ABSCISIC ACID INDUCED DORMANCY AND HARDENING OF *IN VITRO* SASKATOON BERRY

(*Amelanchier alnifolia* Nutt.)

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ABSTRACT

In vitro plantlets of *Amelanchier alnifolia* Nutt. 'Smoky' showed inhibited bud break in response to ABA application to tissue culture growing medium, but did not demonstrate a condition of innate dormancy. Partial formation of terminal buds, and axillary bud transition in phenotype from summer to winter form was produced by inclusion of 50μM ABA in tissue culture growing medium when BA levels were decreased from 11.0μM to 5.5μM and NAA was eliminated.

Addition of 50μM ABA to growing medium approximately doubled the -5°C hardiness levels of control plantlets, but ABA application alone did not generate hardiness levels as high as those seen during low temperature/short day (4°C/8h) acclimation (-27°C). Addition of BA to medium significantly reduced the efficacy of ABA-induced freezing tolerance.

Wounding of plantlets during subculture was found to produce a rapid but transitory increase in hardiness level from -5°C to -9°C within 24 hours of subculture. Newly-subcultured plantlets were found to increase in hardiness more rapidly, and to a greater extent than reports previously published for these plantlets in culture.

While short days (8h) slightly enhanced freezing survival of plantlets under both warm (23°C) and cool (4°C) temperatures, a pre-treatment of short warm days did not increase the rate of plantlet hardening under cold inductive

conditions.

Buds collected from orchard-grown plants demonstrated rising innate dormancy levels in late summer, with maximum levels between early September and late November. From December to the time of natural spring bud break, innate inhibition levels steadily decreased. Similar buds placed in culture were induced to grow at all seasons on BA-supplemented medium and were inhibited on ABA medium. On hormone-free medium, growth responses of de-scaled buds supported on stems above the medium were most similar to the dormancy profile of buds generated outside of culture conditions.

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Acceptance of individuality is a rare gift. Those individuals with the strength and self-confidence to encourage, support or defend difficult or uncommon views are more extraordinary still. It has been my great privilege to work, learn and grow as a student and friend of Dr. Karen Tanino.

I would like to acknowledge the memory of my friend and office partner, David. His death affirmed for me that most of the issues we choose to identify as important in modern science are mere distractions which permit us the luxury of avoiding the questions we find too distressing to confront directly. Placed in the context of life, death and acceptance of ourselves, the questions addressed by science can suddenly show themselves for the empty diversions that they too often are.

We have not the reverent feeling for the rainbow that the savage has, because we know how it is made. We have lost as much as we gained by prying into that matter.

Mark Twain

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1.0 INTRODUCTION

Success of any plant species depends upon both competitive utilization of environmental resources, and reliable survival of potentially lethal climatic events. Low winter temperature is considered one of the most limiting factors in global plant distribution (Sakai and Weiser, 1973). For perennial woody species of cool-temperate regions, a seasonal shift in development is considered an essential aspect of winter survival (Perry, 1971). Prior to winter's onset, the form of woody plants is transformed from one permitting active growth, to one of suppressed growth and a condition broadly defined as dormancy. Once dormant, plants are considered able to acclimate for survival of extreme winter temperatures (Fuchigami *et al.*, 1982). The alignment of both dormancy and hardiness development are critical to survival. While many fully hardened plant species can withstand direct immersion in liquid nitrogen, these same plants may be killed by a light frost while in active growth (Weiser, 1970).

A substantial body of evidence presently exists linking the growth regulator abscisic acid (ABA) to processes of both cold acclimation and dormancy. Exogenous application of ABA to cell cultures has been shown to induce rapid hardening of cells to maximum levels (Chen and Gusta, 1983). Hardening responses have been far less dramatic for ABA applications to whole plants (Fuchigami *et al.*, 1971; Gusta

et al., 1982). Inadequate uptake, rapid metabolism and microbial degradation are suggested as possible reasons for the minor hardiness increases observed in whole plants following ABA application (Chen and Gusta, 1983). Even more inconsistent are the dormancy responses to ABA application.

To minimize the suggested obstacles associated with ABA application to organized plant bodies, the current study employs *in vitro* plantlets for investigation of the effect of ABA on cold acclimation. Tissue-cultured plantlets of saskatoon berry (*Amelanchier alnifolia* Nutt.) possess organized tissues and organs, while lending themselves to hormonal manipulation through the growing medium. The extensive northern geographic range of this species (Packer, 1983), combined with reports by Junttila *et al.* (1983); Kaurin *et al.* (1984); Stushnoff *et al.* (1984); and Friesen (1987) attest to the high natural level of cold hardiness of saskatoon berry. In addition, the saskatoon berry plantlets employed throughout this study were previously shown capable of cold acclimation *in vitro* (Caswell *et al.*, 1986). Thus, the system utilized in this work has potential to investigate hormonal effects on cold acclimation within the context of a more fully-integrated organism than is permitted by cell suspension cultures.

The overall objective of this thesis was therefore to determine whether tissue culture systems offers a practical means by which to investigate dormancy and cold acclimation of woody plant species.

2.0 LITERATURE REVIEW

2.1 The Genus *Amelanchier*

Amelanchier is a genus of shrubs and small trees in the subfamily Pomoideae of Rosaceae. The genus is of extensive north-temperate distribution and is open to much taxonomic speculation. The first known designation of the genus *Amelanchier* was made by Freiedrich Casimir Medicus in his taxonomic description of *Amelanchier ovalis* Medic., a common European shrub. He is thought to have obtained the generic name from the Provençal vernacular name *amelanche* by which this common European species was known (Harris, 1970). A single species, *A. asiatica* Schneid, is native to Asia, being found in Japan, China and Korea (Harris, 1970). In North America, at least one representative of *Amelanchier* is found in each state and province (Jones 1946), as well as both Canadian territories (Packer 1983).

2.2 The saskatoon berry

The saskatoon berry, *Amelanchier alnifolia* Nutt. is accepted as western Canada's only common and widespread representative of the genus. While Looman and Best (1979) list both *A. alnifolia* and *A. florida* Lindl. as native to Alberta, Packer (1983) lists only *A. alnifolia* as native to

that province. In the Great Plains region of North America, *A. alnifolia* is accepted as either the single, or certainly the most common species of *Amelanchier*. The natural range of this species forms a broad triangle across much of North America, from Alaska, Yukon and the Northwest Territories, east to James Bay and south through Minnesota, Nebraska to Arizona, New Mexico and California (Packer, 1983). Throughout much of its range, the plant inhabits open woodlands where it forms a shrub or small tree with a maximum height of about 5 m (Packer, 1983). Saskatoon berry plants may also be found on exposed prairie sites where mature plants may reach heights of only 0.5 m (Stushnoff, 1990).

2.3 Dormancy

2.3.1 Dormancy terminology

In its broadest botanical sense, the general term *dormancy* can refer to any suspension of growth in a particular structure associated with a meristem, where the suspension is temporary, and the capacity for growth is regained (Simpson, 1990). Only a brief examination of existing literature in the field of dormancy is necessary to realize that a multitude of loosely-defined terms have been, and continue to be used as descriptions of the various

aspects of plant dormancy. The field of plant dormancy includes examination of seeds, buds, tubers, bulbs, rhizomes, and turions. For each of these botanical structures, general or specialized terms may be found in the literature to describe the possible origin, control, depth and timing of the particular dormant condition under investigation. Considering possible combinations of botanical structure, control, degree and timing, in addition to revisions in terminology adopted by individual authors through time, the assortment of terms for dormancy listed in the English language literature alone is enormous. With such a vast array of terms, precise communication on the subject of dormancy is seldom assured.

An attempt to standardize dormancy terminology was made in 1985 by Lang *et al.*, with revisions published by Lang (1987). The goal of both publications was the establishment of a simple, yet inclusive three-term system capable of describing all possible states of plant dormancy. In Lang *et al.* (1985) the terms *endodormancy* (Gk *endon* - within), *ecodormancy* (Gk *oikos* - house) and *ectodormancy* (Gk *ektos* - outside) were presented and described. The original definitions were refined by Lang in a 1987 publication, with the Greek prefix *para* (meaning beside) being introduced to replace the earlier used prefix *ecto*. In the final suggested terminology of Lang (1987) the term endodormancy was proposed to denote all dormancy conditions controlled by

physiological factors within the affected structure. Two of the most common pre-existing synonyms for this word would be, *rest* and *innate dormancy*. Ecodormancy was suggested as an inclusive term for the arrest of growth due to environmental factors, and was intended to replace such commonly used terms as *quiescence* and *imposed dormancy*. The most recently introduced term, paradormancy, was recommended as an exclusive term for dormancy controlled by physiological factors within the plant, originating from outside the affected structure. It was offered as a replacement for such terms as *correlative inhibition* and *summer dormancy*.

The main criticism of Lang's terminology was based on failure of the proposed terms to differentiate among differences in degree and timing of the dormancy condition (Simpson, 1990). In any case, adoption of the terminology has not been universal, suggesting that the projected streamlining in terminology of Lang *et al.* (1985) and Lang (1987) contributed three more terms to an already copiously supplied nomenclature.

Throughout this thesis the terms, *dormancy*, *rest*, *quiescence* and *correlative inhibition* will be used in accordance with their usage in the degree growth stage model of Fuchigami *et al.* (1982).

2.3.2 Degree growth stage model (°GS)

One of the most commonly cited models delineating bud dormancy of woody plants is the degree growth stage model (°GS) of Fuchigami *et al.* (1982) based on a system using red-osier dogwood (*Cornus sericea* L.). This model represents the annual developmental rhythms of dogwood plants, by expressing identifiable growth stages around the 360° perimeter of a circle. In this way, cyclical climatic events can be identified with the plant's associated physiological stage. Five distinct developmental phases in the annual growth cycle are designated. These are: spring bud break (0°GS), maturity induction point (90°GS), vegetative maturity (180°GS), maximum rest (270°GS) and end of rest (315°GS).

At spring bud break (0°GS) the plant's newly overwintered buds are released from a state of quiescence by warm temperatures to produce a flush of rapidly elongating shoots. A plant in the rapid growth phases between 0°GS and 90°GS is unresponsive to short photoperiod and cannot be induced to cold acclimate.

When the plant first becomes responsive to short photoperiods following spring bud break, it is said to have reached the maturity induction point (90°GS). Having attained 90°GS, the plant will respond to short or decreasing photoperiods by slowing, and eventually

terminating growth with the formation of a terminal bud. Upon completion of that season's growth, but not yet having realized the next developmental stage, a plant will quickly resume growth in response to defoliation. When a plant has undergone growth cessation in response to short days and no longer resumes growth in response to defoliation, it is considered to have reached the 180°GS stage, or the state of vegetative maturity.

Vegetative maturity (180°GS) is considered to be the onset of rest, and is achieved by plants following exposure to short days with warm temperatures. On a vegetatively mature plant, correlatively inhibited buds no longer break in response to defoliation. The plant will also become fully competent to cold acclimate in response to short day, low temperature conditions. Rest progressively deepens in the buds from 180°GS until the 270°GS is reached.

At 270°GS the plant has achieved the stage known as maximum rest. Here, buds of the plant require the greatest number of hours under chilling temperatures, or the highest concentration of gibberellic acid to prompt recommencement of growth.

From the point of maximum rest, low winter temperatures lead to gradual satisfaction of the chilling requirement, slowly weakening the condition of rest within the plant. Full satisfaction of the chilling requirement prior to spring brings the plant to 315°GS or end of rest. At this

point, buds remain quiescent until warm temperatures again release them to produce another spring bud break (0°GS) and subsequent flush of growth.

2.3.3 Saskatoon berry dormancy

A dormant vegetative bud is defined as a shortened axis with appressed foliar structures (Noodén and Weber, 1978). Most investigations of woody plant dormancy are reported for vegetative rather than floral buds. In the saskatoon berry however, dormant floral buds break to produce a short determinate reproductive shoot on which the lowermost 1 or 2 axils are vegetative, with the uppermost axil reproductive (Steeves and Steeves, 1990.) Such buds are found both laterally and terminally on existing shoots (St. Pierre and Steeves, 1990).

Vegetative maturity has been defined as the stage of dormancy which corresponds to the transition from summer to winter dormancy and to the onset of cold acclimation in dogwood (Fuchigami *et al.*, 1982). Nissila and Fuchigami (1978) found that short days and warm temperatures were required for the onset of vegetative maturity in dogwood. A study of 'Smoky' saskatoon berry growing in Saskatoon, SK (latitude 52°07') found that plants had achieved a state of vegetative maturity by May 29, two months earlier than red-osier dogwood and crabapple (*Malus domestica* Mill. x *baccata*

'Rescue') studied in the same location (Friesen and Stushnoff, 1989). Each of the species studied was assessed as vegetatively mature when intact shoots on the current season's growth no longer broke bud following defoliation. By the onset of vegetative maturity, the saskatoon berry plants used in this study would still have been experiencing daylengths of increasing duration, suggesting that short daylength is not a critical factor in the onset of growth termination and bud dormancy in saskatoon berry.

Dormancy of floral buds for 'Smoky' and 'Pembina' saskatoon berry was found to be greatest for 'Smoky' between September 1 to October 13, and greatest for 'Pembina' between September 1 to October 27 (Kaurin *et al.*, 1984). Dormancy was defined by these authors as the absence of bud break after 28 days in growth-inductive conditions following 0, 7 or 21 days artificial chilling in darkness at 3°C.

2.3.4 Hormonal regulation of dormancy

While the environmental stimuli of photoperiod and temperature as outlined in the degree growth model provide an essential external stimulus for dormancy responses, a secure understanding of the internal regulatory mechanisms affected by these stimuli has proven more elusive.

Of the known plant growth regulators, abscisic acid is most closely associated with adaptations for survival of

environmental stress. Although one of the earliest suggested names for the ABA molecule was *dormin*, conclusive evidence of the role this molecule plays in dormancy of woody plants has remained uncertain for the three decades since its discovery (Addicott, 1983). The body of evidence linking ABA to woody plant dormancy remains highly contradictory. Two primary lines of study have been pursued. The first concerns endogenous inhibitor levels through the growing season, with levels during dormancy of primary interest. The second line involves plant dormancy responses to exogenous inhibitor application.

2.3.5 Endogenous ABA / inhibitor levels

A crucial step in the eventual identification of ABA became possible in the 1950's with the advent of paper chromatography and its extensive use in the study of auxin activity. At that time, researchers of auxin activity consistently observed a chromatographic region with an activity antagonistic to auxin. The region became known as *inhibitor*-β (Addicott, 1983), and much of its biological activity was ultimately shown to be due to the action of abscisic acid (Milborrow, 1967).

The earliest reported investigation of natural inhibitors as factors in bud dormancy pre-dates the discovery of inhibitor-β, however. Extractions from dormant

buds of *Fraxinus excelsior*, were found to contain high levels of inhibitor in autumn, and progressively lower levels in buds collected sequentially through the winter to the time of natural bud break the following spring (Hemberg, 1949). The highest levels of inhibiting substance coincided with the period of elevated dormancy in early winter. This finding led Hemberg (1949) to the first formal proposal that bud dormancy of woody plants may be under the control of hormonal inhibitors. Subsequent work by numerous authors testing Hemberg's 1949 hypothesis has been far less decisive, leading to conflicting conclusions on the role of ABA in bud dormancy.

In 1956, Wareing noted that exposure of the leaves of *Acer pseudoplatanus* to short days results in the formation of resting buds. In *Betula pubescens*, Eagles and Wareing (1964) demonstrated that mature leaves under short days developed higher levels of growth inhibitors than leaves under long days. These experiments led to suggestion of a translocatable factor being generated in leaves exposed to short days. This factor was thought to be active in growth arrest and bud development. Many experiments in the late 1950's and early 1960's either supported or refuted the proposed role of growth inhibitors (ABA) in bud dormancy (Vegis, 1964). In one of the last studies to rely upon bioassay as a means of quantifying inhibitor levels, Tinklin and Schwabe (1970) found that short days generated

accumulation of inhibitors in bud scales of *Ribes nigrum*. The same investigators however found that while chilling resulted in the gradual reduction of bud inhibitor levels, warm controls which were included in the study showed ABA levels comparable to those of chilled plants. This suggested that reduction of ABA was not exclusively a result of chilling temperatures. Since dormancy was also broken in warm controls, the study judged that reduction of ABA levels by chilling was not a prerequisite to the breaking of dormancy.

In an early study using gas chromatography, Lenton *et al.* (1972) measured the ABA content in dormant buds of *Betula pubescens* and *Acer pseudoplatanus*. No increase in ABA level was observed upon transfer of plants from growth-inductive to dormancy-inductive conditions. Similarly, Barros and Neill (1988) found no significant adjustment in ABA level in response to photoperiodically induced dormancy of *Salix viminalis*.

With *Prunus amygdalus*, Leshem *et al.* (1974) concluded that emergence from innate dormancy was associated with a decrease in free ABA content and a corresponding increase in esterified ABA. Harrison and Saunders (1975) found no decline in the levels of free ABA in *Betula pubescens* buds from early September to the time of spring bud break, but the level of esterified ABA rose steadily as buds decreased in dormancy. With *Acer saccharum*, Dumbroff *et al.* (1979)

observed high ABA levels in buds during July and August with a decline to early October and a sharp increase at time of leaf-fall to maximum level in November and December. Here too, conversion of free to bound ABA was noted as winter progressed. Not all studies concerning free and bound ABA agree, however. Where ABA levels were studied in *Acer pseudoplatanus*, Phillips and Hoffmann (1979) discovered that changes in dormancy status were not a result of alterations in free and bound ABA ratios, but changes in dormancy status actually preceded altered ABA levels.

2.3.6 Exogenous inhibitor (ABA) application

In 1963, Eagles and Wareing reported that application of inhibitors extracted from *Betula pubescens* grown under short days, and applied to the same species under 14.5 hour days induced the formation of resting buds. El-Antably *et al.* (1967) described a similar reaction for *Betula pubescens* growing under 18-hour photoperiods following repeated dipping of leaves and shoot tips in a solution of ABA. In the same study, spray applications to *Betula pubescens*, *Acer pseudoplatanus*, *Ribes nigrum* and *Ailanthus glandulosus* under 18-hour photoperiod showed little effect on cessation of growth or bud development. Some reports of exogenous ABA application under long days describe only partial development of buds characteristic of short day-induced

buds. In a study using Massachusetts and Florida races of *Acer rubrum*, dipping of the uppermost expanded leaf into 30 ppm ABA while plants were maintained under long photoperiods caused growth stoppage and abnormal (partial) bud formation in the northern race, with little effect on the southern maple (Perry and Hellmers, 1973). In this same study discontinuation of ABA treatment led to almost immediate growth resumption in the affected trees. Saunders *et al.* (1974) established that application of ABA to *Salix viminalis* would inhibit growth but would not generate the usual short day responses of terminal bud formation. In a similar study by Hocking and Hillman (1973), bud formation was not observed on *Betula pubescens* and *Alnus glutinosa*. Here, application of ^{14}C-labelled ABA demonstrated low uptake, and rapid degradation of active ABA. Soon after its application, less than 10% of applied ABA was detectible in the active form within the plant. When the stems of young apple trees were pressure injected with ABA, slight inhibition of bud break was observed by Sterrett and Hipkins (1980). The use of ^{14}C-ABA in this study demonstrated a significant increase in phloem radioactivity following injection. These authors reported that ABA remained present and active in the plant following injection.

2.3.7 Dormancy conceptualization

Evidence obtained from ABA research into both the endogenous and exogenous lines of investigation remains highly conflicting. Whether ABA application does in fact mimic the genuine environmental stimulus of dormancy remains uncertain. With each contradicting piece of evidence the possibility of additional research at a still-deeper level beckons.

Examining transitory ABA levels and differential hormone sensitivities within individual organelles of specific cells of individual tissues of specific organs of various species at all seasons of the year, with consideration to all levels of plant maturity, quickly leads to a staggering degree in complexity. Adding to this, the very real probability that dormancy, like most plant responses, is under the control of delicate and shifting hormonal balances, the complexity grows to a still more overwhelming proportion. Yet, while technology presently permits simultaneous detection of various hormone levels, studies of single-hormone analysis are still being conducted and published. Certainly, individual investigators can contest that it is impossible to examine every imaginable aspect in dormancy control simultaneously. Yet, we must recognize that an ever-more detailed inspection of innumerable disjointed views may never produce a fully panoramic view.

The word symphony (*sym/phony*) translates literally from its Greek derivation as *together/sound*. One is able to hear a symphony only when each note of each instrument is played in the precise order, at a designated tempo, at a suitable volume for it to combine with the innumerable other sounds which must be correctly blended to generate the complete work. If every oscillation of every wave of every tone made by each instrument were to be heard in a highly precise, and well controlled, but totally isolated or random manner, the music of even our best-known symphonies would most likely be unrecognizable as a cacophony of haphazard noises.

In examination of a scientific question such as dormancy, it is perhaps too easy to become intensely preoccupied with one isolated fragment. With continual deepening and narrowing of ones frame of reference, appreciation for the sophistication of the overall composition may begin to go unseen and unappreciated. If the essence of nature is the true harmony of its parts (Tanino, 1990), it does not necessarily follow that an ever more explicit awareness of one single tone will bestow a richer appreciation for this overall harmony.

2.4 Cold hardening

Passage of hardy woody plants through the seasonal sequence of frost-tender blossoms to a winter-hardy state

capable of surviving temperatures well-below any which are known to occur naturally on this planet, provides an outstanding example of the seasonal adaptations of plants. With so clear a contrast between the hardened and non-hardened condition, matters of terminology have not posed the same difficulty in cold hardiness as with dormancy.

2.4.1 Hormonal regulation of cold hardening

Like dormancy, inductive environmental stimuli are judged to direct development and loss of hardiness through the coordination of hormonal action (Levitt, 1980). Temperature and light are considered primary in this action, with water, nutrient and numerous other environmental influences playing secondary roles (Levitt, 1980).

2.4.2 Endogenous ABA levels

The first suggestions of an endogenous hormonal basis for hardiness regulation began to appear in the late 1960's, soon after ABA's formal description. At that time, the stimulus of light, in the form of short photoperiod was suggested as an influence in hardiness regulation. Irving and Lanphear (1967) established that higher levels of ABA were present in extractions of short-day-exposed *Acer negundo* leaves than in long-day-exposed leaves. Removal or

wrapping the leaves of *Cornus stolonifera* with aluminum foil was also found to inhibit acclimation of dogwood plants (Hurst *et al.*, 1967). When a single pair of dogwood leaves was left exposed to short photoperiods, however, maximum acclimation became possible. A similar influence of leaves in generating a hardening response was found by Fuchigami *et al.* (1971) who described the translocation of a hardiness promoter from short-day-exposed leaves of *Cornus sericea* to defoliated branches with resultant enhancement of hardiness levels of the leafless branches. When girdling was performed to sever phloem connections between foliated and defoliated branches, enhancement of hardening in leafless branches was prevented. In *Prunus cerasus*, ABA levels were found to increase with autumnal hardiness elevation on trees bearing normal foliage. Defoliation of trees prior to this rise in ABA resulted in no detectible increase in that hormone's level (Mielke and Dennis, 1978). While ample evidence exists for an endogenous ABA increase in woody plants under short days, increased levels of ABA have also been found during low temperature hardiness induction of bean leaves (Eamus and Wilson, 1983), potato stem cultures (Chen *et al.*, 1983) and tomato (Daie and Campbell, 1981).

2.4.3 Exogenous ABA application

An array of plant species show enhanced levels of resistance to chilling or freezing stress following ABA application in the absence of normal low temperature hardening conditions. The range of responses to ABA application is wide, however, with reports of hardiness increases ranging from rapid and dramatic to nonexistent. One of the most marked hardening responses seen following ABA application are those established in cell suspension cultures of bromegrass, winter wheat and winter rye (Chen and Gusta, 1983). Within 4 days of ABA application, winter wheat cell suspension cultures under otherwise normal culture conditions hardened from -9.0°C to -30.0°C. Species native to warm climates tested in the same study showed no hardening response to ABA application, suggesting the lack of a genetic capacity to harden in response to ABA.

Numerous studies have been conducted with an ABA application to whole plants, shoot cultures and cell cultures, leading to a variety of moderate hardening responses. Such hardening reactions have been reported with ABA application to maple (Irving and Lanphear, 1968), cucumber (Rikin *et al.*, 1976), apple (Holubowicz and Boe, 1969) cotton (Rikin *et al.*, 1981) and others. Still others report no hardening was seen in response to ABA application. In winter wheat (Gusta *et al.*, 1982) and dogwood (Fuchigami

et al., 1971) ABA application resulted in no hardiness increase. Inadequate uptake, rapid metabolism and microbial degradation are suggested as possible reasons for the minor or nonexistent hardiness increases seen in whole plants following ABA application (Chen and Gusta, 1983).

2.4.4 Saskatoon berry hardiness

The sub-arctic extent of the species' geographic range (Packer, 1983) provides an indication of the saskatoon berry's high level of cold hardiness. Winter hardening of floral buds was found to begin in early September and achieve -44°C by the end of October (Kaurin *et al.*, 1984). Maximum bud hardiness levels were reported in the range of -60°C (Junttila *et al.*, 1983; Kaurin *et al.*, 1984), with no low-temperature exotherm of flower buds observed (Junttila *et al.*, 1983; Kaurin *et al.*, 1984). Further evidence of bud hardiness was reported by Friesen (1987), who found fully hardened winter buds easily amenable to cryopreservation. During anthesis and early fruit development, however, floral buds are highly susceptible to frost damage at temperatures of only -3 to -4°C (Olson and Steeves, 1982; Olson, 1983). Increased susceptibility to frost damage was correlated with advancing spring phenophase (Friesen and Stushnoff, 1985) and increased moisture content of buds (Junttila *et al.*, 1983; Friesen and Stushnoff, 1985).

In addition to studies using orchard-grown plant material, shoot cultures of saskatoon berry were found to harden *in vitro*. In response to cold acclimating conditions, Caswell *et al.* (1986) found that 'Smoky' saskatoon berry increased in hardiness by 8°C over controls, to a maximum hardiness level of -18.5°C.

3.0 DORMANCY AND GROWTH INHIBITION OF *IN VITRO* AND ORCHARD-GROWN SASKATOON BERRY BUDS

3.1 INTRODUCTION

For woody perennial species growing in cool-temperate regions, a seasonally-mediated shift of developmental forms is essential to survival (Perry, 1971). While annual and herbaceous perennial species are largely able to avoid the stress of low winter temperature either through forming a seed, or through moderated temperatures afforded by soil and snow cover, the long-lived superstructure of woody perennials requires that these plants tolerate, rather than avoid, extended periods of severe low temperatures.

An essential element in tolerance of low winter temperatures is the developmental shift away from a summer phenotype to a form better-suited to survival of low winter temperatures. This rhythmic seasonal shift requires effective recognition of those cues associated with the approach of winter for the establishment of a rigorously maintained state of growth termination known as dormancy. The shift toward dormancy is thought to be directed by internal controls triggered by the environmental cues of

daylength and temperature (Van Huystee *et al.*, 1967; Howell and Weiser, 1970; Fuchigami *et al.*, 1982). Release of the dormant condition is also believed to be internally controlled and environmentally regulated by exposure of dormant organs to suitable periods of chilling temperature (Weiser, 1970; Perry, 1971; Fuchigami *et al.*, 1982).

While substantial evidence exists linking the growth regulator abscisic acid (ABA) to cold acclimation of plants at the cellular level, evidence of the role played by ABA in the processes of growth termination and bud dormancy in woody plants remains contradictory and confusing. Exogenous applications of ABA have shown highly contradictory dormancy responses (Hocking and Hillman, 1973; Perry and Hellmers, 1973; Saunders *et al.*, 1974). Inadequate uptake, rapid metabolism and microbial degradation are suggested as possible obstacles to ABA uptake in whole-plant systems (Chen and Gusta, 1983). Since bud dormancy is a phenomenon of intact organs on intact plants, the obstacles of uptake, metabolism and degradation present themselves in investigations of ABA's role in dormancy induction and maintenance.

To minimize the suggested obstacles associated with ABA application to organized plant bodies, this work employs *in vitro* plantlets. Tissue-cultured plantlets of saskatoon berry (*Amelanchier alnifolia* Nutt.) possess organized tissues and organs, while lending themselves to hormonal

manipulation through the growing medium. Thus, the system utilized in this work has the potential for investigation of hormonal effects on bud dormancy within the context of a more fully-integrated organism than is permitted by cell suspension cultures.

3.1.2 Objectives

The overall objective of this study was to determine if tissue culture offers a viable means to investigate dormancy regulation in hardy woody plants. The study included an examination of hormonal regulation of bud inhibition *in vitro*. In addition, the study investigated the natural dormancy level of orchard-grown saskatoon berries through fall and winter, and determined the effect of ABA, BA and NAA on breaking and maintenance of bud dormancy. The effect of bud scales on dormancy was also examined.

3.2 MATERIALS AND METHODS

3.2.1 Standard culture conditions

All tissue culture plantlets used in this work originated from a singe winter resting bud of 'Smoky' saskatoon berry (*Amelanchier alnifolia* Nutt.). The line had been maintained in culture for approximately 10 years prior to commencement of this study with no obvious sign of mutation during that time. Shoot cultures were grown on proliferation medium consisting of (MS) Murashige and Skoog (1962) salts supplemented with vitamins of B5 medium (Gamborg *et al.*, 1968), 3.0% sucrose, 0.6% agar, 11.0 µM benzylaminopurine (BA) and 0.54 µM naphthaleneacetic acid (NAA). The pH of all media used throughout the study was adjusted to 5.8 with KOH just prior to addition of agar and dispensing to individual culture tubes for sterilization at 121°C and 138kPa pressure for 20 minutes.

Plantlets were grown on 10 ml of the above medium in 50 ml glass culture tubes. Cultures were produced under standard growth room conditions of 23°C with internal tube temperatures alternating between 28 and 23°C day/night as a direct result of photoperiod heat accumulation. Equal numbers of cool-white and Gro-Lux® fluorescent tubes furnished 99 $\mu mol \cdot m^{-2}s^{-1}$ to the culture tubes for a 16-hour photoperiod.

Throughout the course of this work, subculturing was performed routinely to provide a steady supply of vigorous, multi-branched plantlets 4 to 6 weeks in age. From 5 to 7 uniform shoots 10 to 15 mm in length were obtained per subcultured plantlet. These shoots comprised the standard experimental unit used throughout the *in vitro* portion of this work. In most cases, experimental plantlets were maintained on 10 ml of medium in 25 ml glass culture tubes. Plantlets with an experimental treatment period greater than 6 weeks were placed on 15 ml of medium to avoid nutrient depletion during treatment.

3.2.2 Media hormone treatments

In addition to standard proliferation medium containing BA and NAA, 2 additional hormone formulations were extensively used throughout this work. Hormone-free medium was identical to proliferation medium except for the exclusion of all phytohormones. An abscisic acid (ABA) treatment medium consisted of a racemic mixture of ± cis-trans ABA (Sigma Chemical Co.) added to standard proliferation medium for a final ABA concentration of 50.0 µM. Crystalline ABA was dissolved in 2 to 3 drops 1N NaOH prior to inclusion in medium. The 50.0 µM ABA concentration was adopted following a dosage-response experiment over 6 ABA concentrations ranging through, 0.0, 100.0, 50.0, 10.0,

1.0, 0.1, and 0.01 µM ABA. Percent plantlet water content, rate of axillary bud break, leaf senescence, growth inhibition and callus development were evaluated to determine a suitable treatment concentration of ABA.

3.2.3 Hormone codes

For ease of accurate hormone labelling and description in text, the 3 phytohormones employed throughout this work were consistently listed in the order: ABA/BA/NAA. A 3-digit code was then employed where the digit "1" indicated a normal hormonal concentration, "5" denoted half the normal concentration and "0" indicated zero hormone. For example, 011 specified proliferation medium with zero ABA and normal concentrations of both BA and NAA. Hormone-free medium was assigned the code 000, while ABA medium was denoted 111. This three-digit code system was used consistently throughout this work, and was especially valuable for representation of hormone levels during factorial studies.

Table 3.1. Media codes and hormone concentrations of commonly used media formulations.

Medium	**Code**	**Hormone Concentration**		
		ABA µM	BA µM	NAA µM
Standard proliferation	011	0	11.0	0.54
Hormone-free	000	0	0	0
ABA-treatment	111	50.0	11.0	0.54

3.2.4 Experimental design

All tissue culture experiments were conducted in randomized complete block design, with blocks being repeated over time. Each experiment was replicated either 3 or 5 times. Treatment effects on rate of bud break were evaluated by ANOVA.

3.2.5 *In vitro* bud break determination

Plantlets for bud break determination were defoliated by cutting petioles and removing shoot apices. Most ABA-treated plantlets did not require cutting of the petioles because a well-formed abscission layer permitted the leaves to fall easily away from the plantlets as they were removed from ABA-treatment medium. Defoliated plantlets were placed on 10 ml of hormone-free medium (000) and held under standard growing conditions for a 30-day observation period. During this time, the number of days required for the first bud to break was noted. Bud break was judged to have occurred when the first leaf to emerge from an axillary bud had reflexed away from the plantlet and was clearly visible. Bud break may or may not have been followed by continued shoot growth from the broken bud. Because some ABA-treated plantlets would neither break bud nor resume growth within the 30-day observation period, a 6-point rating scale was

used to rank the rate of bud break. Treatment means for bud break ratings were analyzed by ANOVA and compared using LSD at $p = 0.05$.

Table 3.2. Days to bud break and corresponding rating scale used for *in vitro* bud break determination.

Days to Bud Break	1-5	6-10	11-15	16-20	21-25	>25
Rating	1	2	3	4	5	6

3.2.6 *In vitro* dormancy / bud break experiments

Time course

Using a concentration of ABA high enough to assure an observable response without inducing a toxicity effect (100µM ABA), a time course experiment was undertaken to determine an appropriate treatment period of saskatoon berry plantlets for production of a distinct effect from ABA treatment.

Five replications were conducted with 4 plantlets per treatment subcultured to 100 µM ABA treatment-medium (111) and an equal number of control plantlets subcultured to standard proliferation medium (011). Treatment times were

1, 7, 14, 21 and 28 days during which time plantlets were assessed visually for leaf abscission and callus development. The trend toward greater leaf abscission of ABA-treated plantlets could be seen by giving culture tubes a sharp tap. On plantlets treated with higher levels of ABA, this tapping of the culture tube would cause leaves to fall away from plantlets at the natural point of abscission.

Following each treatment period, plantlets were defoliated (if necessary), apical buds were removed and plantlets were placed on hormone-free (000) medium for evaluation of the rate of axillary bud break as outlined in section 3.2.5. Control plantlets were treated in a similar manner to ABA-treated plantlets, but because all control plantlets had broken bud and resumed growth by about 10 days into treatment, controls were not monitored for bud break on those treatment periods exceeding a 2 week duration. Buds were rated on the 1 - 6 scale previously outlined and treatment effects compared by ANOVA.

Concentration gradient

A concentration gradient was undertaken to determine the lowest ABA concentration that would produce a clear inhibition of bud break, without permanently stopping plantlet growth, or producing excessive callus development. The initial concentration gradient was repeated three times.

Each replication consisted of a serial dilution of ABA producing concentrations of 100.0, 10.0, 1.0, 0.1, 0.01μM ABA added to standard proliferation medium (011). Standard proliferation medium (011) containing 11μM BA and 0.54μM NAA was used as a control. In each of the three replications, 8 plantlets were placed on each of the 6 different media for each of 1, 14 and 28 days. Evaluation of bud break was performed on 4 plantlets from each medium following the procedure outlined previously. The remaining 4 plantlets from each treatment medium were used for determination of percentage plantlet water content at the 1-, 14- and 28-day-treatment times.

Because a large percentage of plantlets treated with ABA at 100.0 μM showed permanent bud inhibition and substantial callus development, while plantlets at the next lowest ABA level (10.0 μM) did not demonstrate adequate bud inhibition, a second smaller concentration gradient experiment was undertaken. This experiment used the hormone treatments: 0.0, 100.0, 10.0 μM ABA with the added intermediate ABA concentration of 50.0 μM. The procedure followed was essentially identical to that used for the first concentration gradient experiment except for the exclusion of plantlet water content determinations. Visual observation of ABA's effect on plantlets also played an important role in determining the results of this supplementary experiment.

The effect of ABA/BA/NAA concentration on bud break

A hormone factorial was conducted to determine the individual effect of each of the 3 phytohormones used in the study on plantlet bud break. The factorial consisted of medium with the standard concentrations of ABA (50.0 μM) either present or absent. Under both of these conditions, BA was either present at the regular concentration of 11.0μM, at one-half this rate (5.5μM), or absent from the medium. At each of the possible 6 combination of these, NAA was either present at the normal rate of 0.54μM, at one-half this concentration (0.27μM), or was absent. This produced a total of 18 (2 x 3 x 3) separate hormone treatments.

For each replication, 13 plantlets were placed on each of the 18 hormone combinations, requiring 234 plantlets per replication. With 3 replications, the total number of plantlets used in this experiment was 702. All plantlets were given a 2-week treatment time under standard temperature and photoperiod conditions. Following the 2-week treatment-period, 3 plantlets from each medium were defoliated and 30-day bud break evaluations were begun. The remaining 10 plantlets were subjected to a freezing test for appraisal of LT_{50} as described in section 4.2.3 of this thesis.

3.2.7 Dormancy studies in orchard-grown buds

All experiments with orchard-grown material were conducted with buds collected from simple long shoots as defined by St. Pierre and Steeves (1990) of 'Honeywood' saskatoon berry cultivated under orchard conditions at the horticulture field plots, University of Saskatchewan, Saskatoon, latitude 52°07′ (SE ¼, sec.35, tp.36, rge.5, W 3rd mer). 'Honeywood' was used for this portion of the work because a large quantity of highly uniform buds were available from the approximately 10-year-old plantation. Bud collection was performed every 14 to 20 days through 3 consecutive winters, with collection during the first winter begun in early December. The two following winters, collection commenced in late summer. All three years, collection of samples continued through to natural spring bud break. At each sampling time, approximately 20 shoots were collected 1 to 2 m above the ground on the north and south exposures of 2 to 3 m bushes growing in clean-cultivated, drip-irrigated rows running east-west. The uppermost shoots of plants were not collected. Each collected shoot consisted of 150 to 200 mm of the previous season's growth and included 5 to 8 lateral reproductive buds and 1 terminal. Within 30 minutes of collection, scales were detached from 6 to 8 randomly selected buds. Bud scales, inner portions of de-scaled buds, intact buds

and adjacent internode segments were placed in separate 2.0 ml cryogenic vials and stored in LN_2 for subsequent hormonal analysis.

Dormancy determination of orchard-grown buds

Five intact simple long shoots were randomly selected from each collection period to serve in dormancy determination. Shoots collected prior to natural leaf abscission were defoliated by cutting petioles. Dormant shoots were placed in a warm greenhouse conducive to bud break under days not less than 16h long. Samples were held in 200 ml disposable plastic cups. A small hole was drilled approximately 20 mm from the bottom of each cup. Cups were filled once or twice a day during routine greenhouse watering, with leakage from the hole restoring water to the desired 20 mm level within several minutes. This facilitated frequent water replacement while preventing waterlogging of buds due to submersion. Stems were re-cut on a weekly basis.

The number of days required under growth-inductive conditions for the first bud on each sample to break was recorded. Bud break was judged to have occurred when the first of 2 or 3 pre-formed leaves within the bud had separated and reflexed away from the bud's central floret cluster (Figure 3.1.). Reflexing of the first leaf fell

between *tight cluster* and *loose cluster* phenophase as defined for saskatoon berry by Friesen (1987).

Figure 3.1. Reflexing of the first leaf away from the floret cluster as the indication of bud break for orchard-grown saskatoon berry resting buds.

Orchard-grown bud placement in culture

At each sampling time during the final 2 seasons of collection, buds were cut from orchard-grown shoots leaving each axillary bud attached to approximately 20 to 30 mm of its underlying internode. Approximately 60 buds with intact stem segments were surface sterilized in 200 ml of 0.6% sodium hypochlorite solution (10% Javex® bleach) which was vigorously stirred with a magnetic stir bar for 15 minutes. Sterilized buds were rinsed 4 times with autoclaved distilled deionized water and given a final rinse in a small quantity of 70% ethanol. Fifteen buds were then placed on each of the 3 media previously described as 000, 011 and 111. On each medium, 5 buds were excised from internode segments, scales removed and inner portions consisting of 2 to 3 preformed leaves with a condensed inflorescence placed directly on the growing medium. The other 10 buds placed on each of the different media were supported above the medium by the attached internode segments. Five of these buds were de-scaled, while 5 were left intact. Over a 3-month period following placement in culture, buds were evaluated for shoot growth under standard culture growing conditions (Figure 3.2.). Growth was assessed on the basis of leaf counts, and callus development was noted.

Figure 3.2. Orchard-grown saskatoon berry bud supported above hormone-free medium by an internode segment. The bud shown was collected in mid-March, and placed in culture on hormone-free medium with scales attached. It shows 20-days growth.

3.3 RESULTS

3.3.1 *In vitro* plantlet bud break

Length of time in ABA treatment was significant in inhibiting bud break (Table 3.3.). The average bud break score after a 14-day 100μM ABA treatment and subsequent placement on hormone-free medium (000) was significantly higher than the scores of plantlets treated for 1 and 7 days respectively. After 14 days, ABA-treated plantlets did not break bud until around 24 days. (Figure 3.3.). Conversely, by 14 days in treatment, ABA-free control plantlets had already broken bud and resumed growth. Abscisic acid treatment-periods of 21 and 28 days did not show significant increases in bud inhibition over those of the 14-day treatment-period. The average scores for the 21 and 28-day ABA treated plantlets were 4.2 and 4.6 translating to an average of 21 and 22 days to bud break respectively.

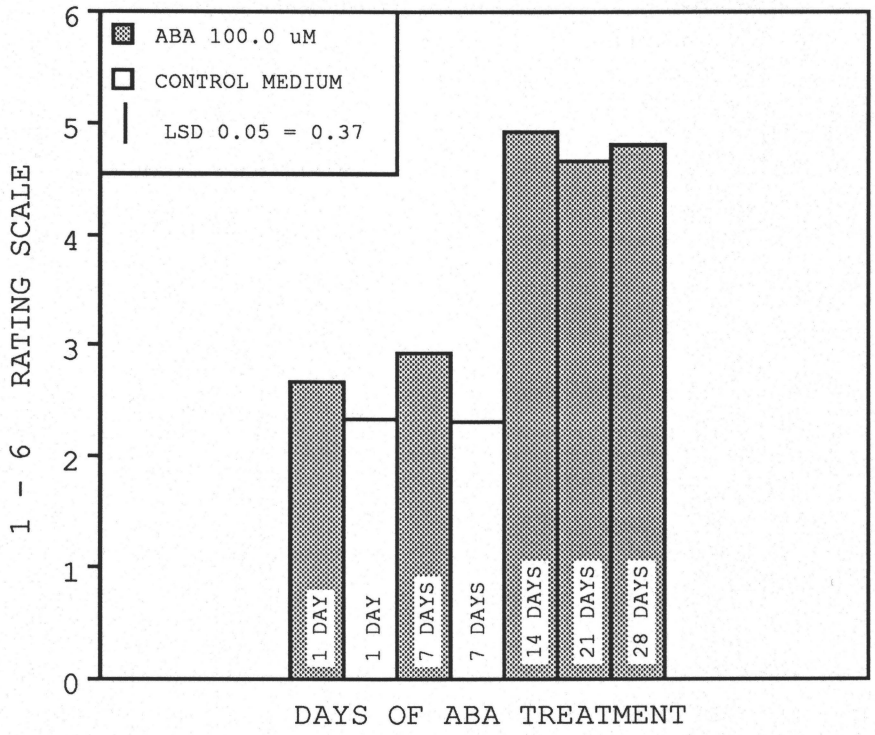

Figure 3.3. Bud break on a 1 to 6 rating scale following 1, 7, 14, 21 or 28 days treatment on 100µM ABA and control medium (011).

Table 3.3. Analysis of variance for square root transformed data on bud break ratings of tissue culture-grown saskatoon berry plantlets on hormone-free medium following ABA treatment at 100µM for 1, 7, 21 and 28 days.

Source	df	SS	MS	F	p
Replication	4	0.70075	0.17519	2.13	ns
Treatment	6	3.09723	0.51620	6.27	0.000 **
Error	24	1.97669	0.08236		
Total	34	5.77467			

LSD = 0.370 cv% = 15.9

3.3.2 ABA concentration gradient and plantlet bud break

A concentration gradient experiment was undertaken to determine an optimum level of ABA for inhibition of bud break in plantlets without imposing toxic or other detrimental effects. Concentration, time and concentration x time were all significant (Table 3.4.).

The inclusion of ABA at 0.01 and 0.1µM to standard proliferation medium did not lead to inhibition of bud break after 1, 14 or 28 days treatment (Figure 3.4.). An ABA concentration of 1.0µM produced a slight inhibitory effect over controls at day 14, but a non-significant effect after 28 days.

The two highest concentrations of ABA used were 10.0 and 100µM. These showed no inhibition of buds following a single day in treatment, but both produced significant bud inhibition after 14 and 28 days. For the 14-day treatment,

the 10.0μM concentration produced ratings of 3.1 while the higher concentration rated 5.6. After 28 days, the ratings in the same order were 3.5 and 5.8. While the 100μM ABA treatment showed excessive bud inhibition, very few of these plantlets ever resumed growth on hormone-free medium. Because of these findings a supplementary experiment was conducted where an intermediate ABA concentration (50μM) was supplied, and plantlets were assessed for bud break and callus effects. Visual observation of plantlets in association with bud break data led to the acceptance of the 50μM ABA concentration as suitable for the system. This concentration showed a high degree of leaf abscission, and growth prevention, without permanently halting plantlet growth. The selection of this concentration was largely based upon visual observation of plantlets. Following selection of the 50μM ABA treatment, essentially all further ABA application was carried out using this concentration. Unless otherwise stated, ABA was used at this concentration from the remainder of this work.

Table 3.4. Analysis of variance for bud break ratings of tissue culture-grown saskatoon berry plantlets on hormone-free medium following ABA-treatments of 0.0, 100.0, 10.0, 1.0, 0.1 and 0.01 µM for 1, 7, 21 and 28 days.

Source	df	SS	MS	F	p	
Replication	2	1.6770	0.8385	3.05	0.061	
Concentration	5	40.5676	8.1135	29.48	0.000	**
Time	2	12.8404	6.4202	23.33	0.000	**
Conc. x Time	10	15.2219	1.5222	5.53	0.000	**
Error	34	9.3563	0.2752			
Total	53	79.6631				

LSD = 0.870 cv% = 18.5

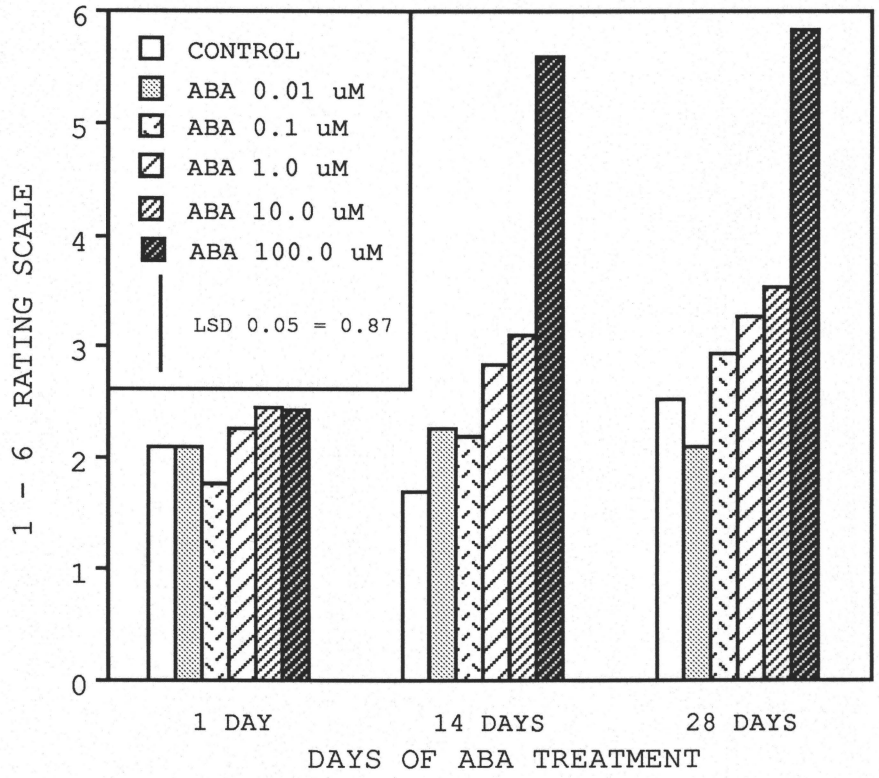

Figure 3.4. Bud break on a 1 - 6 point rating scale following ABA treatments at concentrations from 0.0, 100.0, 10.0, 1.0, 0.1, 0.01µM ABA for 1, 14 or 28 days.

3.3.3 The effect of ABA/BA/NAA concentration on plantlet bud break

The hormones ABA and BA both show significant effects on the rate of plantlet bud break (Table 3.5.). On the 6-point rating scale used for *in vitro* bud break evaluation, all plantlets grown on medium containing ABA at the standard 50μM concentration showed significant delays in bud break over plantlets grown on medium containing BA at either one-half, or full concentration. Where plantlets were grown on medium containing neither ABA nor BA, average bud break ratings were consistently higher than for those plantlets grown on ABA-medium which included BA. NAA showed no effect on plantlet bud break, appearing to neither inhibit nor promote breaking of buds.

Plantlets maintained on hormone-free medium (000) expressed an average bud break rating of 4.7 after 2 weeks of treatment (Table 3.6.). Addition of ABA to this medium (100) resulted in an increased rating for days to bud break, with a score of 6.0. Addition of a half the normal level of BA to 000 medium resulted in a decrease in the time to first bud break from 4.7 to 1.3. A further addition of BA to the full normal level of this hormone did not alter the rate of bud break over the half-strength treatments, with an average score of 1.3 again being found. A combination of BA and ABA treatments produced bud break ratings intermediate between these individual applications.

Table 3.5. Analysis of variance for square root transformed data on bud break ratings of tissue culture-grown saskatoon berry plantlets following 14 days treatment on ABA, BA and NAA factorial hormone treatments.

Source	df	SS	MS	F	p	
ABA	1	1.6058	1.6058	13.36	0.001	**
BA	2	10.8755	5.4377	45.25	0.000	**
NAA	2	0.0840	0.0420	0.35	0.707	ns
ABA x BA	2	0.1264	0.0632	0.53	0.595	ns
ABA x NAA	2	0.0237	0.0119	0.10	0.906	ns
BA x NAA	4	0.1606	0.0401	0.33	0.853	ns
ABA x BA x NAA	4	0.1288	0.0322	0.27	0.897	ns
Error	36	4.3258	0.1202			
Total	53	17.3307				

LSD for ABA = 0.191; LSD for BA = 0.233; cv% = 15.9

Table 3.6. Scores on a 1 - 6 rating scale for bud break of tissue culture-grown saskatoon berry plantlets after 2-weeks on differing hormone combinations under standard growing conditions. Codes read in the order ABA/BA/NAA, where 1 = a standard concentration, 5 = ½ the standard concentration and 0 = absence of that hormone.

ABA-Free Medium		ABA-Treatment Medium	
Media Composition	Rating	Media Composition	Rating
000	4.7	100	6.0
005	3.7	105	6.0
001	4.7	101	6.0
050	1.3	150	2.0
055	1.3	155	2.0
051	1.7	151	2.0
010	1.3	110	3.0
015	1.3	115	2.0
011	1.0	111	1.7

LSD for BA effect = 0.233; LSD for ABA effect = 0.191

3.3.4 Dormancy profile of orchard-grown buds

Buds collected from late-summer to the time of natural spring bud break from orchard-grown 'Honeywood' saskatoon berry plants showed an increasing level of bud inhibition from July (averaging 30 days to bud break) to August (averaging 39 days to bud break), with the highest level of inhibition recorded in the months of September, October and November (Figure 3.5.). Data for samples taken during these months were sparse because the extended suspension of growth prior to bud break provided a large window of opportunity for samples to decay and bud viability to be lost. Of 60 individual samples collected for the dates in September, October and November, only 20 percent had broken bud by the time buds were considered non-viable. Data from November through the end of April demonstrated a steadily declining level of bud inhibition to the point of buds breaking in the field during approximately the first week of May.

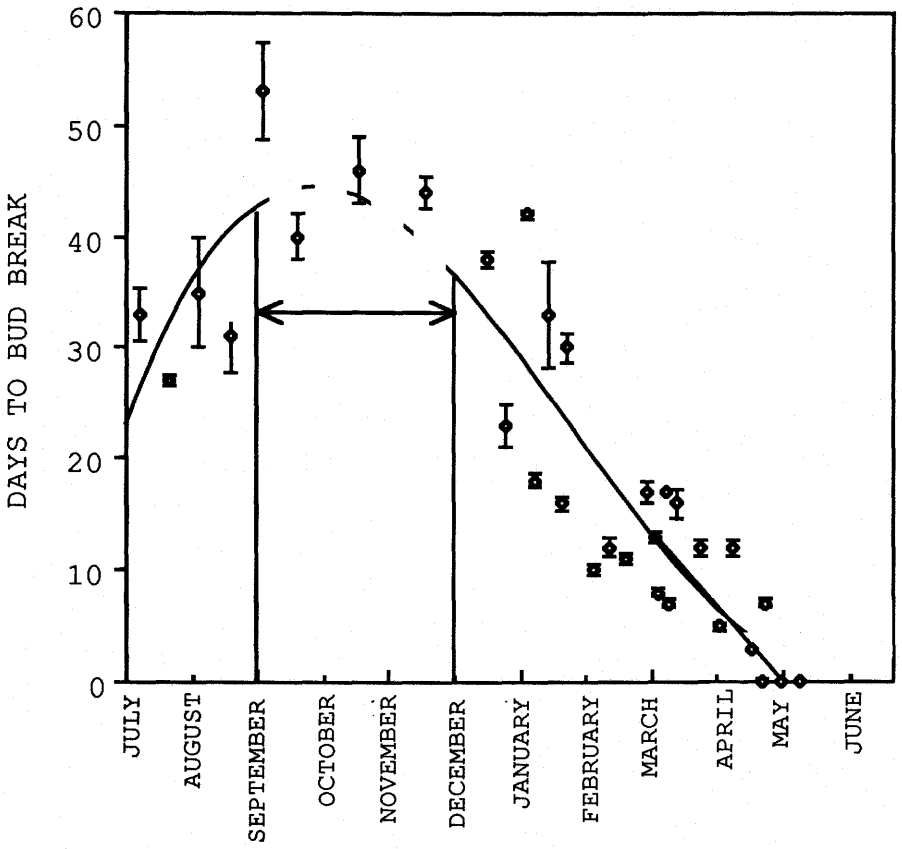

Figure 3.5. Annual dormancy profile of 'Honeywood' saskatoon berry buds collected from orchard-grown plants from July to time of natural bud break the following spring over 2 full collection periods.

3.3.5 Concentration of ABA/BA/NAA and effect on bud morphology

Plantlets treated in a factorial with hormone combination 150 (50.0µM ABA/5.5µM BA/0 NAA) were consistently observed to develop an altered bud morphology by the end of the 2-week treatment period (Figures 3.6., 3.7., 3.8., 3.9., 3.11.).

Examination of these buds showed that stipules had become more rigid and upright than those normally seen on tissue cultured saskatoon berry plantlets (Figure 3.7.). The stipules became red-brown in colour and became somewhat sheath-like, tending to partially cover the apex of the plantlet (Figures 3.8, 3.9). Petioles were reduced in length, and expanded in width, while the leaf laminae were greatly reduced in size. Laminae often abscised from the petiole at the petiole-lamina interface, rather than at the normal zone of abscission. Axillary buds became larger than normal and developed a red coloration prior to an arrest in their visible development. A small number of plantlets (10 - 15%) displayed at least partial formation of what appeared to be a terminal bud, while about 80 - 90% of treated plantlets showed axillary bud modifications. The sensitivity window of individual plantlet buds appeared to be quite narrow, as individual buds on the same plantlet often showed a differing extent of transformation.

Figure 3.6. Brown-red and rigid stipules on leaves toward the plantlet apex following treatment with ABA medium in combination with half the normal dose of BA, and zero NAA.

Figure 3.7. Saskatoon berry plantlet apex showing red-brown stipules and reduced lamina just prior to laminar abscission following ABA treatment.

Figure 3.8. ABA-treated (150) plantlet showing red and swollen axillary buds.

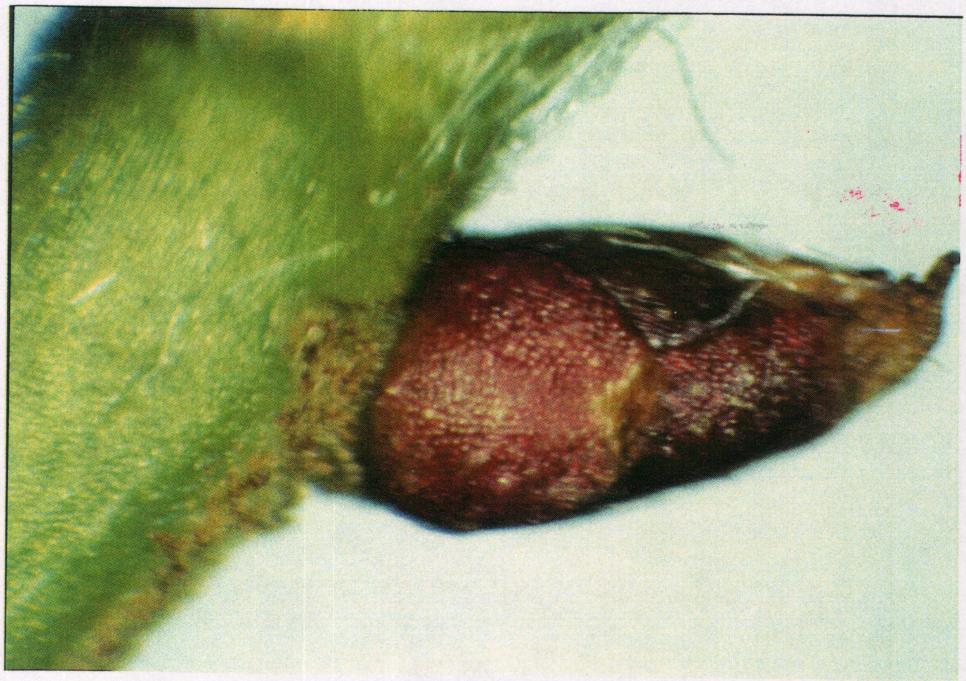

Figure 3.9. Axillary buds of tissue cultured saskatoon berries following treatment with ABA medium (150).

Figure 3.10. Saskatoon berry axillary bud collected from orchard-grown plant in early July for comparison to "dormant appearing" buds produced in tissue culture.

Figure 3.11. Saskatoon berry plantlet terminal bud formed *in vitro* following ABA (150) application to growing medium.

3.4 Orchard-grown bud placement in culture

3.4.1 Scales removed with buds placed directly on medium

Scales were removed from orchard-grown saskatoon berry resting buds through the months of September to April and central portions of buds were placed on growing medium of hormone compositions 000, 011 and 111. Proliferation medium (011) containing BA and NAA at standard concentrations elicited growth at each sampling period (Figure 3.12). At all collection times during the season, interior portions of buds which were placed in direct contact with 011 medium rapidly grew to form plantlets similar to those already in culture. De-scaled buds on hormone-free (000) medium, showed an increasing trend toward growth coinciding with the satisfaction of chilling requirement of previous tests. Bud growth on hormone-free medium, however, was limited to 1, 2 or 3 pre-existing leaves, which developed to a size more natural for these plants in nature (Figure 3.2.). When de-scaled buds were placed on proliferation medium to which ABA had been added (111), no growth was observed from buds at any point during the collection season.

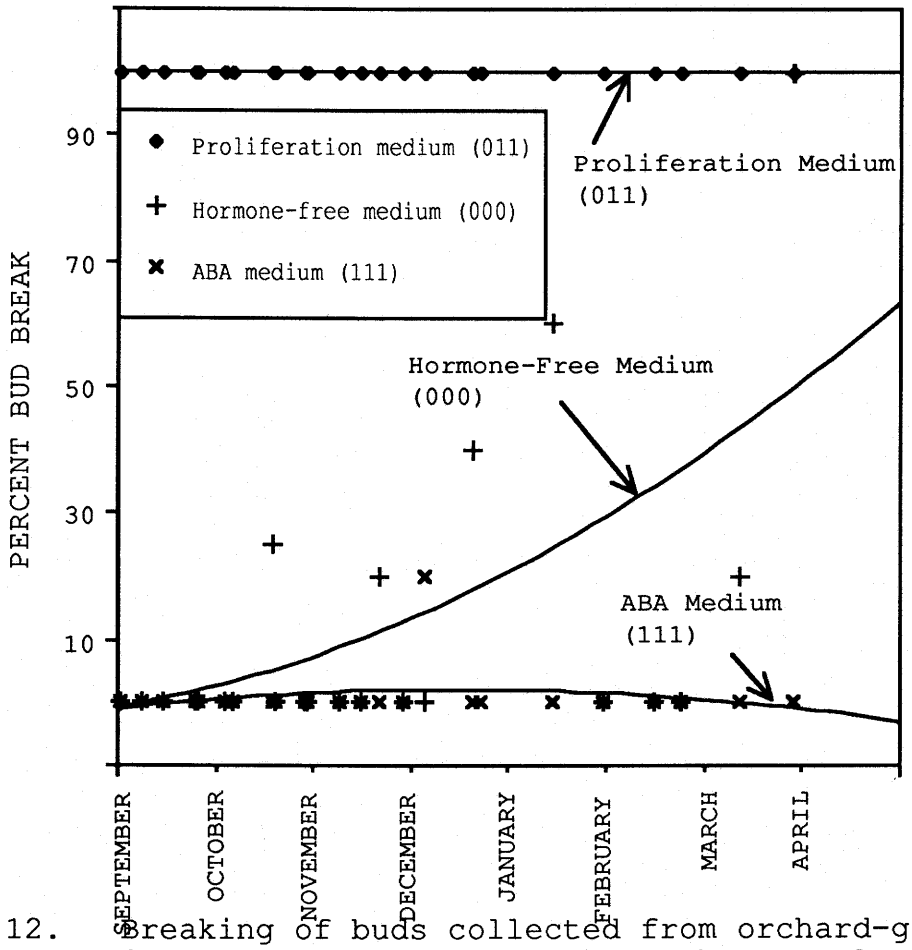

Figure 3.12. Breaking of buds collected from orchard-grown 'Honeywood' saskatoon berry, where scales were removed prior to placement directly on proliferation (011), hormone-free (000) and ABA treatment-medium (111).

3.4.2 Scales removed with buds supported above the medium

Where scales were removed from orchard-grown resting buds, and buds were supported above the medium on internode segments to prevent direct media contact, de-scaled samples on proliferation medium did not show growth for all collection times as they did when buds were in direct contact with the medium (Figure 3.13.). On both proliferation (011) and hormone-free medium (000), buds showed a trend of decreasing capacity for growth from September to December, with an increased competence for growth from December toward the date of natural spring bud break in the field. Again, ABA-supplemented medium produced essentially no growth of buds collected at any time during the fall, winter or early spring.

3.4.3 Scales intact with buds supported above the medium

Where buds were supported over the same three growing media and scales were left intact, in general, fewer buds grew than observed when buds had been de-scaled (Figure 3.14.). The trends in growth from fall and winter to spring remained the same as the de-scaled treatment, but were less pronounced.

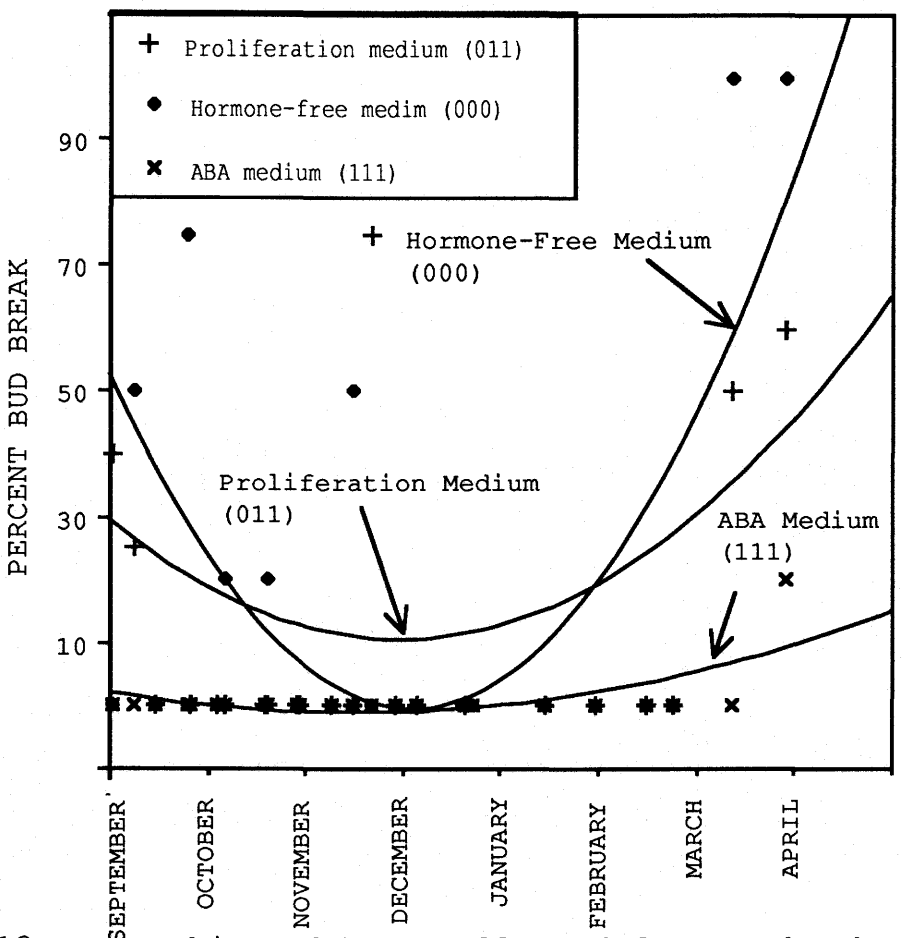

Figure 3.13. Breaking of buds collected from orchard-grown 'Honeywood' saskatoon berry plants. Scales were removed and buds supported by internode segments above proliferation (011), hormone-free (000) and ABA (111) medium.

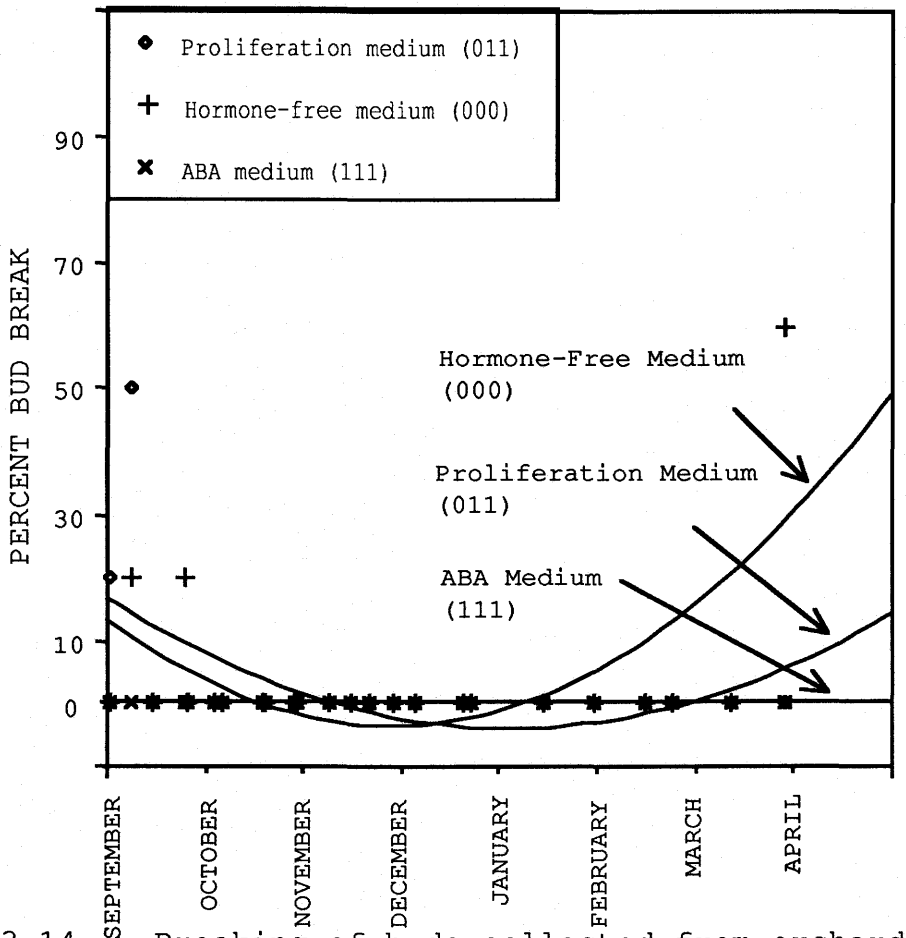

Figure 3.14. Breaking of buds collected from orchard-grown 'Honeywood' saskatoon berry plants. Scales were left intact and buds were supported by internode segments above proliferation (011), hormone-free (000) and ABA (111) medium.

3.5 DISCUSSION

3.5.1 *In vitro* plantlet bud break

In this study, *in vitro* bud break responses to ABA treatment were time-dependent with significant inhibition of buds after treatment periods of 14 days or greater. The response to ABA was also concentration-dependent with the highest tested concentration of ABA (100μM) inducing the greatest inhibition of bud break. An intermediate 50μM dose was also effective in inhibition of bud break. Although no statistically significant interaction was detected, the counteractive influence of BA on ABA-induced bud inhibition became apparent. Rate of bud break was not influenced by NAA, however. Application of cytokinin is known to release dormancy of buds in woody species and turions of aquatic species (Noodén and Weber, 1978). Injection of ^{14}C BA resulted in hastened bud break in apple when pressure injected to young dormant apple trees (Sterrett and Hipkins, 1980).

The inherent problem with this *in vitro* system was the inability to separate growth cessation in the form of non-breaking axillary buds from a condition of true bud dormancy that would be associated with winter survival. With this system, however, a combination of 50μM ABA, 5.5 μM BA and 0μM NAA (hormone code 150) consistently altered bud

morphology in plantlets, generating a high percentage of buds with altered morphology and colour. This has also been observed with ABA application to whole plants (Eagles and Wareing, 1963; El-Antably *et al.*, 1967). The window of sensitivity of an individual bud appeared to be quite narrow, however, with the level of response of different buds on the same plantlet being highly variable. This observed shift in plantlet morphology bore at least some resemblance to the natural transition from summer to winter resting buds (Figures 3.6., 3.7., 3.8., 3.9., 3.10., 3.11.).

Steeves and Steeves (1990) suggested that an endogenous inhibitor/promoter balance most likely played a role in the transition from scale-leaf (cataphyll) to photosynthetic leaf formation in saskatoon berry. This statement was supported by the morphological shifts seen during hormone factorial studies in this thesis. On hormone medium coded 150, a partial to full shift in foliar development was seen from photosynthetic-leaf to scale-leaf morphology. In general, lamina became reduced and often fell away, petioles became broad and short, while stipules acquired a red-brown coloration, became rigid, upright and at least somewhat sheathing. These findings are in agreement with earlier work done by Eagles and Wareing (1963); El-Antably *et al.* (1967) who applied ABA to plants and found at least partial bud development. The present study is the only one in which a factorial of inhibitor/promoter application was able to

establish bud development at least partially similar to resting buds. Furthermore, it is the first study to demonstrate bud scale development *in vitro*.

3.5.2 Dormancy profile of orchard-grown saskatoon berry

Buds collected from late-summer to the time of natural spring bud break showed an increasing level of inhibition from July, through August, with the highest levels of inhibition recorded in the months of September, October and November. Bud break data for sampling dates during these months were sparse, however, because the extended periods for which collected samples were held in water during this time provided a large window of opportunity for sample decay, desiccation and viability loss. Data from November through the end of April demonstrated a steadily declining level of bud inhibition with bud break in the field around the first week of May.

Fuchigami *et al.* (1982) defined vegetative maturity as the point at which woody plants entered rest, where correlatively inhibited buds no longer broke in response to defoliation under growth-inductive conditions and plants became competent to cold-acclimate. Vegetative maturity in 'Smoky' saskatoon berry was reported by Friesen and Stushnoff (1989) to occur on May 29. By this date, selected shoots on field-grown plants no longer recommenced growth in

response to defoliation. Shoots of these plants also displayed cold-acclimating capability in response to artificially imposed short day/low temperature conditions. At the same time, comparison species of 'Rescue' crabapple and red-osier dogwood had not attained vegetative maturity, and their shoots were incapable of elevating basal levels of hardiness when placed in cold-acclimating conditions.

Friesen and Stushnoff's (1989) reported date for vegetative maturity onset in saskatoon berry is not consistent with the requisite short day/warm temperature conditions specified as necessary to induce the condition by Fuchigami *et al.* (1982). On the reported date, plants growing outdoors in Saskatoon would have been experiencing increasing photoperiods, with daylengths continuing to extend for another 3-weeks. If, by definition, vegetative maturity is generated exclusively in response to shortening warm days, the state specified as vegetative maturity in saskatoon berry by Friesen and Stushnoff (1989) cannot in fact be precisely the same condition of vegetative maturity as defined by Fuchigami *et al.* (1982). In subsequent communication with L.H. Fuchigami, however he stated that the condition could still be referred to as vegetative maturity, and suggested that the condition may be induced by a critical photoperiod.

It was not a goal of the dormancy profile constructed for presentation in this thesis to determine the date of

vegetative maturity onset for saskatoon berry. But rather, to follow the seasonal dormancy levels for this species in comparison with ABA levels in buds collected at each sampling time. All material collected for hormonal analysis was accidentally destroyed prior to analysis commencement, however. 'Honeywood' material observed for bud break in this part of the study nevertheless corroborated the autumnal rise, early-winter peak and late-winter decline in levels of bud inhibition for woody species, as described by Fuchigami *et al.* (1982); Weiser (1970) and others. By the commencement of sample collection for dormancy determination in July, the plants did not exhibit bud break in response to defoliation, suggesting an existing condition of rest within the buds, well-known as a condition associated with buds in the vegetatively mature state. Collections from July through the end of summer demonstrated a deepening degree of rest, again in agreement with the degree growth stage model of Fuchigami *et al.* (1982). These findings corresponded to the segment event between vegetative maturity (90°GS) and maximum rest (270°GS).

Kaurin *et al.* (1984) established the threshold point for satisfaction of chilling in 'Smoky' and 'Pembina' saskatoon to be mid- and late-October respectively. 'Smoky' buds exposed to continual warm temperatures were reported as showing 0 to 30% bud break on samples collected in mid-September and mid-October. For 'Pembina,' equally low rates

of bud break were reported for mid-September, mid- and late-October. Data reported from the study did not, however, state whether 0% bud break within the 28-day observation period was followed by losses of bud viability. In determining the dormancy profile for 'Honeywood' saskatoon berry, non-breaking buds from autumn collections were usually lost due to desiccation, or decay before bud break could be reported. This suggests that accounts of 0% bud break made by Kaurin *et al.* (1984) on samples collected in autumn, may well have indicated death of the buds after this time.

Maximum rest for the 'Honeywood' saskatoon berries studied in this thesis was found to lie within the months of September, October and November. The large percentage of buds that lost viability prior to breakage, made determination of an exact point for maximum rest difficult. These finding were in accordance with the findings of Kaurin *et al.* (1984) however, who established maximum rest for 'Smoky' and 'Pembina' as falling within the dates of September 1 and October 27.

Junttila *et al.* (1983) establish that 'Smoky' and 'Pembina' were no longer in deep rest by February 23, with each cultivar showing 90 to 100% bud break within 21 days under growth-inductive conditions. The number of days to bud break for this date are in agreement with the 'Honeywood' findings in the present study, where buds

collected in February broke after an average of 15 days in growth-inductive conditions. By March 30, Junttila *et al.* (1982) reported buds of 'Smoky' and 'Pembina' to have broken in 12 to 16 days. 'Honeywood' buds collected from similar calendar dates broke within an average of 11 days. The three genotypes observed for dormancy were all selected from natural populations in different regions of Alberta and Saskatchewan, ranging from Beaverlodge, in north-western Alberta for 'Smoky,' central Alberta for 'Pembina' and central Saskatchewan for 'Honeywood.' The differing regions of origin for these named cultivars could at first suggest the possibility of ecotypic variation in responses to photoperiod for the different cultivars growing in a common garden. The May 29 date of vegetative maturity onset for 'Smoky' growing south of its natural range is still not properly accounted for, however, as daylength is still increasing at this time. If vegetative maturity were seen for this cultivar even a few days after the summer solstice, photoperiod could be suggested as one possible stimulus for growth termination. Because photoperiods were still increasing at this time, however, a high level of responsiveness to shortening days does not account for the date of growth termination. In addition, close agreement in the depth of bud dormancy between the three genotypes in the common garden suggest that ecotypic variation is not playing a role in the developmental timing of saskatoon berry.

While the degree growth model of Fuchigami *et al.* (1982) has been widely embraced as a means of describing the sequential annual phases on woody plant development, it should be recognized that the model is based upon extensive observations utilizing the single wide-ranging woody plant species, *Cornus sericea* L. Variations in growth habit, form and annual developmental sequences between differing species may lead to less-than-ideal agreement for all temperate woody plants with a model based upon dogwoods. Care must be taken to avoid the tendency of generalizing of these results to all woody species.

A 1990 paper by Steeves and Steeves detailed a morphological study of the cyclical annual events in vegetative and floral bud development in saskatoon berry. Results of this study demonstrated a fundamental difference between dogwood and saskatoon berry plants, in that saskatoon berry has a determinate shoot growth habit. By contrast, dogwood is indeterminate, with shoots continuing to grow in the presence of long days with no natural pause detected prior to a short-day stimulus (Fuchigami *et al.*, 1982).

Growth of an organism is defined as an irreversible increase in size, accomplished by a combination of cell division and cell enlargement, with cell expansion not necessarily following in immediate succession to division (Steeves and Sussex, 1989). The obvious signs of spring

growth in saskatoon berry are accomplished mainly by enlargement of cells which divided during differentiation of floral and vegetative primordia the previous year. In saskatoon berry, cells formed in the summer of one year remain relatively inactive through to the following spring, when rapid expansion brings about what we perceive as that season's growth (Steeves and Steeves, 1990). Growth processes in both vegetative and flowering shoot apices were studied by Steeves and Steeves (1990) with the finding that cataphylls (bud scales) were being initiated by saskatoon berry plants concurrently with anthesis and expansion of pre-formed leaves in May. Cataphyll initiation was fully complete by late June. Cataphylls themselves are modified leaves which function in protection of growing points, usually by forming imbricately or valvate buds. The cataphylls which are initiated as primordia during anthesis in saskatoon berry will enlarge later to enclose the un-expanded but differentiated floral and vegetative organs at the end of that growth period. Once they have expanded to enclose the living meristem and pre-formed primordia, a bud is formed.

The study by Steeves and Steeves (1990) also found that by mid-August all primordial development of the following year's photosynthetic leaves was complete, with floral structures fully established by mid-September. Other than leaf abscission, no further obvious physical changes were

recorded until the first week of April the following spring, when bud swelling became apparent with the onset of cell expansion, and the development of an abscission zone at the bases of each cataphyll. This coincides with the phenological development and water content of buds reported by Friesen (1987). Within 5 or 6 weeks of bud break, shoot expansion is complete for that year, terminating with the enlargement of earlier-differentiated cataphylls (Steeves and Steeves, 1990). This date corresponds well with the May 29 date of vegetative maturity reported by Friesen and Stushnoff (1989), suggesting that the observations made were correct, although the condition is not photoperiodically induced as it would be in dogwood.

A term used by many experienced prairie horticulturists to describe cultivars of tender woody plants of superior or exceptional hardiness is, "hardy to the tip." (personal communication, Dieter Martin, horticulturist extraordinaire). In the Parkland series of roses, for example, the cultivar 'Centennial' may be described as hardy to the tip, while 'Adelaide Hoodless' may regularly suffer tip dieback above the snow-line (personal observation). While dieback at the tips may be a function of various factors, morphological differences between older (basal) and younger (upper) portions of the plant are one of the most conspicuous differences. Axillary buds toward the bottom of a rose are produced earlier in the season, and therefore

have a longer period of time in which to complete the shift necessary for winter survival. On upper buds where the shift to a winter phenotype may be only partially complete by the end of the growing season, winter survival is less certain.

3.5.3 Orchard-grown bud placement in culture

When bud scales were removed from orchard-grown 'Honeywood' buds, and interior portions of buds placed directly onto the various media, proliferation medium (011) always induced 100% of buds to grow, while ABA medium (111) consistently inhibited bud growth. Hormone-free medium (000) demonstrated growth resumption in a similar pattern to the dormancy profile generated when field-sampled buds were held in water under greenhouse conditions. On both sets of these buds, the percentage of buds surviving and growing increased from December to spring. The effect of proliferation (011) and ABA (111) media on field-bud growth was similar to the effects of these media on *in vitro* plantlet bud break. Proliferation medium promoted bud break, while ABA medium inhibited it.

When de-scaled buds were supported above each of the media on internode segments, the bud break profile on hormone-free medium (000) became still more similar to that of field-sampled buds under greenhouse conditions. Here

again, the addition of ABA to the growing medium (111) inhibited all growth on samples. Conversely, the bud break profile of internode-elevated buds on proliferation medium (011) was altered such that it did not enhance bud break over hormone-free medium and never induced the 100% bud break of those buds placed directly on the culture medium with scales removed. Suggestions have been made that a barrier may exist between buds and vascular tissue during dormancy (Chalker-Scott, 1992). However, since ABA exerted an inhibitory effect whether the buds were supported above, or placed directly on the medium, and since there was bud break produced by proliferation medium both on, and above the medium, bud inhibition does not appear to be the result of vascular blockage. Hormonal uptake into buds supported above the medium is more dependent on transpiration, than the diffusion-promoted uptake of buds directly place on the medium. However, it is not known why proliferation medium would decrease the incidence of bud break below that of the hormone-free treatment.

Bud scales markedly decreased bud survival and growth in culture throughout the seasonal sampling on all treatment media. Similar consistently-low viability levels were not observed on all samples held in the greenhouse for bud break determination. Under tissue culture conditions, scales appear to induce rapid rotting of buds. In all cases, where buds with scales contacted the medium, buds were killed.

Evidence linking bud scales to development and maintenance of bud dormancy has been published by Schneider, 1968; Tinklin and Schwabe, 1970; Iwasaki and Weaver, 1977; Swartz *et al.* 1984. In our studies, however, bud scales had a detrimental effect on bud break by greatly lowering bud viability. Such a finding should not be interpreted as scales playing a role in bud inhibition, but should be interpreted only as a sign of bud scale non-adaptiveness to the highly unusual conditions of tissue culture. Mal-adaptiveness in a tissue culture situation speaks only of the unnatural character of the system of study. Bud scales do not have this deleterious effect in nature. Such observations should serve as a reminder to researchers that the system of study can easily impose effects which could be misinterpreted.

The profile which most closely simulated the bud break of field-sampled bud under the traditional greenhouse test was the treatment where bud scales were removed and buds were maintained on internode segments above hormone-free (000) medium. It therefore appears that endogenous factors within the buds are more directly involved in dormancy and dormancy release than bud scales which are also detrimental traits under culture conditions.

3.5.4 Conclusions for dormancy studies

Application of 50 μM ABA inhibited bud break and growth of tissue culture plantlets. In no case, however, was ABA-application shown to induce a condition of innate bud dormancy. Where ABA was added to growing medium in combination with reduced levels of BA and exclusion of NAA, buds demonstrated a partial transition in form, with photosynthetic leaves being converted to scale-leaf form, leading to partial formation of terminal buds in some cases.

Buds collected from plants growing under orchard conditions demonstrated increasing levels of endogenously controlled dormancy during autumn, with levels reaching a maximum between the beginning of September and the end of November. The level of innate rest decreased steadily from December to May. Orchard-collected buds placed on culture medium demonstrated similar trends in endogenously-controlled inhibitor levels.

4.0 COLD HARDENING OF *IN VITRO* SASKATOON BERRY PLANTLETS

4.1 INTRODUCTION

The inability to survive low winter temperature is a primary limiting factor in global plant distribution (Sakai and Weiser, 1973). The seasonal alternation of woody plants demands a high level of synchrony of plant with environment. Imperfect alignment of even the hardiest of woody perennials to season can easily result in injury from freezing temperatures. Perhaps most striking is the tolerance of hardened woody plants to direct immersion in liquid nitrogen, while being killed by only a few degrees of frost in the non-hardened state (Weiser, 1970).

A substantial body of evidence presently exists to link the growth regulator abscisic acid (ABA) to cold acclimation of plants at the cellular level. Exogenous application of ABA to cell cultures can induce rapid hardening of cells to maximum levels (Chen and Gusta, 1983; Tanino *et al.*, 1990). Still, applications of ABA to whole plants have consistently shown far less dramatic hardening responses than cells (Holubowicz and Boe, 1969; Fuchigami *et al.*, 1971; Gusta *et al.*, 1982). Inadequate uptake, rapid metabolism and

microbial degradation are suggested as possible reasons for the minor hardiness increases seen in whole plants following ABA application (Chen and Gusta, 1983). To minimize the suggested obstacles associated with ABA application to organized plant bodies, the current study employs *in vitro* plantlets for investigation of the effect of ABA on cold acclimation. Tissue-cultured plantlets of saskatoon berry (*Amelanchier alnifolia* Nutt.) possess organized tissues and organs, while lending themselves to hormonal manipulation through the growing medium.

The extensive northern geographic range of saskatoon berry (Packer, 1983), combined with reports by Junttila *et al.* (1983); Kaurin *et al.* (1984); Stushnoff *et al.* (1984); and Friesen (1987) attest to the high natural level of cold hardiness of the species. In addition, the same line of saskatoon berry plantlets employed throughout this study were previously shown capable of cold acclimation *in vitro* (Caswell *et al.*, 1986). Thus, the system utilized in this work has potential to investigate hormonal effects on cold acclimation within the context of a more fully-integrated organism than is permitted by cell suspension cultures.

4.1.2 Objectives

The overall objective of this study was to determine if tissue culture could offer a viable system with which to

examine the regulation of acclimation in woody plants. In studying acclimation, a system was first developed to determine the rate and depth of *in vitro* plantlet hardening in response to cold acclimating growing conditions. The effects of temperature and photoperiod on plantlet hardening were then investigated. A second objective was to determine if ABA application to the growing medium increases plantlet hardiness, and if so, to determine the degree to which ABA showed an effect. Finally, the study attempted to determine the effect of growth-promoting hormones on ABA-induced hardiness.

4.2 MATERIALS AND METHODS

4.2.1 Standard culture conditions

Standard culture conditions, media hormone treatments and hormonal codes are as described in section 3.2.1, 3.2.2 and 3.2.3 of this thesis.

4.2.2 Freezing tests

Caps of culture tubes containing plantlets to be frozen were sealed with several layers of Parafilm® laboratory film immediately prior to equilibrating in a Neslab LT-50 low temperature bath containing ethylene glycol maintained at -2°C. Tubes were immersed to a depth of 120 mm leaving only the lids above the surface of the circulating liquid. During several preliminary freezing tests, a thermocouple was placed within a tube containing an expendable plantlet to monitor temperature within the tube at plantlet height. Internal temperature was observed to follow bath temperature with an approximately 60 second lag as bath temperature dropped. Because of consistent close agreement between temperatures inside and outside of the culture tube, a thermocouple was not included in later freezing runs, and plantlet temperature was accepted as essentially equal to

the digitally displayed bath temperature.

To permit ice nucleation while maintaining culture sterility, the base of each equilibrated tube was touched to a small container of LN_2. After several seconds contact between the chilled tube and the metal container of LN_2, ice crystals could be seen to have formed within the culture medium. Nucleated samples were then quickly returned to the bath and left overnight at -2°C to permit plantlet nucleation. The following morning, bath temperature was lowered at a standard rate of 2°C per hour with samples removed after 1 hour at each test temperature. Samples removed from the freezing bath were transferred directly to a refrigerator at 4°C to thaw overnight.

4.2.3 Viability determination

Thawed plantlets were transferred to 5 ml of fresh proliferation medium (011) and held for 14 days under standard growing conditions. Plantlets were then rated as *live* or *dead* and LT_{50} scores were estimated (Figure 4.1.). An LT_{50} rating can be defined as the lowest temperature at which 50% plantlet survival was found following a freezing test. To be rated as *live*, a plantlet must have initiated new growth following freezing. In some cases, ABA-treated plantlets maintained green leaf segments following freezing. Such plantlets were rated *dead* because all growing points

were brown and dead, preventing regrowth. Extended observation of such plantlets verified this evaluation with eventual total browning and no sign of regrowth.

Figure 4.1. Typical regrowth of a surviving tissue cultured saskatoon berry plantlet 14 days after freezing.

4.2.4 Experimental design

All tissue culture experiments were conducted in randomized complete block design, with blocks being repeated over time. Each experiment was replicated either 2, 3 or 5 times. Treatment effects on LT_{50} were evaluated by ANOVA.

4.2.5 Initial hardening study

To ascertain the rate and magnitude of *in vitro* plantlet hardening in response to cold-acclimating conditions, plantlets were placed in conditions of 4°C with an 8h photoperiod for 2, 4, 6 and 8 weeks prior to freezing. Control plantlets were held for similar time periods under standard culture conditions of 23°C with 16h days. Both replications began with plantlets being subcultured to standard proliferation medium immediately prior to plantlet placement in treatment- and control-growth conditions. Initial hardiness ratings were determined by freezing a subset of mother plantlets from which treatment plantlets were obtained. The experiment was replicated twice, with 4 plantlets used at each of the 5 freezing temperatures per treatment time, resulting in a total of 200 plantlets frozen for the experiment. Determination of LT_{50} and data analysis were accomplished following the standard procedures outlined in section 4.2.2.

4.2.6 Photoperiod effect on plantlet hardiness

This experiment was undertaken to help separate the individual roles played by photoperiod and temperature on plantlet acclimation. In each of three replications, plantlets were maintained on standard proliferation medium under 4 different photoperiods at two different temperatures. Photoperiod treatments included 0, 8, 16 and 24 hours of light per day, with analogous sets of plantlets being held under temperature treatments of 4°C and 23°C for three weeks. A small preliminary test had previously demonstrated that photoperiod effects required a 3-week treatment-period before an effect became apparent in hardiness levels. The photoperiod experiments therefore adopted a 3-week treatment period, rather than the standard 2-week treatment period used for most other *in vitro* studies of this work.

Following photoperiod treatments at both temperatures, plantlets were frozen according to the standard freezing procedures for determination of LT_{50} levels. Three replications were conducted. Four freezing temperatures were used per treatment, with 2 plantlets per freezing temperature. With treatment periods at 0 and 3 weeks, the experiment required a total of 384 plantlets.

4.2.7 Photoperiod pre-treatment and hardening rate

This experiment was undertaken to determine if plantlet exposure to short days in combination with warm temperatures (8h/23°C) for 3 weeks would lead to an accelerated rate of subsequent plantlet hardening upon transfer to cold-acclimating conditions. Plantlets maintained under standard growing conditions of 16h days at 23°C were used as controls. All plantlets were subcultured to fresh proliferation medium prior to pre-treatment for 3 weeks at both photoperiods. Hardiness levels were determined prior to placement in cold-acclimating conditions. Sub-samples were then frozen after 3 and 14 days in cold-acclimating conditions. Data for the LT_{50} scores were plotted in raw form rather than as averaged values of the replications. This provided a larger number of data points with which to generate a line of best fit. Regression analysis was conducted to determine if the slopes of the short- and long-day pre-treated plantlets showed significant differences. The experiment used 2 plantlets for each of 4 freezing test temperatures at each of 3 treatment periods. It was replicated twice, requiring a total of 96 plantlets.

4.2.8 ABA-induced hardening and subculture effect

This experiment addressed two questions. Primarily, it was undertaken to determine the rate and level of plantlet hardening in response to the addition of 50.0μM ABA to the growing medium under otherwise standard culture conditions. A second set of controls was included in the experiment, however, because earlier observations during the establishment of a suitable freezing procedure had shown newly-subcultured plantlets grown at normal room temperatures had unexpectedly high hardiness levels. Subculturing of plantlets involved the removal of 5 to 7 uniform shoots from highly-branched mother plantlets. Individual excised shoots were then placed in separate culture tubes on new growing medium. Because hardiness differences had been noted following the wounding incurred during subculture, 2 different sets of control plantlets were included in this experiment. A non-subcultured set was included, as well as a set of control plantlets subcultured to standard proliferation medium at the same time as plantlets were subcultured to ABA-treatment medium.

The experiment was repeated three times. With each replication, a subset of mother plantlets was tested for hardiness at 0 time. Non-frozen mother plantlets of the same age were then subcultured to ABA (111) and control (011) medium, with these new plantlets frozen at 1, 3, 7, 10

and 14 days following subculture. Plantlets which were non-subcultured, subcultured to proliferation medium and subcultured to ABA-treatment medium were all held under standard growing conditions for hardiness determination at each of the treatment times. Two plantlets were used for each of the 3 treatments at each of 5 freezing temperatures, per treatment time, per replication, bringing the total number of plantlets frozen for this experiment to 450. All freezing, survival ratings and data analysis were conducted following the standard procedures previously outlined.

4.2.9 The effect of ABA concentration on plantlet water content

Percentage water data were collected from plantlets treated for 2 weeks at ABA concentrations of 100.0, 10.0, 1.0, 0.1, and 0.01μM and control plantlets on standard proliferation medium. After treatment for 2 weeks at standard growth room temperature and photoperiod, plantlets were removed from culture, dried of agar by rolling in a clean absorbent tissue and fresh weights were determined. Following 24 hours in a drying oven, percentage water content was calculated as: $\frac{\text{fresh weight - dry weight}}{\text{fresh weight}} \times 100$

4.2.10 The effect of ABA/BA/NAA concentration on plantlet hardiness

A hormone factorial was conducted to determine the individual effect of each of the 3 phytohormones used in the study on plantlet hardiness. The factorial consisted of 18 media combinations as described in section 3.2.6 of this thesis. All plantlets were given a 2-week treatment time under standard temperature and photoperiod conditions. Following the 2-week treatment-period, 10 plantlets from each medium were frozen for appraisal of LT_{50}. Because each of these freezing runs consisted of 180 individual plantlets and the circulating bath could accommodate only 66 culture tubes when fully loaded, freezing runs for the factorial were split and conducted over 3 successive days. Freezing runs were conducted on days 13, 14 and 15, of the 2-week treatment period, with companion samples from plantlets frozen on day-13 included in the day-15 freezing run. This yielded an average treatment time of 14-days to all samples.

4.2.11 Rate of plantlet deacclimation upon transfer from cold-inductive to growth-inductive conditions

The rate of hardiness loss was monitored following movement of plantlets from cold-acclimating conditions to growth-inductive conditions for plantlets on 5 different

hormone treatments. Plantlets were subcultured to standard proliferation medium and placed in cold acclimating conditions for 14 days for establishment of initial hardiness levels. After 2 weeks, samples were transferred (not subcultured) to 1 of 5 different media compositions and placed under standard growing conditions of 23°C/16h days. Sub-samples were frozen for hardiness determination at 0, 3, 6 and 9 days to determine initial hardiness levels and the rate of hardiness abatement. Media tested were: 000, 111, 100, 011 and a fifth medium consisting of standard proliferation medium (011) to which $2gl^{-1}$ of activated charcoal had been added. This medium was given the code "011C" and was used only in this experiment. Charcoal-supplemented medium was included in the experiment because previous small freezing experiments with plantlets on charcoal-supplemented medium had demonstrated elevated levels of plantlet hardiness at room temperature. Two replications were done with a total of 160 plantlets per replication and a total plantlet number of 320 for the experiment.

4.3 RESULTS

4.3.1 Initial hardening study

Time by temperature interaction was significant. Plantlets placed into short day/low temperature conditions (8h/4°C) directly after subculture onto proliferation medium showed a rapid and significant increase in hardiness (Table 4.1., Figure 4.2.). Incremental elevations in hardiness with each 2-week treatment period were significant between weeks 0 to 2, with a gain of 10.5°C; between weeks 2 to 4, with a gain of 5.8°C; and between weeks 4 and 6, with a gain of 5°C. Hardiness increases of treated plantlets between weeks 6 and 8 were not significant, increasing by only 0.8°C.

Throughout the 8-week study period, hardiness of control plantlets remained unchanged at -6°C. Although treated plantlets were able to harden to an absolute temperature of -27°C, no signs of winter resting buds were observed, and rapid regrowth was observed for all surviving plantlets following freezing.

Table 4.1. Analysis of variance for cold hardening of tissue culture-grown saskatoon berry plantlets in response to short day/low temperature (8h/4°C) over 8 weeks.

Source	df	SS	MS	F	p
Time	1	825.61	825.61	595.04	0.000 **
Temperature	4	364.33	91.08	65.64	0.000 **
Interaction	4	301.33	75.33	54.29	0.000 **
Error	10	13.88	1.39		
Total	19	1505.14			

** = significant at p = 0.01; cv%= 9.4;
LSD for interaction = 2.63 at 0.05

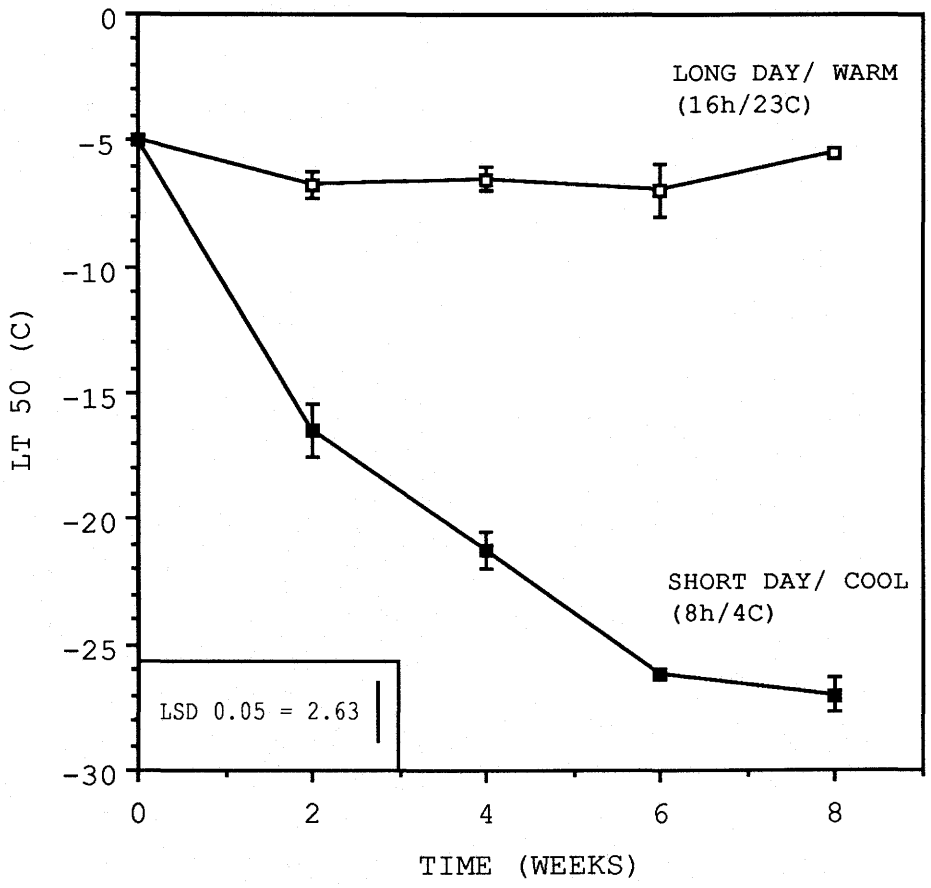

Figure 4.2. Rate and depth of saskatoon berry plantlet acclimation *in vitro* in response to short day/low temperature growing conditions (8h/4°C).

4.3.2 Photoperiod effect on plantlet hardiness

Plantlets on standard proliferation medium (011) held for 3 weeks under cool temperatures (4°C) with daily light exposures of 0, 8, 16 and 24 hours all showed hardiness increases over plantlets grown with similar photoperiods under warm temperatures (23°C) (Table 4.2., Figure 4.3.). The overall average LT_{50} rating for cool-treated plantlets was -21.6°C, while warm-grown plantlets showed an overall hardiness average of -5.4°C.

Photoperiod and photoperiod x temperature interaction were significant (Table 4.2.). Under warm growing temperatures the only significant differences observed in hardiness level were between plantlets given short days (8h) and plantlets under continual lighting. Plantlets under short days showed hardiness levels of -7°C while plantlets given 24 hours of light per day were less than half as hardy with LT_{50} ratings of -3°C. Under warm temperatures, there were no hardiness differences between plantlets grown in total darkness, and plantlets grown under either 8 or 16-hour photoperiods.

Of cool-grown plantlets, those kept in continual darkness were the least hardy, displaying hardiness levels of -17°C which was significantly lower than plantlets exposed to any daily period of light. Plantlets given short days in combination with low temperature showed the greatest

elevation of hardiness levels (-25°C), which was significantly more hardy than both plantlets treated with continual darkness (-17°C) and continual light (-21.3°C) at the same temperature.

Photoperiods of 8 and 16 hours did not generate differing levels of plantlet hardiness at cool temperatures.

Table 4.2. Analysis of variance for photoperiod effect on tissue culture-grown saskatoon berry plantlet hardiness at warm (23°C) and cool (4°C) temperature.

Source	df	SS	MS	F	p
Temperature	1	1568.17	1568.17	418.18	0.000 **
Photoperiod	3	71.00	23.67	6.31	0.005 *
Interaction	3	60.83	20.28	5.41	0.009 *
Error	16	60.00	3.75		
Total	23	1760.0			

** = significant at p = 0.01; * = significant at p = 0.05
LSD for interaction = 3.35; cv%= 14.3

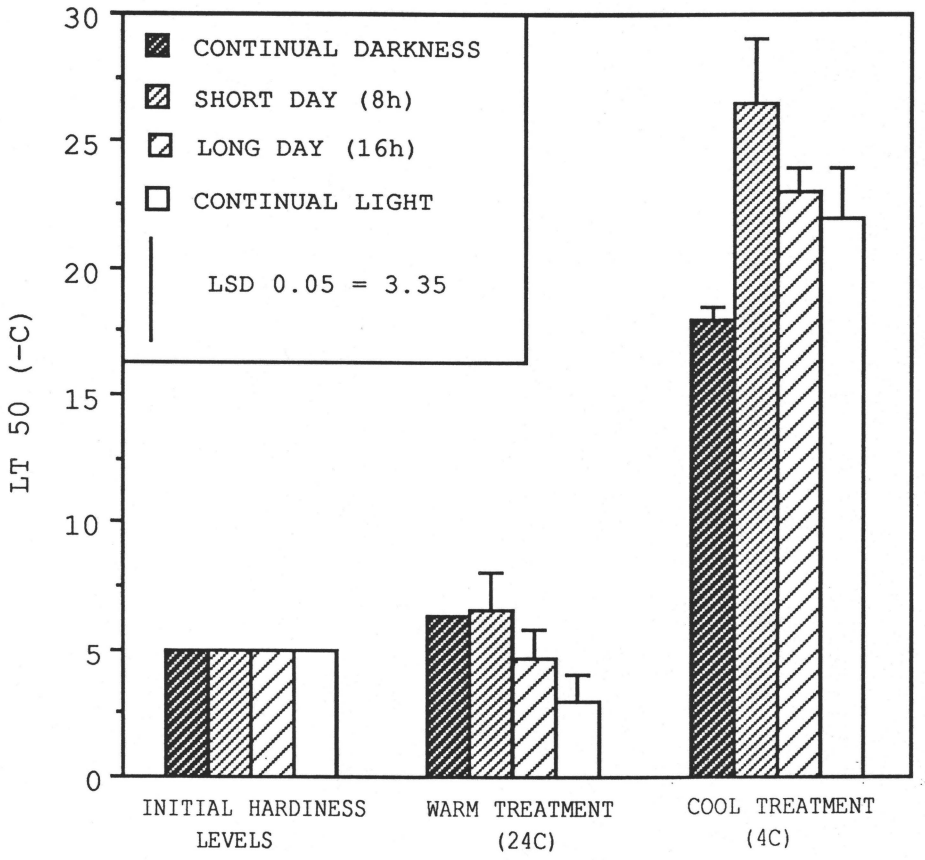

Figure 4.3. Saskatoon berry plantlet hardiness levels at warm (23°C) and cool (4°C) temperatures under photoperiods of 0, 8, 16 or 24 hours for 3 weeks.

4.3.3 Effect of short day/warm temperature pre-treatment on plantlet hardiness

Following both long day and short day pre-treatments, plantlet cold hardiness increased significantly after 14 days in 8h/4°C acclimating conditions (Figure 4.4., Tables 4.3. and 4.4.). At warm temperatures (23°C), plantlets grown under 8h days did not show an enhanced rate of acclimation over plantlets grown at the same temperature with 16h days, when both were moved to cold-acclimating conditions. Raw data for LT_{50} were plotted and regression curves generated (Tables 4.5. and 4.6). Comparisons of regression curves showed no difference in slope at 0.05, indicating no difference in hardening rate following a pre-treatment of short days with warm temperatures.

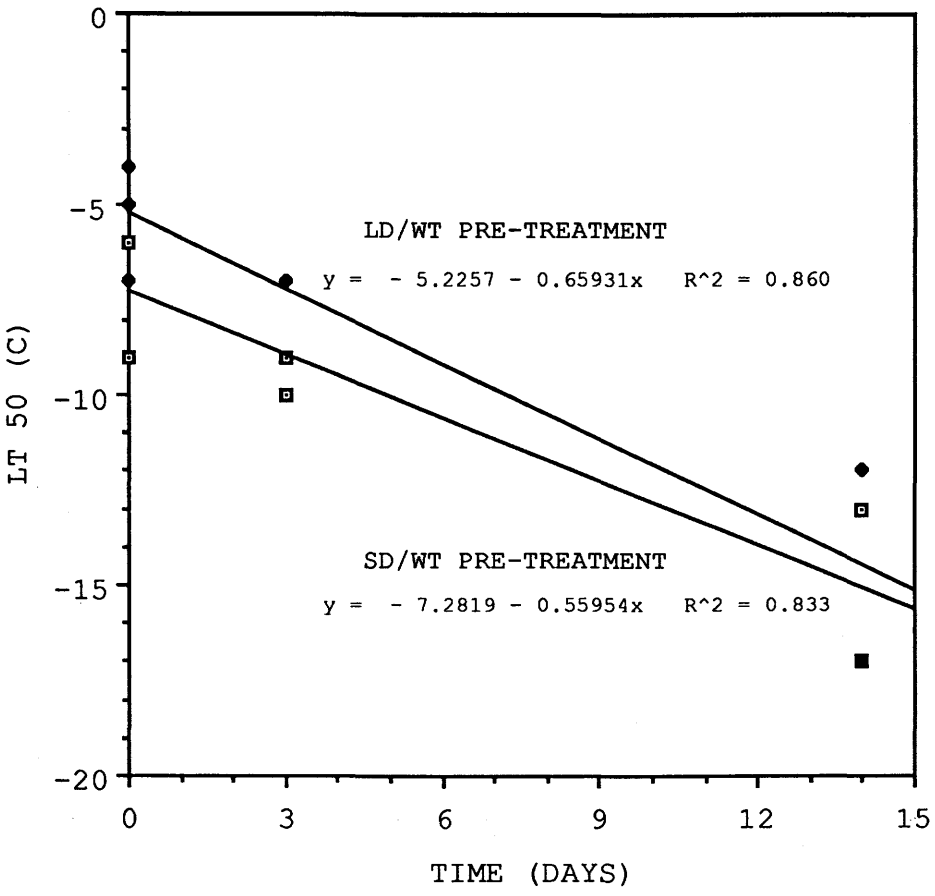

Figure 4.4. Regression curves for saskatoon berry plantlet acclimation rate following short day/warm (8h/23°C) and long day/warm (16h/23°C) pre-treatments for 3 weeks prior to placement in cold acclimating conditions (8h/4°C).

Eight hour pre-treatment

Table 4.3. Regression analysis of hardening rate of tissue culture-grown saskatoon berry plantlets at 8h/4°C following a 3-week pre-treatment of short day/warm temperature (8h/23°C).

Predictor	Coef	Stdev	t-ratio	p
Constant	7.2824	0.8569	8.50	0.000 **
C1	0.5595	0.1120	5.00	0.004 **

s = 1.752 R-sq = 83.3% R-sq(adj) = 80.0%
** = significant at p = 0.01
y = 7.28 + 0.560x b_2 = 0.560

Table 4.4. Analysis of variance for hardening rate of tissue culture-grown saskatoon berry plantlets at 8h/4°C following a 3-week pre-treatment of short day/warm temperature (8h/23°C).

Source	df	SS	MS	F	p
Regression	1	76.653	76.653	24.97	0.004 **
Error	5	15.347	3.069		
Total	6	92.000			

** = significant at p = 0.01

Sixteen hour pre-treatment

Table 4.5. Regression analysis of hardening rate of tissue culture-grown saskatoon berry plantlets at 8h day/4°C following a 3-week long day/warm temperature pre-treatment (16h/23°C).

Predictor	Coef	Stdev	t-ratio	p
Constant	5.2264	0.9094	5.75	0.002 **
C1	0.6593	0.1188	5.55	0.003 **

s = 1.859 R-sq = 86.0% R-sq(adj) = 83.2%
** = significant at p = 0.01
$y = 5.23 + 0.659x$ $b_2 = 0.659$

Table 4.6. Analysis of variance for hardening rate of tissue culture-grown saskatoon berry plantlets at 8h/4°C following a 3-week pre-treatment of long day/warm temperature (16h/23°C).

Source	df	SS	MS	F	p
Regression	1	106.43	106.43	30.78	0.003 **
Error	5	17.29	3.46		
Total	6	123.71			

** = significant at p = 0.01

4.3.4 ABA-induced hardening and effect of subculture on plantlet hardening

Non-subcultured control plantlets growing on standard proliferation medium (011) show no variation in hardiness over the 2-week study period with an average hardiness rating of -5°C (Figure 4.5.). Plantlets subcultured to the same medium and maintained under similar conditions of temperature and photoperiod, however, significantly increased in hardiness 24-hours after subculture. The hardiness of these plantlets continued to increase for 3 days following subculture at which time subcultured control plantlets achieved maximum hardiness levels of -9°C. Hardiness levels then rapidly declined, returning to the level of non-subcultured controls by 7 to 10 days after subculture.

Like plantlets subcultured to proliferation medium (011), plantlets subcultured to ABA-treatment medium (111) followed a pattern of rapid hardiness development 1-day after subculture (Figure 4.5., Table 4.7.). On the day following subculture to ABA-treatment medium, plantlets had significantly increased in hardiness to a level of -8°C, nearly doubling the -5°C hardiness levels of non-subcultured controls. Unlike plantlets subcultured to proliferation medium, ABA-treated plantlets maintained elevated hardiness levels from days 1 through to the end of the study at day

14. The average LT_{50} rating for ABA-treated plantlets between days 1 and 14 was -8.4°C, with no significant alteration in hardiness levels between these days.

The experiment demonstrates that addition of 50.0µM ABA to standard culture medium increased the hardiness of plantlets at normal room temperatures, under conditions of long days. The absolute hardiness levels attained by plantlets in response to ABA-treatment medium under warm conditions, were not as pronounced as those of plantlets on proliferation medium under short day/low temperature (8h/4°C), however. Also, no morphological signs of resting bud formation were detected. The experiment also indicates that the act of subculture can induce rapid transitory but significant levels of hardiness increase.

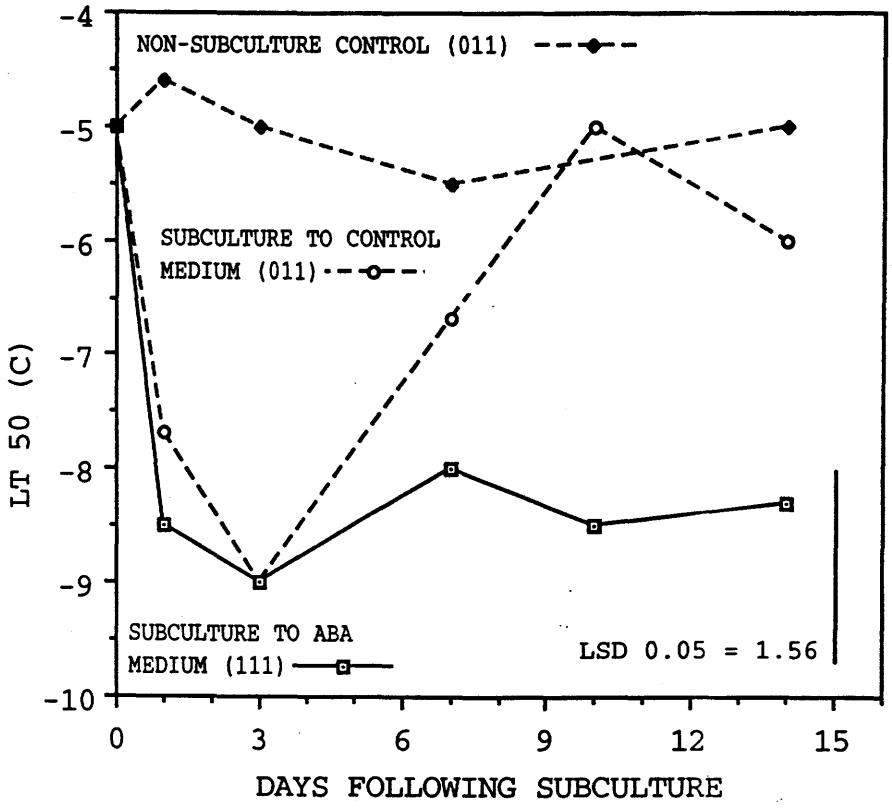

Figure 4.5. Hardiness increase and loss following subculture of saskatoon berry plantlets to ABA-treatment medium (111), and control medium (011).

111 = 50µM ABA, 11.0 µM BA + 0.54 µM NAA
011 = 0µM ABA, 11.0 µM BA + 0.54 µM NAA

Table 4.7. Analysis of variance for rate of ABA-induced acclimation at long day/warm temperature (16h/23°C) and effect of subculture on hardiness of tissue culture-grown saskatoon berry plantlets.

Source	df	SS	MS	F	p
Hormone	2	69.4815	34.7407	39.08	0.000 **
Time	5	35.8704	7.1741	8.07	0.000 **
Interaction	10	34.0741	3.4074	3.83	0.000 **
Error	36	32.0000	0.8889		
Total	53	171.4259			

** = significant at p = 0.01; cv% = 14.65;
LSD for interaction = 1.556

4.3.5 The effect of ABA concentration on plantlet water percentage

The analysis of variance demonstrated that none of the ABA concentrations produced significant alterations in plantlet water content (Table 4.8.). After 1 and 14 days treatment, no significant differences were found between plantlet water content at any of the ABA concentrations (Figure 4.6.). By 28 days in treatment, both control and treated plantlets showed a significant decrease in water content (Table 4.8.). This finding supported the suitability of a 14-day-treatment period for most *in vitro* studies done in this work. Since control plantlets showed a similar decrease in water content to ABA-treated plantlets, the difference in water content was more a factor of overall drying of the growing medium than of ABA treatment.

Table 4.8. Analysis of variance for arcsine transformed percentage water data of tissue culture-grown saskatoon berry plantlets following treatments of 0.0, 100.0, 10.0, 1.0, 0.1 and 0.01μM ABA for 1, 14 or 28 days at standard growing conditions.

Source	df	SS	MS	F	p
Concentration	5	54.95	10.839	2.29	0.066 ns
Treatment Time	2	233.131	116.565	24.60	0.000 **
Interaction	10	28.126	2.813	0.59	0.016 ns
Error	36	170.588	4.739		
Total	53	486.041			

ns = not significant at p = 0.05; ** = significant at p = 0.01; cv% = 3.6; LSD for treatment time 1.470

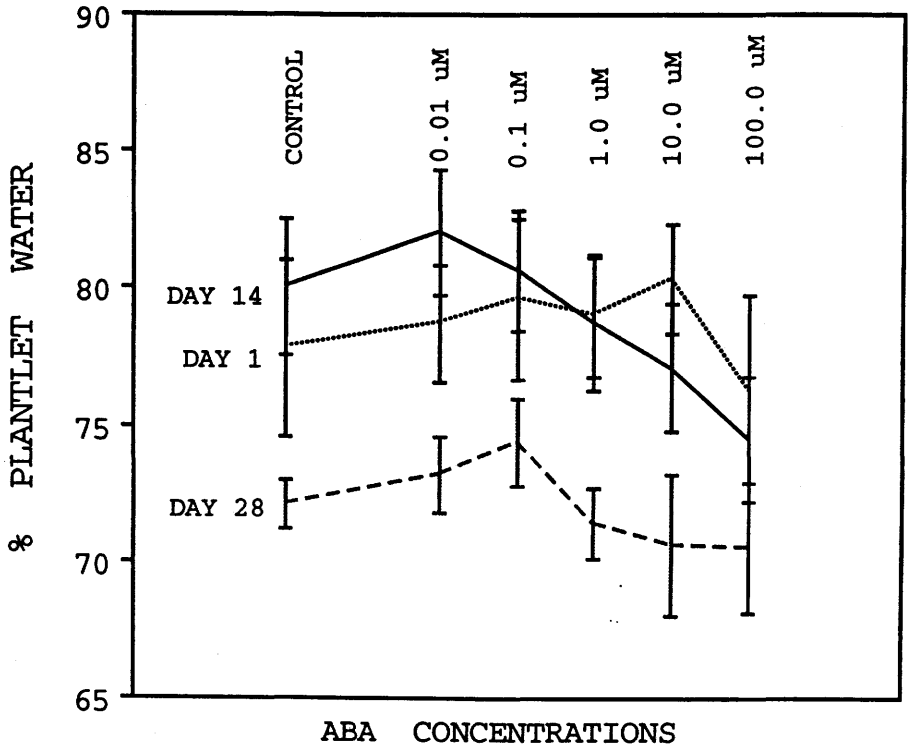

Figure 4.6. Percentage saskatoon berry plantlet water content following 1, 14 or 28 days treatment on culture media containing 0.0, 100.0, 10.0, 1.0, 0.1, 0.01 µM ABA under standard growing conditions.

4.3.6 The effect of ABA/BA/NAA concentration on plantlet hardiness

A significant effect on plantlet hardiness was seen in response to ABA and BA but not NAA (Table 4.9, Table 4.11). In all cases, addition of 50.0 μM ABA to growing medium elevated plantlet hardiness levels after 2 weeks under standard growing conditions (Tables 4.10. and 4.11.). Average LT_{50} values for all plantlets on media combinations containing ABA were -9.5°C, with plantlets on ABA-free media combinations exhibiting an average plantlet hardiness rating of -6.6°C.

Addition of BA to the growing medium either at the normal 11.0μM, or one-half the normal dose (5.5μM), always produced a significant reduction in plantlet hardiness levels (Table 4.10). The overall hardiness rating for plantlets grown on media combinations that did not include BA was -11.3°C. For plantlets on media combinations including BA at half the normal concentration, hardiness levels dropped to -6.9°C, while at the full concentration of BA, plantlet hardiness declined slightly to -5.9°C

NAA showed no effect on plantlet hardiness in any of the three concentrations tested.

Plantlets maintained on medium without hormones (000) expressed an LT_{50} of -10.5°C after 2 weeks of treatment (Table 4.11.). Addition of ABA to this medium (100)

resulted in a slight increase in hardiness to -12.7°C. Addition of a half the normal level of BA to 000 medium resulted in a significant drop in hardiness from -10.5°C to -5.3°C. A further addition of BA to the full normal level of this hormone did not significantly decrease hardiness over the half-strength treatments, decreasing hardiness by only an additional 0.6°C. A combination of BA and ABA treatments produced LT_{50}'s intermediate between individual applications of either hormone.

Table 4.9. Analysis of variance for the effect of media hormone on LT_{50} for saskatoon berry plantlets after a 2-week treatment period under standard growing conditions.

Source	df	SS	MS	F	p
ABA	1	111.227	111.227	46.83	0.000 **
BA	2	296.843	148.421	62.49	0.000 **
NAA	2	2.787	1.394	0.59	0.561 ns
ABA x BA	2	0.176	0.088	0.04	0.964 ns
ABA x NAA	2	0.676	0.338	0.14	0.868 ns
NAA x BA	4	3.130	0.782	0.33	0.856 ns
ABA x BA x NAA	4	10.796	2.699	1.14	0.355 ns
Error	36	85.500	2.375		
Total	53	511.134			

** = significant at p = 0.01; cv%= 19.14
ns = not significant at p = 0.05;
LSD for ABA effect = 0.848; LSD for BA effect = 1.038

Table 4.10. Overall average LT_{50} values for ABA- and BA-treated saskatoon berry plantlets after 2-weeks treatment at standard growing temperature and photoperiod.

BA (μM)	-ABA (0μM)	+ABA (50.0μM)	Mean
0	-9.9	-12.7	-11.3
5.5	-5.4	-8.4	-6.9
11.0	-4.4	-7.3	-5.9
Mean	-6.6	-9.5	

Table 4.11. LT_{50} values for saskatoon berry plantlets after 2-weeks on differing hormone combinations under standard growing conditions. Codes read in the order ABA/BA/NAA, where 1 = a normal concentration for that hormone, 5 = ½ the normal concentration and 0 = absence of that hormone.

ABA-Free Medium		ABA-Treatment Medium	
Code	LT_{50} (°C)	Code	LT_{50} (°C)
000	-10.5	100	-12.7
005	-10.3	105	-12.3
001	-9.0	101	-13.0
050	-5.3	150	-10.0
055	-5.3	155	-8.0
051	-5.7	151	-7.3
010	-4.7	110	-7.0
015	-4.3	115	-7.3
011	-4.3	111	-7.7

LSD for BA effect = 1.038; LSD for ABA effect = 0.848

4.3.7 Rate of plantlet deacclimation in response to media hormones (ABA/BA/NAA)

Plantlets acclimated on standard proliferation medium for 2 weeks under short day/low temperature conditions (8h/4°C) achieved hardiness levels of -16.5°C (Figure 4.7.). These plantlets all showed a rapid decrease in hardiness when transferred (not subcultured) to media of differing hormonal constitution and placed in standard growing conditions of long days/warm temperatures (16h/23°C). There was a strong hormone, time and hormone x time interaction (Table 4.12.).

The rate and magnitude of hardiness-loss was identical for all treatments for the first 3 days. All plantlets lost more than half of the initial hardiness level, declining in hardiness to -9.5 within 72 hours.

By 6 days, hardiness of plantlets on standard proliferation medium dropped to -5°C while the combined average LT_{50}'s of all other hormone treatments was -8°C. Furthermore, each individual treatment was significantly more hardy than those plantlets on proliferation medium. Also by day-6, plantlets on medium containing only ABA (100) had sustained a hardiness level of -9.5°C, making them significantly more hardy than all other treatments.

Those trends generated by 6 days under growth-inductive conditions showed no changes by 9 days. Throughout the

experiment, hormone-free medium (000), ABA-treatment medium (111) and charcoal-supplemented proliferation medium (011C) all demonstrated equal rates and magnitudes of hardiness loss (Figure 4.7.). None of the hormone treatments could maintain the initially high hardiness levels induced under short day/low temperature conditions.

Table 4.12. Analysis of variance for the effect of ABA, BA, NAA, hormone-free and charcoal-supplemented medium on the rate of hardiness-loss when hardened plantlets are placed under growth-inductive (16h/23°C) conditions.

Source	df	SS	MS	F	p
Hormone	4	25.400	6.350	8.19	0.000 **
Time	3	532.475	177.492	229.02	0.000 **
Interaction	12	27.400	2.283	2.95	0.016 *
Error	20	15.500	0.775		
Total	39	600.775			

** = significant at p = 0.01; * = significant at p = 0.05
cv% = 8.5; LSD for interaction = 1.836

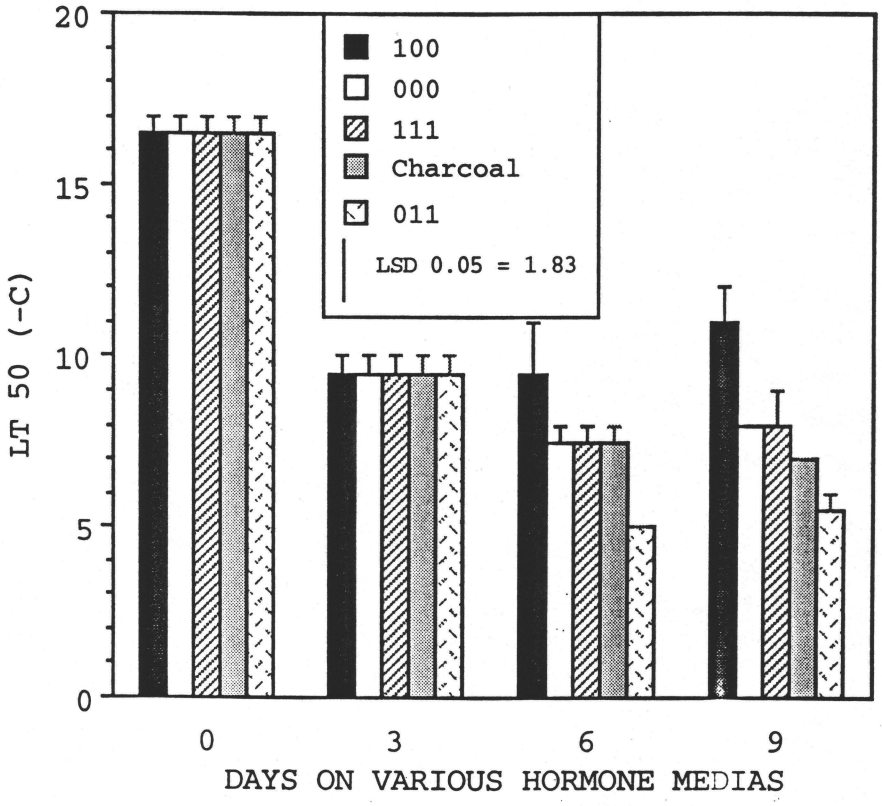

Figure 4.7. Hormonal effect on rate of saskatoon berry plantlet hardiness loss upon transfer from cold-inductive (8h/4°C) to growth-inductive (16h/23°C) growing conditions.

4.4 DISCUSSION

4.4.1 *In vitro* acclimation

There are numerous reports of *in vitro* hardening. Some genera which have been shown to harden *in vitro* in response to cold-acclimating conditions include: *Populus* (Sakai and Sugawara, 1973; Duda and Kacperska, 1983), *Chrysanthemum* (Bannier and Steponkus, 1976), *Solanum* spp. (Chen *et al.*, 1979; Van Swaaij, 1987), *Pinus* (Hellergren, 1983), *Malus* and *Amelanchier* (Caswell *et al.*, 1986) and *Rubus* (Zatylny *et al.* 1993). Advantages of *in vitro* hardening systems are said to include: the use of such systems for screening large populations for hardiness selection; facilitation of the uptake of compounds in investigation of physiological processes, prevention of rapid hormonal degradation by microbial action and presentation of a more controllable system.

In the present study, saskatoon berry plantlets significantly cold-hardened under short day/low temperature conditions (Fig 4.2.). The LT_{50} of control plantlets maintained a consistent -5°C throughout the 8-week test period. By contrast, cold-acclimated plantlet hardiness increased to -17°C in 2 weeks and by 8 weeks had reached -27°C. These hardiness levels are greater than observed by

Caswell *et al.* (1986).

Caswell *et al.* (1986) is particularly worthy of attention because it is based upon the exact cell line of *Amelanchier alnifolia* used throughout the *in vitro* portion of this thesis work, and the same growing medium. It has also been cited by subsequent authors as designating a suitable method for *in vitro* plantlet acclimation. In the 1986 publication, Caswell *et al.* described hardiness levels of control plantlets of -10.5°C, with cold acclimated plantlets showing maximum hardiness levels of -18.5°C following 8-weeks of cold-inductive treatment. This represents a hardiness elevation of cold-acclimated plantlets over controls of only 8°C, or a gain of only 1°C per week of hardening treatment. Control plantlets were also reported to have maintained hardiness levels of -10.5°C under standard growing conditions, although they were only tested for hardiness after 10-weeks in culture. These controls show approximately double the hardiness levels of control plantlets found in all cases throughout the present study (-5°C).

The high level of hardiness found by Caswell *et al.* (1986) in control cultures may have been a function of plantlet desiccation, nutrient or hormone depletion. Evidence indicates that desiccation leads to enhanced hardiness levels (Chen *et al.* 1975; Tyler *et al.* 1981). Similar evidence supports nutrient depletion as a factor in

moderate hardiness elevations (Christersson, 1975; Beattie and Flint, 1973; Pellet, 1973). In the present work, a small freezing trial was conducted using some very old (approximately 16 weeks) visibly desiccated cultures. The experiment was undertaken only as a preliminary step for determination of an appropriate temperature range if desiccation studies were conducted later. The experiment was neither blocked over time, nor designed with internal replicates. Desiccated plantlets were frozen both slowly and rapidly to -8, -10, -12 and -14°C. Slow freezing was conducted as previously described with nucleated culture tubes containing individual plantlets being held at -2°C overnight before the temperature was dropped at the standard rate of 2°C per hour for 1 hour at each of the test temperatures. The culture tubes for rapidly frozen plantlets were placed directly into the circulating bath at each of the test temperatures. These plantlets would have been experiencing temperatures dropping from standard growing conditions to each of the freezing temperatures within moments, imposing a sudden hard frost. They were left in the bath for the standard 60 minutes before being thawed in a refrigerator at 4°C overnight. Both rapid and slow freeze techniques produced identical results, with survival at -8, -10 and -12°C, and death at -14°C, establishing rough LT_{50} levels for desiccated plantlets around -13°C. With Caswell's 10-week-old plantlets,

desiccation may well have played a role in generating LT_{50} levels of -10.5°C.

A second small experiment performed during the course of this work also indicated that if the culture tubes were tightly closed, and sealed with Parafilm®, water content of the enclosed medium had not altered after 8 weeks under standard conditions. Water content of the medium had significantly decreased, if the caps were left slightly ajar as was recommended during the initial stages of this work. The means of tube closure and sealing was not specified by Caswell *et al.* (1986).

Although not directly measured, depletion of BA from the growing medium could also have been responsible for the elevated hardiness levels displayed by Caswell's controls at the end of the 10-week growing period. Caswell's reported hardiness levels of -10.5°C exactly matches the hardiness level of plantlets from hormone-free (000) medium frozen during the factorial work in the present study. For plantlets grown on all hormone compositions that did not include BA or ABA, the average overall LT_{50} was -9.9°C, corresponding quite closely to the -10.5°C hardiness levels of control plantlets in Caswell *et al.* (1986). Also, observation of plantlets used in the present study demonstrated that after 10 weeks in culture, plantlet branching had greatly slowed, or stopped, suggesting a possible decline in cytokinin levels. Since NAA was not

found to affect plantlet hardiness, depletion of NAA from the treatment medium after 10 weeks was most likely not a factor in the high hardiness levels of Caswell's control plantlets.

The list of the most obvious reasons for differences in plantlet hardiness between this work and that of Caswell *et al.* (1986) demonstrates that even when a well-specified, highly regulated system is being employed, numerous small variations in laboratory procedure can impose considerable differences in the responses of the plant material under study. A seemingly insignificant factor such as the degree to which a threaded cap for a culture tube is turned and tightened, may introduce variations in the growing environment which are not detected or accounted for, yet able to introduce significant inconsistency into results. This underscores the fact that the high degree of control offered by laboratory systems should not be accepted without cautious appreciation for the minute level of variation that can generate notable differences in response.

4.4.2 Photoperiod effect on plantlet hardiness

It is noteworthy that even under tissue culture conditions, a day/night photoperiod appears to have a direct influence on enhancing low temperature-induced cold hardiness. Plantlets grown with continuous lighting or

continuous darkness, were significantly less hardy than plantlets given short day treatments. There was no significant difference between short and long day treatments, however. Under natural conditions, photoperiod serves to mediate several plant responses including production of inhibitors (Hurst *et al.*, 1967; Fuchigami *et al.*, 1971), photosynthesis and transpiration. It is not clear if these or other factors are influencing the hardening response observed in these plantlets.

4.4.3 The effect of subculture on plantlet hardiness

Our studies show a significant subculture effect in enhancing hardiness (Figure 4.5.). Subculturing alone enhanced hardiness levels to -9°C in three days. Thereafter, hardiness levels began to decrease until control levels (-5°C) were reached between 7 and 10 days. The act of subculturing induces a wounding response. Wounding of plant material is known to induce elevated levels of ethylene (Boller, 1988) and ABA (Sánchez-Serrano *et al.*, 1991) leading to rapid alteration in gene expression and hardiness levels (Sánchez-Serrano *et al.*, 1991). As opposed to the Caswell *et al.* (1986) study, our acclimation system directly imposed the acclimating conditions of 8h/4°C immediately after subculture. Caswell *et al.* (1986) held plantlets for 4 weeks after subculture under positive

growing conditions to permit plantlets to resume growth before exposures to acclimating conditions were begun. Although plantlets were placed under the well-established dormancy/hardiness inductive regime of short day/warm temperatures (8h/20°C) for the last 2 of these 4 weeks, our studies indicate no growth cessation under these conditions. Thus, their plantlets were probably actively growing and had overcome any initial subculture effect by the time acclimation treatments were applied. The direct imposition of short day/low temperature conditions (8h/4°C) following subculture in this study appeared to capture the hardiness increases associated with subculture wounding, and permit plantlets to begin hardening without having to first slow growth in response to low temperatures.

The lack of response to a pre-exposure to short photoperiods *in vitro* demonstrates that consistent maximum levels of hardiness may not be established where controlled artificial conditions are imposed in an attempt to mimic natural conditions. Where growth is simply stopped by subculture shock, and plantlets placed directly into conditions of short day/low temperature (8h/4°C), hardening is much more rapid. These findings may also serve as a reminder that *in vitro* plantlets cannot be assumed to be acting in a fully comparable manner to fully functional plant material *in situ*. Hardening regimes produced using field-grown plants may not provide appropriate standards for

plantlets *in vitro*, and researchers wishing to develop *in vitro* systems to model the authentic plant environment must recognize that responses observed in culture do not necessarily parallel those seen in nature. The appealing level of control offered by laboratory systems may in fact introduce spurious factors to which plant material is reacting. Where researchers are trained to place an extremely high value on the control offered by laboratory systems, it perhaps becomes too easy to overlook the fact that a fully specified growth medium in combination with a highly precise climate-controlled growth chamber almost certainly does not succeed in producing responses fully analogous to those which would be seen in a natural environment. Highly specialised equipment and refined laboratory techniques are almost certainly better-able to deceive the researcher than they are to deceive the plant under investigation. If a basic aim of science is an enhanced and accurate understanding of nature, the value of natural systems which provide a lower degree of control, but a higher degree of authenticity in response should not be overlooked. In the process of scientific training, it is perhaps too easy to mistake precision for accuracy.

4.4.4 Hardening of plantlets in response to ABA

Since ABA has demonstrated its effectiveness in hardening plants cells and cultures under warm temperature

conditions, the hormone has proven beneficial in helping separate the effects of low temperature from other effects of acclimation. Studies examining ABA-induced cold hardiness on *in vitro* grown plantlet include reports on potato plantlets (Chen and Li, 1982), cell suspension cultures (Chen and Gusta, 1983; Tanino *et al.*, 1990). As well, whole plants have been found to harden in response to ABA application (Irving and Lanphear, 1968; Holubowicz and Boe, 1969; Rikin *et al.*, 1981).

In this study 2 weeks represented the minimum time under 100μM ABA treatment to induce a significant inhibition of plantlet growth without conspicuous toxicity effects. A subsequent concentration dose-response test determined 50μM to be the minimum ABA concentration required for growth inhibition evaluated on the basis of lateral bud break. Under these conditions, hardiness increased to a maximum of -9°C in 2 weeks as compared to the -5°C hardiness of control plantlets. When growth was inhibited by placement of plantlets on hormone-free (000) medium, hardiness levels increased from -5 to -10.5°C. This is close to the level under ABA treatment and may either indicate a non-specific hardiness increase due to growth cessation or a stress-induced endogenous ABA production under 000 medium conditions. Internal ABA levels were not measured in these experiments. The cytokinin BA plays a prominent role in promoting axillary bud break, and clearly inhibits ABA-

induced acclimation of plantlets in this system. The auxin NAA, also a growth promoting substance, has no effect on ABA-induced hardiness in this system. This could be due the relatively low concentration of NAA used throughout this work. At the standard concentrations used in this work, BA (11.0μM) and ABA (50.0μM) are two orders of magnitude greater than the standard level of NAA (0.54μM). While NAA did not show an effect on hardiness at this concentration, other work demonstrated that NAA was playing a role determining bud morphology.

The hardiness levels attained under ABA treatment at warm temperatures averaged -9.5°C for all treatments in which ABA was applied. These do not approach those of low-temperature-induced acclimation (-27°C). This indicates that ABA alone cannot replace the effect of low temperature acclimating processes, indicating that factors other than ABA level are involved in hardening. Furthermore, the deacclimation study indicated that although ABA slowed the rate of hardiness loss, it was unable to maintain the hardiness levels induced under low temperature acclimation. Plantlet hardiness quickly declined to the point expected for ABA-induced freezing tolerance under standard growing conditions.

In general, *in vitro* cell suspension cultures demonstrate a greater magnitude of hardening in response to ABA application. While bromegrass cells increase in

hardiness from -8°C to -25°C with ABA application (Chen and Gusta, 1983), plantlets tend to show less dramatic responses to media-applied ABA. Plantlets retain a higher order of organization than suspension cultures, and the relatively low hardiness increases observed in these studies may be a reflection of this higher order, where ABA uptake and responses between cells of suspension cultures and shoot cultures are different. For example, Tanino *et al.* (1990) demonstrated that ABA enhanced uptake of ^{14}C sucrose into acclimating bromegrass cell suspension cultures bathed in a 3% sucrose solution. Although plantlets in the present study were also maintained on a 3% sucrose solution, uptake to plantlet cells via the stem is likely much lower than the direct and relatively unrestricted flow possible to the cells of a suspension culture. Therefore it is improbable that tissue culture systems using plantlets, let alone cell suspension cultures, will replicate low temperature-induced cold hardening of whole plants.

Finally, *Amelanchier alnifolia* has a high genetic potential to acclimate to cold under natural conditions so the inability of ABA to cold harden these plantlets cannot be attributed to a inherent inability to cold acclimate.

5.0 SUMMARY AND CONCLUSIONS

The system used for these studies was able to demonstrate that the addition of 50μM ABA to tissue culture growing medium inhibited breaking of both tissue culture-grown and orchard-grown saskatoon berry axillary buds. In addition, ABA was found to enhance plantlet survival of freezing temperatures when added to medium containing BA. Finally, addition of ABA in specific combination with the cytokinin BA and the absence of the auxin NAA was found to induce a partial developmental shift away from photosynthetic leaf-form to scale leaf-form. A similar developmental shift is seen in naturally growing plants during bud formation and growth termination as an initial stage in the development of innately dormant buds.

An association between non-breakage of buds and plantlet survival of freezing was demonstrated where plantlets in active growth showed the lowest survival levels following freezing (-5°C). Where bud break was slowed or stopped by hormonal manipulation, wounding or low temperature, freezing survival of the plantlets increased. Bud break could be inhibited at warm temperatures by addition of ABA to proliferation medium (111), elimination of hormones (mainly BA) from the medium (000) or by wounding. These means of inhibiting bud break increased freezing survival levels to -10°C (±2°C). Low temperatures

(4°C) offered the most effective means of both inhibiting bud break and elevating freezing survival. Even on proliferation medium (011) where bud break is being strongly promoted by hormones, no bud swelling or burst was observed when plantlets were held at low temperatures. These conditions also led to the greatest enhancement of freeze-tolerance levels (-27°C).

Buds collected from plants growing under orchard conditions demonstrated increasing levels of endogenously controlled dormancy during autumn, with levels reaching a maximum between the beginning of September and end of November. The level of innate rest decreased steadily from December to May. Collected buds placed on culture medium demonstrated similar trends in endogenously controlled inhibitor levels. Where bud scales were not removed from collected material, bud survival in culture was greatly reduced.

Buds of plantlets grown under tissue culture conditions were never found to develop an innate dormancy, or if the condition was present, it was not detected. Because normal growth for these plantlets was dependent upon media-supplied BA and NAA, the hormone-free medium on which bud break was determined did not provide a appropriately neutral condition under which to determine bud break.

Throughout this thesis, the words *plant* and *plantlet* were kept scrupulously separated so that tissue culture-

grown specimens were referred to only as plantlets, while saskatoon berries growing under nearly natural (orchard) conditions were referred to as *plants*. Freezing survival rates among tissue culture plantlets were reported throughout this work by stating that saskatoon berry plantlets were hardy to -*X*°C.

Without looking to the published literature on saskatoon berry hardiness, where buds were subjected to precise laboratory procedures for determination of exact hardiness levels, it is still reasonable to state that naturally existing saskatoon berry plants are reliably hardy to at least -40°C. Ample evidence for this statement exists in the name of this city, personal observation of winter temperatures, and springtime bud break of overwintered saskatoon berry plants.

Culture systems are often used as model systems for application of treatments permitting an examination of hardening mechanisms. The systems are useful, however, only if they are able to reliably mimic some aspect of natural response. During the course of this work, several thousand saskatoon berry plantlets were frozen and assessed for regrowth. Of these plantlets, hundreds were acclimated to levels well beyond the -5°C level typical of controls. No acclimated plantlet ever developed a terminal bud, but all had numerous axillary buds. In all cases, plantlet regrowth following freezing to extreme temperatures was entirely a

function of survival in the apical meristem. Hardening of axillary buds and vascular tissue above standard control levels was unseen. Reports of hardiness for these plantlets should therefore be specified in such a manner that the tendency to misinterpret *in vitro* freeze-survival as equivalent to the condition of *in situ* survival found in overwintering plants is avoided. Following a typical Saskatchewan winter, field-grown saskatoon berry plants emerge with essentially all vascular tissue intact; with axillary and terminal buds poised for rapid growth. This growth resumption is driven by the plants' internal levels of hormone, moisture and carbohydrate. Following freezing, tissue culture plantlets in this study were essentially bathed in a warm, moist, sterile, sucrose-saturated environment, heavily laden with growth promoters. If a report from this work were to be published stating simply that plants were hardy to -27°C, it may create a far more sweeping impression of plantlet freezing tolerance than a more specific report stating that plantlet apical meristems were able to regrow in tissue culture following a single controlled freeze to -27°C. For the sake of brevity, however, most authors report only statements similar to the first. Here, concise wording also at least partially screens the high degree of artificiality in the findings from view. Without careful reporting, plantlets in which a small number of surviving cells within the non-terminated

apex can be nursed back into health under conditions which are not unlike intensive care, can become identified with fully autonomous, highly-adaptive plants developing the ability to survive several consecutive months of extremely low temperature before resuming growth from all meristems in spring.

No condition of innate dormancy was detected in the axillary buds of plantlets, yet 50% of fully hardened plantlets were able to recover from freezing to -27°C. If the conclusion were drawn from this observation that dormancy and hardiness are unrelated, and careful attention were not payed to emphasizing the fact that this conclusion was derived from a model system, one natural progression may then be for readers to generalize that fully autonomous plants do not require dormancy for winter survival. Such a conclusion would be based upon non-appreciation for plant/plantlet differences and acceptance of *in situ* and *in vitro* survival ratings described by the single term *hardiness*. While the system was able to demonstrate a five-fold increase in the level to which plantlets could be frozen and recover. In no case, did it demonstrate survival of vascular tissue or buds at temperatures much below control levels. The system did not, therefore demonstrate survival effects adequately similar to what plants under natural conditions would rely upon during winter survival. Also, because plantlets required BA and NAA to demonstrate

normal growth in culture, the hormone-free medium (000) used for bud break determination did not represent a neutral means by which to examine time to bud break following hormonal bud inhibition. Hormone-free medium imposed an inhibition on its own. The system was therefore not particularly well-suited to examination of cold hardiness or dormancy. The strongest point in favour of this system was the means it provided of generating a partial transition from summer to winter bud form. Here, the high level of control permitted hormonal manipulation to mimic an aspect of dormancy responses which would be observed in nature. Here too, however, one must be careful to specify that these affected buds demonstrated only a partial alteration in appearance, and were never found to possess an innate condition of dormancy.

6.0 FINAL THOUGHTS

It is essential that all scientific findings be considered with consideration given to the contradictions we impose in our systems of study. No matter what system is devised, it almost certainly imposes some well-defined and some barely intelligible differences on the plant material. Use of the same word to report adaptiveness to winter survival and the ability to recover from a single controlled freezing event conducted under highly artificial conditions, perceptually diminishes the degree to which these processes may differ. The terms we select in reporting findings also, therefore, demonstrate the level of our willingness to embrace simple systems as faithful representations of a much more complex natural world. An integral part of scientific training is the development of an appreciation for carefully controlled experiments where a single factor can be precisely altered for determination of an exact effect. Yet, if the findings from even the most carefully designed and managed experiments are not reported with an equal vigilance payed to the precision in the language of reporting, impressions drawn within the reading audience can be as inaccurate as they would be from a poorly designed experiment. I believe that responsible reporting of this study requires a clear distinction be drawn between these

findings and what one would find under natural conditions.

In asking research questions, and reporting findings, where we do and do not choose to draw distinctions may reveal as much about ourselves as it does the plants that we study. Although the objectivity of science is often suggested as providing a neutral stance from which to determine the truths which are supposed to grant us a richer sense of what reality is, our pre-existing notions of reality undoubtedly play a role in directing both the questions we ask, and the means by which we seek to answer them. If this same study were conducted without the inclusion of the field-grown buds, it is somewhat dismaying but not unreasonable to suggest that a student could have completed this project, graduated and published papers on this subject without ever having seen a saskatoon berry plant growing under natural conditions. Investigations into many aspects of plant science have reached a point where it is possible for a student to complete postgraduate study working entirely within an artificial system. When a researcher accepts an artificial system as a faithful representation of whole plants, simple basic differences can begin to go unappreciated. In this study, the difference between a forceful springtime flush of growth and a feebly emerging single leaf from a moribund bud were reported as the same thing. Both were described as bud break. Having never seen authentic spring bud break, a student unfamiliar

with saskatoon berry plants would perhaps not appreciate the difference between bud break seen under artificial conditions and seen *in situ*. It is possible that a student could accept this *in vitro* system as a fully legitimate means by which to identify ABA's effect on dormancy and hardening. Such an acceptance, however, would demonstrate a level of detachment between researcher and plant which could prove problematic when the researcher is later called upon to interpret the findings within the context of the natural world.

The elegant hormonal balances which direct all plant development are not often witnessed by students first-hand. For me, the most personally satisfying aspect of this system was its ability to demonstrate that an inhibitor/promoter balance at least partially generated a shift in development similar to what is seen as buds on this species mature and prepare for winter. Among the plantlets being assessed for bud break, in the very first group to be evaluated on ABA-supplemented medium, a single axillary bud on a single plantlet appeared somehow similar to a dormant winter bud for this species. On the first of many data sheets used in preparation of this work was written the comment, "one very good looking bud." That bud's rate of breaking was similar to the others, but its appearance suggested a difference. Following that single initial observation, two years of work passed with thousands of plantlets, and tens of thousands of

axillary buds being observed. During this time, no other such buds were seen until the condition again appeared during factorial studies.

My earlier suggestion that it is possible to study plant dormancy and hardiness without seeing a natural representative of the plant upon which ones conclusions are to be based, leads to the question: If a student unfamiliar with natural saskatoon berry plants were to have completed this work, would the differences noted in the first modified bud, or the differences seen during factorial studies have been recognized as somehow associated with how plants appear during dormancy? At first this question may seem absurd. Unfortunately, however, it does not strike me as an impossibility that a student adhering strictly to a prescribed research method, focusing only on determination of days to bud break and LT_{50} scores for replicated sets of ABA-treated plantlets, having never worked with, examined, touched or experienced the plant in its natural condition, could overlook the significance in altered bud appearance. The ability to simply notice a bud's appearance receives very little attention in the current dormancy literature. In fact, throughout the Degree Growth Model of Fuchigami *et al.*, (1982) the word *bud* is used in a fully interchangeable manner with the word *shoot*. While it would not be unreasonable to base ones work upon the comprehensive Fuchigami model of dormancy, employing this model as a

foundation for ones work, a student would read that, "Growing buds characteristically elongate rapidly during the spring flush of growth, and more slowly as the summer progresses, [various factors] eventually cause the bud to stop growing. When a bud stops growing it is dormant." In describing the maturity induction point, the model states, "In dogwood, growth of buds exposed to short days slows and stops. Growth of buds exposed to long days continues." If a saskatoon berry plant has never actually been observed, it is not unreasonable for a person to conceive of bud growth and development as entirely an on/off process. Appreciation for differences seen between buds at different points in their seasonal development requires more than simple precise attention to hormone levels. Where students are taught to trust only those well-defined systems which permit the (appearance of) complete control, where an appreciation for the unaffected organism of study is neither expected nor required, it can become too easy to overlook the degree to which our trust in simple systems isolates us from the world where plants and people do in fact coincide.

Rapid advancements in technology have provided plant scientists with the means by which to view and quantify aspects of plant growth and function that would have been almost impossible for earlier researchers to imagine. One seldom-considered consequence of the arrival of highly technical and precise machinery, however, has been the

further isolation of plant scientists from plants. Questions and problems are neither generated nor solved by machines. Both questions and solutions are generated in our minds. Machines (and model systems) simply provide us a means by which to confirm our solution. Certainly, many things cannot be seen or experienced without technological assistance. As students, however, it is often far easier to transcribe a highly precise, digitally-displayed readout from a machine, than to accurately translate our impressions into meaningful descriptions of what we observe, sense or feel to be happening. Some would say that feelings should not play a role in science. Still, without a deep and genuinely-felt sensitivity to the organism of study, I believe our investigations cannot become much more than vast inventories of cold and largely incomprehensible facts on subjects which may neither inspire our interest nor arouse our enthusiasm.

The questions we choose to ask, and the means by which we choose to ask them possibly reveals as much about ourselves as the things we attempt to study. A masters thesis almost invariably ends with a list of undetermined factors and the highly predictable and dutifully-polite statement that more research is necessary. Before I could truthfully state that I consider more research to be necessary in this or many other fields, I would have to first suggest that each person involved in a research career

carefully contemplate exactly why it is that he or she is addressing their particular research questions. Once our personal reasons for focusing on our questions are better understood, consideration should be given to the role our research has in a broader context. As individuals working at what is suggested to be the forefront of human knowledge and understanding, researchers play a primary role in directing the prevalent attitudes among non-scientific members of society. Because this little-considered leadership role which all researchers play influences the widespread conceptions of our world, we must continually be re-evaluating our work in terms of both our basic personal motivations and the influence we are exerting in a more universal sense. In both performing research, and training successive generations of researchers, we must begin to acknowledge a deeper sense of obligation to the broader role our work plays and the wide-ranging impact our work can have at a variety of levels.

Where science involves the continual description of facts with little consideration payed to interpretation, our role becomes little more than that of a technician. We may possess abilities which make us rare and highly specialized, but where little in-depth consideration is given to the facts we generate, our technician status remains. While some facts require a greater technical expertise, the production of factual information is not an especially rare

talent. Almost anyone can generate a valid, scientifically proven fact. It is only at the level of interpretation where a more remarkable ability is demonstrated.

If we consider a convex lens and two parallel rays of light, it is easy to visualize the rays passing though the lens, being refracted toward one another, and eventually converging at the focal point. Similarly, in experimental science, two factors are brought together in such a way that their combined effect becomes distinct. Their relationship is brought into sharp focus. At the precise point of focus, clear images permit distinct conclusions to be drawn with outright certainty. For scientists as technicians, the focal point, or place where factual certainty is possible, represents the end-point of an investigation.

As interpretive scientists, however, we must appreciate that a focal point is not a point of closure, but merely a point of intersection from which the rays continue in a constantly divergent pattern. We must look beyond our work. We must look beyond the focal points of various known facts. Beyond the focus, clarity declines, and with it fades the reassuring sense of comfort we all derive from scientific certainty. We must recognize that as the two rays which emerge from this single fact continue to diverge, they quickly begin to intersect with innumerable rays of innumerable other focal points where some fact has become known. Here the significance of a broadly-based knowledge

and deeper sense of understanding becomes most evident. If we have been taught to orient ourselves by only the two factors which we combined to form our single perfectly-known speck of scientific truth, advancement beyond our point of focus can suddenly leave us with the very uncomfortable sensation that what was earlier a clear understanding, has now suddenly turned upside down. It is beyond the point of focused experimental certainty where a broadly based knowledge, combined with a deep and sincerely-felt sense of trust for ones own feelings grants the interpretive scientist the courage to venture beyond the certainty of individual unconnected facts. Here, such a person can begin to speculate on how hundreds of factors interact as a whole. Such speculation can never be based on a single sharply-focused view, but must rely upon a slightly blurred, but broader vision of many intellectually known facts in combination with a keen and trusted awareness of ones feelings and perceptions.

If students being trained as scientists are not expected to feel a deep sense of connection with the their work; if training includes subtle suggestions that we need not care for or notice the actual organism we study; if training involves only a routine application of the scientific method; if we are taught only to accept what we can isolate, immobilize and measure; if we are taught to trust only rational truth, and doubt visceral truths; the

courage to trust ourselves and step beyond the comfortable level of scientific certainty cannot be expected to develop. To sense nature's intricate harmony, and attempt to communicate its essence requires that science be learned in connection with a passionate and personal sense of connection. Until we recognize this need for passion, we will continue to produce only technicians.

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