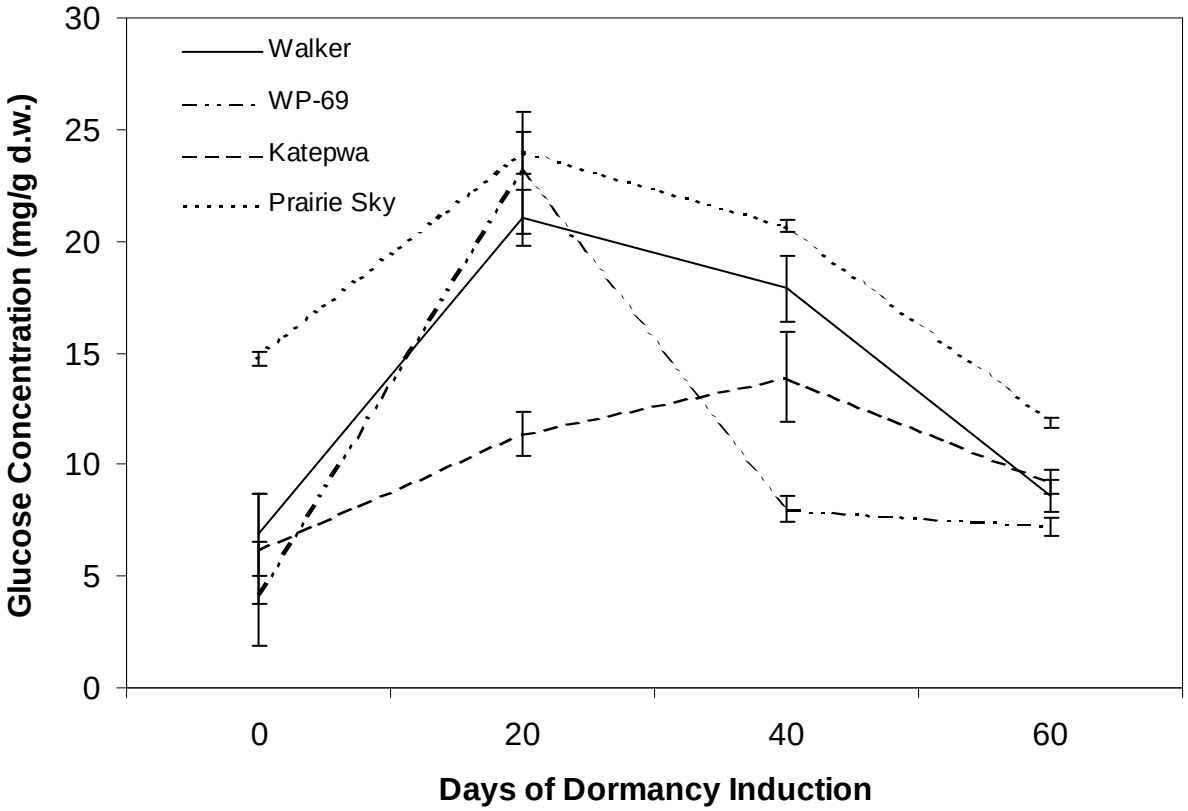
Although statistically insignificant, there were a few notable observations. Samples with low dormancy appeared to have higher sucrose accumulation than samples with higher dormancy development. ‘Prairie Sky’ and clones within the coolest night temperature (18.5ºC /3.5ºC)



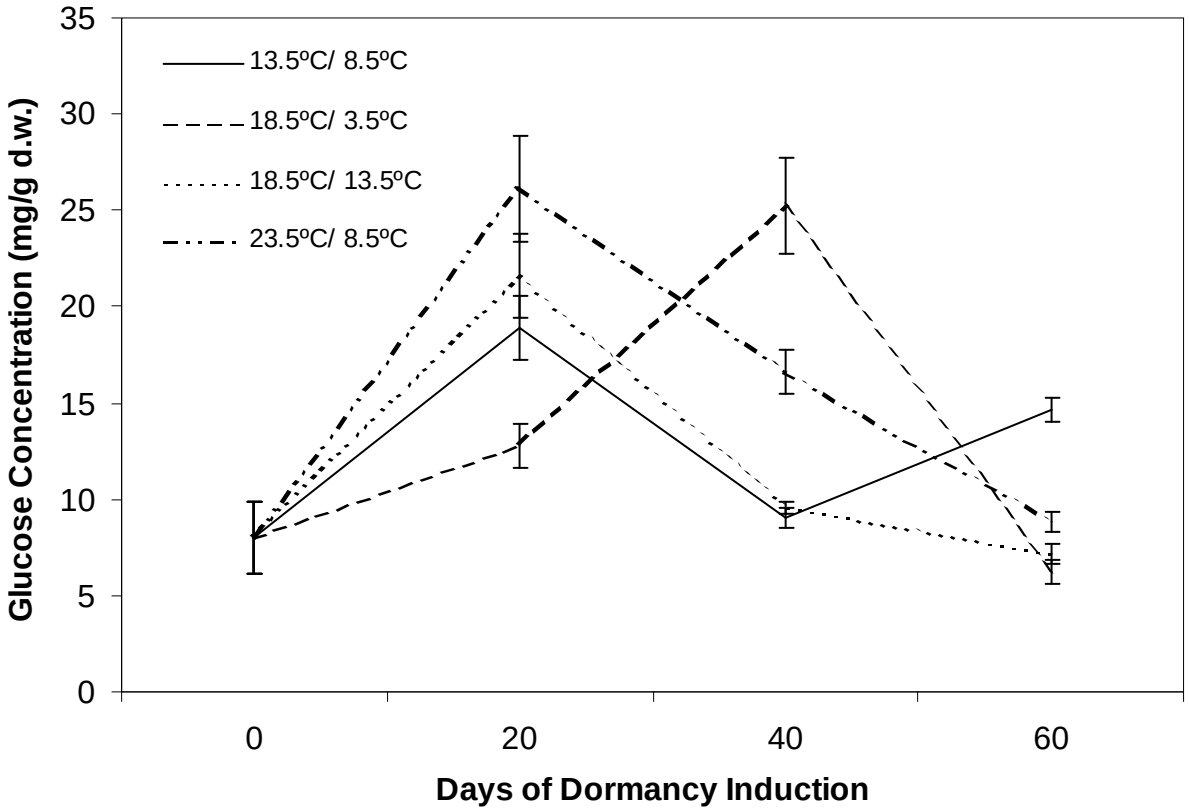
Sucrose concentration (mg/g dry weight) of four poplar (*Populus sp.)* clones ('Walker', 'WP-69', 'Katepwa' and 'Prairie Sky') after 0, 20, 40 and 60 days of dormancy induction. Error bars denote ± SE.



Sucrose concentration (mg/g dry weight) of poplar stems for four different temperature regimes (13.5°C/8.5°C, 18.5°C/3.5°C, 18.5°C/13.5°C and 23.5°C/8.5°C day/ night temperatures). Error bars denote ± SE.



Glucose concentration (mg/g dry weight) of four poplar (*Populus sp.*) clones ('Walker', 'WP-69', 'Katepwa' and 'Prairie Sky') after 0, 20, 40 and 60 days of dormancy induction. Error bars denote ± SE.



Glucose concentration (mg/g dry weight) of poplar stems for four different temperature regimes (13.5°C/8.5°C, 18.5°C/3.5°C, 18.5°C/13.5°C and 23.5°C/8.5°C day/ night temperatures). Error bars denote ± SE.

showed the highest sugar concentration. This may be a result of the effect of the trees being exposed to acclimating conditions in the cool night treatment once growth cessation had occurred. Rather than actual increases in sucrose in actively growing samples, it is possible that the difference between the warm night temperature (18.5ºC /13.5ºC) and cool night temperature (18.5ºC /3.5ºC) was a result of earlier and more extensive wood formation in the warm night treatment (data not shown). Wood would contain extra dry weight with low soluble sugar content. Differential wood formation may account for some of the observed discrepancies in the data.

If this experiment were to be repeated, separation of the vascular tissue from the woody section would be useful in getting more accurate results. In addition to separation of tissue types, larger sample sizes may also aid in increasing accuracy of sugar samples. Sample size was as large as possible for this experiment and was pushing the limits of detectability in the HPLC in spite of concentrating the sample 9:1. Lastly, sampling from different regions in the plant including sugars within the bud and stem tissue directly adjacent to axillary buds may yield better results than sampling sugars from the tip of the plant directly below the axillary mersitem.

C.4 Conclusion

Soluble sugars did not accumulate during dormancy induction in hybrid poplar. Changes in stem soluble sugar content may be attributed to cold hardiness rather than dormancy development. Changes during dormancy induction that result in increases in freezing tolerance are likely not related to sugars. These changes may be closer related to proteins, cell-wall changes or decreases in water content.

APPENDIX D. FUTURE WORK

While this project contributed towards identifying how photoperiodic induction in hybrid poplar, it did not identify how dormancy and cold acclimation is affected by temperature under field conditions. Future work could include identifying how dormancy and cold hardiness levels are affected by autumn temperature. Since warmer conditions induce earlier growth cessation and subsequent increases in dormancy and cold hardiness levels under constant photoperiod, clones with shorter critical photoperiods may be more competitive since growth is better synchronized with the available growing season. Identifying critical photoperiod of each clone and determining whether there is a shift in competitive advantage under warming conditions, towards clones that have shorter critical photoperiods would be advantageous for breeding and forest conservation programs.

Aside from whole plant responses to temperatures, using this plant system of contrasting clonal responses enables the separation of dormancy and cold hardiness. Using 'Prairie Sky', which does not reach endodormancy, in conjunction with 'WP-69', which is not affected by temperatures and 'Walker', which is affected by temperature would be useful to explore gene expression, hormonal levels and water status corresponding to endodormancy. Using available micro-array technology and contrasting clonal responses, candidate genes that are affected by temperature can be identified and used to select genotypes that are less responsive to temperature for future adaptability to climate change. Additionally, exploring expression of phytochrome related genes in response to temperature may strengthen the hypothesis that phytochrome is the underlying physiological process by which temperature impacts dormancy induction in hybrid poplar. The use of NMR could be further developed to explore changes in water mobility between plant tissues possibly using tracer elements and dyes to identify blockages in tissue-tissue and cell-cell communication of metabolites. Hormonal studies, specifically looking at GA levels and translocation between plant organs would complement the work with phytochrome and water. These studies would strengthen emerging theories on the regulation of dormancy in woody plants.

APPENDIX E. ANOVA TABLES

Analysis of Variance of temperature and clone on dormancy development (DBB) after 20 days of dormancy induction.

Source	DF	SS	MS	F	P
Temperature	3	7.063	2.354	3.99	0.010
Clone	3	3.975	1.325	2.24	0.087
Interaction	9	9.231	1.026	1.74	0.089
Error	112	66.158	0.591		
Total	127	86.428			

Analysis of Variance of temperature and clone on dormancy development (DBB) after 40 days of dormancy induction.

Source	DF	SS	MS	F	P
Temperature	3	176.126	58.709	65.79	<0.001
Clone	3	262.596	87.532	98.09	<0.001
Interaction	9	107.121	11.902	13.34	<0.001
Error	112	99.940	0.892		
Total	127	645.782			

Analysis of Variance of temperature and clone on dormancy development (DBB) after 40 days of dormancy induction.

Source	DF	SS	MS	F	P
Temperature	3	1093.18	364.39	193.77	<0.001
Clone	3	1090.47	363.49	193.29	<0.001
Interaction	9	317.68	35.30	18.77	<0.001
Error	112	210.62	1.88		
Total	127	2711.95			

Analysis of Variance of temperature and clone on cold hardiness (LT_{50}°C) after 20 days of dormancy induction.

Source	DF	SS	MS	F	P
Temperature	3	270.27	90.09	9.67	<0.001
Clone	3	585.27	195.09	20.94	<0.001
Interaction	9	627.45	69.72	7.48	<0.001
Error	240	2235.88	9.32		
Total	255	3718.86			

Analysis of Variance of temperature and clone on cold hardiness (LT_{50}°C) after 40 days of dormancy induction.

Source	DF	SS	MS	F	P
Temperature	3	1844.7	614.9	22.71	<0.001
Clone	3	4322.3	1440.8	53.20	<0.001
Interaction	9	1348.9	149.9	5.53	<0.001
Error	240	6499.3	27.1		
Total	255	14015.2			

Analysis of Variance of temperature and clone on cold hardiness (LT_{50}°C) after 60 days of dormancy induction.

Source	DF	SS	MS	F	P
Temperature	3	7072.4	2357.5	51.08	<0.001
Clone	3	12110.5	4036.8	87.46	<0.001
Interaction	9	4604.6	511.6	11.08	<0.001
Error	240	11077.6	46.2		
Total	255	34865.1			

Analysis of Variance of temperature, clone and days of dormancy induction on T_1 relaxation times (ms) in the vascular tissue region.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Clone	1	195659	195659	195659	4.72	0.040
Temperature	1	747	747	747	0.02	0.894
Day	2	716367	716367	358184	8.64	0.001
Clone *Temperature	1	6834	6834	6834	0.16	0.688
Clone * Day	2	196900	196900	98450	2.37	0.115
Temperature * Day	2	109408	109408	54704	1.32	0.286
Clone *Temperature *Day	2	70744	70744	35372	0.85	0.439
Error	24	995341	995341	41473		
Total	35	2292000				

Analysis of Variance of temperature, clone and days of dormancy induction on T_1 relaxation times (ms) in the vascular transition region.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Clone	1	57680	57680	57680	4.87	0.037
Temperature	1	4032	4032	4032	0.34	0.565
Day	2	145403	145403	72702	6.14	0.007
Clone *Temperature	1	9571	9571	9571	0.81	0.378
Clone *Day	2	128042	128042	64021	5.41	0.012
Temperature*Day	2	2181	2181	1091	0.09	0.912
Clone *Temperature*Day	2	32598	32598	16299	1.38	0.272
Error	24	284250	284250	11844		
Total	35	663758				

Analysis of Variance of temperature, clone and days of dormancy induction on T_1 relaxation times (ms) in the lower axillary bud region.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Clone	1	2085	2085	2085	0.81	0.378
Temperature	1	16	16	16	0.01	0.938
Day	2	14225	14225	7112	2.75	0.084
Clone *Temperature	1	2635	2635	2635	1.02	0.323
Clone *Day	2	42630	42630	21315	8.24	0.002
Temperature*Day	2	4 25	425	212	0.08	0.921
Clone *Temperature*Day	2	11902	11902	5951	2.30	0.122
Error	24	62047	62047	2585		
Total	35	135965				

Analysis of Variance of temperature, clone and days of dormancy induction on T_1 relaxation times (ms) in the upper axillary bud region.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Clone	1	1	1	1	0.00	0.987
Temperature	1	14360	14360	14360	5.76	0.025
Day	2	8462	8462	4231	1.70	0.205
Clone*Temperature	1	4467	4467	4467	1.79	0.193
Clone* Day	2	25872	25872	12936	5.19	0.013
Temperature*Day	2	26052	26052	13026	5.22	0.013
Clone*Temperature*Day	2	6634	6634	3317	1.33	0.283
Error	24	59875	59875	2495		
Total	35	145722				

Analysis of Variance of temperature, clone and days of dormancy induction on Apparent Diffusion Coefficient ($mm^2 \cdot sec^{-1} \times 10^{-4}$) in the vascular tissue region.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Clone	1	6.362	6.362	6.362	7.05	0.014
Temperature	1	0.356	0.356	0.356	0.40	0.536
Day	2	68.499	68.499	34.250	37.96	0.000
Clone*Temperature	1	11.919	11.919	11.919	13.21	0.001
Clone*Day	2	7.404	7.404	3.702	4.10	0.029
Temperature*Day	2	7.365	7.365	3.682	4.08	0.030
Clone*Temperature*Day	2	14.748	14.748	7.374	8.17	0.002
Error	24	21.655	21.655	0.902		
Total	35	138.310				

Analysis of Variance of temperature, clone and days of dormancy induction on Apparent Diffusion Coefficient ($mm^2 \cdot sec^{-1} \times 10^{-4}$) in the vascular transition region.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Clone	1	48.812	48.812	48.812	50.21	0.000
Temperature	1	5.682	5.682	5.682	5.85	0.024
Day	2	103.833	103.833	51.917	53.41	0.000
Clone*Temperature	1	8.204	8.204	8.204	8.44	0.008
Clone*Day	2	13.969	13.969	6.984	7.18	0.004
Temperature*Day	2	18.472	18.472	9.236	9.50	0.001
Clone*Temperature*Day	2	8.517	8.517	4.258	4.38	0.024
Error	24	23.330	23.330	0.972		
Total	35	230.818				

Analysis of Variance of temperature, clone and days of dormancy induction on Apparent Diffusion Coefficient ($mm^2 \cdot sec^{-1} \times 10^{-4}$) in the lower axillary bud region.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Clone	1	10.3253	10.3253	10.3253	11.78	0.002
Temperature	1	5.8822	5.8822	5.8822	6.71	0.016
Day	2	67.1477	67.1477	33.5738	38.29	0.000
Clone*Temperature	1	6.0692	6.0692	6.0692	6.92	0.015
Clone*Day	2	1.8055	1.8055	0.9027	1.03	0.372
Temperature*Day	2	8.1236	8.1236	4.0618	4.63	0.020
Clone*Temperature*Day	2	0.9497	0.9497	0.4749	0.54	0.589
Error	24	21.0420	21.0420	0.8767		
Total	35	121.3451				

Analysis of Variance of temperature, clone and days of dormancy induction on Apparent Diffusion Coefficient ($mm^2 \cdot sec^{-1} \times 10^{-4}$) in the upper axilalry bud region.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Clone	1	0.0565	0.0565	0.0565	0.10	0.758
Temperature	1	1.2545	1.2545	1.2545	2.15	0.155
Day	2	3.6767	3.6767	1.8384	3.15	0.061
Clone*Temperature	1	0.0686	0.0686	0.0686	0.12	0.735
Clone*Day	2	6.3574	6.3574	3.1787	5.45	0.011
Temperature*Day	2	1.8768	1.8768	0.9384	1.61	0.221
Clone*Temperature*Day	2	6.4286	6.4286	3.2143	5.51	0.011
Error	24	13.9977	13.9977	0.5832		
Total	35	33.7168				