PHYSIOLOGY AND DORMANCY OF THE AQUATIC ANGIOSPERM *POTAMOGETON CRISPUS* L. TURIONS

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Dedication

This dissertation is dedicated to my family; Joel, Otto, Octavia, and Opal. Their patience and tolerance these last five years has been appreciated. Also, I would like to dedicate this work to Christ. This project would not have gotten done without divine intervention.

Comprehensive Abstract

Vegetative buds (turions) are the major source of propagation for the aquatic invasive angiosperm, *Potamogeton crispus* L. (Potamogetonaceae). An understanding of the regulation of turion dormancy could lead to better methods of population control. The majority of *Potamogeton crispus* turions remain dormant over the summer and sprout in the autumn, while a small subset of turions remain dormant for an unspecified time. Hormonal control of dormancy in aquatic plant vegetative propagules is not well understood. For this study turions were divided into two different age groups, newly formed (current season) and older than one year (overwintered). The effect of varying light durations and temperatures on sprouting was monitored in these different groups. Non-structural carbohydrates, photosynthesis, and aerobic respiration were measured to determine metabolic activity. We also measured abscisic acid concentrations and sprouting levels in turions that were exposed to various hormones, temperatures, and light durations to elucidate hormonal control of dormancy. Current season turions were found to sprout mainly in response to day length and were metabolically active over a 6 week period. They are in a semi-dormant state and 60-70% will sprout in the autumn. The remaining current season turions presumably go into a state of deep dormancy and remain dormant over the winter. Turions that have overwintered are not photosynthetically active, have stable carbohydrate levels, and can remain dormant but viable for several years. Under laboratory conditions, they sprouted mainly in response to an increase in water temperature. These different age groups correspond to different turion physiological states and can explain sprouting variability recorded by other

researchers. Current season turions produce and sprout in response to changes in ABA levels. A reduction of ABA in new turions is correlated with the breaking of dormancy. Overwintered turions do not sprout in response to ABA or GA changes. The results indicate two different pathways utilized by *P. crispus* turions to maintain and break dormancy.

Table of Contents

List of Tables

Chapter 2:

Chapter 3:

Appendix A:

Appendix B:

Appendix C:

List of Figures

Chapter 1:

Chapter 2:

Prologue

Aquatic angiosperms propagate primarily by asexual reproduction (Sculthorpe 1967). A subset of aquatic angiosperm species has the potential to become invasive in their non-native ranges. The invasive potential of a species is partially related to a species ability to reproduce quickly. A better understanding of the physiology of aquatic vegetative propagules could aid managers in promoting or eliminating specific aquatic species. In this dissertation, I have investigated factors that impact dormancy and sprouting in the vegetative propagules (turions) of the submersed aquatic angiosperm, *Potamogeton crispus* L.

CHAPTER 1 – Review of Vegetative Reproduction in Aquatic Angiosperms

In Chapter One, I review the physiology of aquatic angiosperm vegetative propagules commonly found in freshwater ecosystems. All aquatic vegetative propagules are modified stems; however they vary in morphology and physiology. Unlike the comprehensive studies on potato tuber morphology and physiology, aquatic vegetative propagules have been studied sporadically with contradictory results. I discuss general characteristics of five types of aquatic vegetative propagules and clarify common terminology within the aquatic plant reproductive field. The importance of propagule ecology under various conditions is addressed as well as details of the abiotic and hormonal controls of vegetative propagule development and dormancy. Turions, a type of vegetative propagule, have been studied more extensively than other propagules and are given special consideration within this review.

My research focuses on turions of the North American invasive *Potamogeton crispus*. This species of aquatic angiosperm has a unique life cycle that contributes to its invasive ability. Therefore, I introduce the species life cycle, general physiology, and invasive potential to better aid the reader in understanding why my specific research was conducted.

Chapter One will be submitted as an aquatic propagule review article with the addition of a table that illustrates species, propagule type, and dormancy duration if known. Additional information on propagules other than turions will be highlighted and the *P. crispus* physiology will be removed.

CHAPTER 2 – Dormancy Regulation in *Potamogeton crispus* Turions

In Chapter Two I present my research into turion physiology and abiotic factors that trigger dormancy. Turions were separated into two groups based on age, newly formed (Current Season) and older than one year (Overwintered). Both groups were monitored under various light durations and temperatures, and differences in sprouting were described. The glucose and starch contents were determined. Photosynthesis and respiration were also recorded for both types of turions. Newly formed turions were found to be metabolically active and sprout in response to light duration changes, while older turions were not found to be metabolically active and sprouted in response to an increase in temperature.

Chapter Two was submitted to a journal, Aquatic Botany, in its current form and is still under review.

CHAPTER 3 – Hormonal Regulation of Dormancy in *Potamogeton crispus*

In Chapter Three, I continue my investigation into turion dormancy by attempting to determine the hormonal control of dormancy in the two different types of turions. Abscisic acid (ABA) was measured by GC-MS in the adult plant and axillary buds during different developmental time points of turion formation. The results indicated a correlation between the initiation of turion formation and an increase in ABA concentration, suggesting that ABA is required for turion formation and maturation. Dormancy in newly formed turions is most likely maintained by ABA based on a correlation between a reduction of internal ABA concentrations and increased sprouting. Older turions do not respond to endogenous ABA changes nor do they sprout in response gibberellins.

Chapter Three will be submitted for publication.

CHAPTER 4- Conclusions and Future Directions

I conclude the dissertation will a summary of key findings from Chapters Two and Three. The possible overall impacts of my research on the field of aquatic plant management are discussed. Alternative hypothesizes and experiments are addressed to further the understanding of dormancy of *Potamogeton crispus* turions.

Chapter 1 - Asexual Reproduction in Freshwater Aquatic Angiosperms

Aquatic plant communities are both a curse and a blessing to human activities. Both submersed and emergent vegetation prevent erosion of shorelines (Coops et al. 1996) and canal systems (Boedeltje et al. 2003) by breaking up wave action. Additionally, their presence in wetlands of river basins control flooding of homes and businesses (Emmett et al. 1996). Aquatic communities also act as a natural water filtration system and can play a part in phytoremediation of environmental contamination from industrial waste water (Srivastava et al. 2011; Xue et al. 2010) Submersed plants uptake and sequester heavy metals such as cadmium from the surrounding water column (Emmett et al. 1996; Sivaci et al. 2008) and indirectly improve water clarity (Bakker et al. 2010; Heuschele 2006). Conversely, aquatic plants propensity for rapid and dense growth has made them a nuisance to anthropogenic activities. Heavy growth can block navigational channels (Murphy 1988) and impede recreation costing billions of dollars to control (Knight and Hauxwell 2009). Exotic aquatics can change the plant communities and lead to a loss of diversity (Knight and Hauxwell 2009).

Overall aquatic plant species are very cosmopolitan. Sculthorpe (1967) identified that 60% of know aquatic angiosperms are found on multiple continents, for example *Potamogeton crispus* is native throughout Asia, Europe and portions of Africa. Very few species of aquatic plants are endemic to a specific area; even then, the ranges are large compared to terrestrial plants (Camenisch and Cook 1996). The large ranges of these plants are attributed to multiple reasons (Santamaría 2002). One hypothesis is that these

plants have reproductive plasticity allowing for quick colonization and long term maintenance (Santamaría 2002).

Plants have the ability to reproduce asexually. This is achieved by meristematic cells capable of cellular differentiation present in many plant tissues such as modified leaves (Herrera and Nassar 2009) and stems (Spencer et al. 2000). Unlike sexual reproduction, asexual reproduction involves the replication of the parent chromosomes and thus is an identical copy of its progenitor. In angiosperms asexual reproduction is often accomplished through the production of runners, rhizome, sucker, tuber, offset or bulbs depending on the species. Collectively these structures are referred to as vegetative propagules and are generally derived from stems.

Although freshwater aquatic plants represent only 1-2% of known angiosperms (Sculthorpe 1967), they generally have more asexual verses sexual reproduction compared to terrestrial angiosperms (Barrat-Segretain et al. 1998; Grace 1993; Sculthorpe 1967). The abundance of vegetative propagules in aquatic plant communities has been shown to aid in successful colonization, establishment, and maintenance (Barrat-Segretain et al. 1998; Grace 1993). Vegetative propagation is important in an aquatic environment where sexual reproduction can be difficult because of the inherent dynamics of growing in a liquid environment and compounded by a lack of reliable pollenating vectors.

Understanding the physiology and dormancy mechanisms of an aquatic angiosperm's primary source of reproduction, vegetative propagules, would aid managers in promoting or inhibiting plant growth of a community depending on the goal of the

project. This review explains vegetative propagule physiology and dormancy in some well-known freshwater angiosperms.

General Characteristics of Vegetative Propagules

The terminology used to differentiate aquatic vegetative propagules in the literature can be confusing. For instance, Steward (1969) identifies both above and below ground reproductive structures of *Hydrilla spp.* as turions, while Sastroutomo (1980) indicates that *Hydrilla spp.* produces both tubers (below ground) and turions (above ground). The term hibernaculum has also been used to define tubers, turions, or dormant buds (Best and Soekarjo 1976; Haag 1979; Sculthorpe 1967). For the purposes of this review the types of propagules are defined as follows: Tubers are highly compressed stems containing starch and multiple buds that form below the soil substrate. Turions are also modified stems, but they develop in the leaf axils and at the apical tip. The stem becomes swollen with starch and separate from the parental plant. If leaf structures are still attached to the turions, the starch content is higher in modified structures compared to actively growing leaves. Dormant buds, also known as winter buds, are similar to turions. They are modified stems that develop at the apical tips, and generally do not separate from the parental plant. The buds are composed of tightly packed leaves with very short internodes. The tight packing of the leaves protect the apical meristem from desiccation and freezing. The buds do not contain high levels of starch compared to turions (Spencer and Anderson 1987). Fragments contain at least one node from a stem or root, but are usually comprised of multiple nodes. Fragmentation

can occur with above ground stems as in *Myriophyllum spicatum* (Nichols and Shaw 1986), modified stems like the pseudostolons of *Luronium natans* (Nielsen et al. 2006) or underground stems like the rhizomes of *Nuphar spp.* (Barrat-Segretain 1996).

The types of vegetative propagules found are species specific. Some species, like *Hydrilla spp.*, have multiple types of vegetative propagules (Netherland 1997), while other species have only one type (Catling and Dobson 1985). Propagules, such as turions and tubers, are used for continued population establishment after poor environmental conditions have occurred, such as overwintering and low nutrient ability (Appenroth 2010; Wehrmeister and Stuckey 1992), while fragments are used for immediate colonization.

Most aquatic vegetative propagules are either neutrally buoyant or sink. Buoyancy is thought to be due to the starch content within the propagule (Woolf and Madsen 2003). However, the increase of intercellular lacuna (gas spaces) coincides with turion floating in *Myriophyllum verticillatum* (Weber and Noodén 2005). Changes in internal pressure cause a propagule to float or sink. Sinking into soil substrate may be a mechanism for maintaining propagule dormancy and/ or protection from freezing. Microorganisms can contribute to a soil's ability to maintain warmer temperatures then the surrounding water (Barko et al. 1991; Smith 1979). Propagules are then exposed to various types of microbes both beneficial and pathogenic. The various levels of phenols and tannins found in propagules (Spencer and Ksander 1994) of some species are speculated to be a method of propagule defense. Vegetative propagules that float or are suspended by stalks ,such as *Utricularia vulgaris* turions, freeze in or on top of the ice.

These propagules use the insulating features of snow and the suns radiant heat to prevent the temperature from becoming too extreme for extended period of time (Winston and Gorham 1979).

Ecology

Environmental cues influence selection of which reproductive strategy, sexual or asexual, will yield the most reproductive success (Obeso 2002). The allocation of resources to favor a specific method has been modeled as a tradeoff between seed production and vegetative propagules (Wang et al. 2010). Within the model seeds are produced when resources, such as nitrogen and phosphorus, are low or at toxic levels, while at all other times vegetative reproduction occurs (Wang et al. 2010). However most aquatic angiosperms utilize both asexual propagation and limited sexual reproduction (Barrett et al. 1993) irrespective of the nutrients present compared to terrestrial angiosperms. The lack of sexual success might be linked to unsuitable environmental conditions for seed production, germination and seedling establishment (Barrett et al. 1993). While seed production has been recorded for all known *Potamogeton* species, observations of germinating seeds have been limited to the laboratory (Boedeltje et al. 2003; Combroux and Bornette 2004; Yeo 1966). Asexual reproduction might also contribute to the limited success of sexual reproduction by limiting the exposure of ovules to sexually compatible pollen (Charpentier et al. 2000). *Potamogeton crispus* inflorescences produce four ovules (Zhang et al. 2009) but many

times not all of the ovules are fertilized (Rogers and Breen 1980; Yeo 1966), possibly due to sexual incompatibility.

Combroux and Bronette (2004) investigated reproductive plasticity by comparing vegetative propagules and seeds in the substrate (propagule bank) to actively growing plant populations in various types of disturbed river systems. They found low seed presence within sites that flooded compared to sites with stable water levels. They concluded that the lack of seeds indicated large germination events were being used to maintain populations. Stem fragments present were significantly higher in the sites with stable water levels than the flooded sites, indicating that asexual reproduction was the major driver in maintaining plant populations. The authors concluded that disturbance dictated which type of primary reproduction was selected. The propagule banks were representative of the emergent species present, but not the submersed species. They concluded that the submersed species must be reproducing from unspecified fragments that were transported downstream. Boedeltje and coworkers (2003) also found little similarity between submerged vegetation and the propagule bank within a canal system. The low levels of seeds and high levels of plant fragments indicate that submerged plants either do not produce seeds and propagules, or that the seeds and propagules are utilized at different rates.

The plasticity of regeneration of submersed plants is a tradeoff between colonization and establishment. After fragmentation aquatic plants either develop roots rapidly and establish themselves or develop propagules that can be dispersed (Barrat-Segretain et al. 1998). Not all propagules have equal competitive ability. While *Hydrilla*

verticillata turions and tubers are anatomically similar (Pieterse 1981), turions are smaller and naturally buoyant. They sprout faster than tubers allowing them to compete with other aquatic plants for colonization. *H. verticillata* tubers establish slower than turions, but produce more robust adults that in turn produce more turions and tubers for the next growing season, ensuring long term establishment (Spencer and Rejmánek 1989).

Timing of vegetative dispersal also plays a part in plasticity. Establishment of *Luronium natans* fragments are significantly increased in the autumn compared to fragmentation that occurs in the spring (Nielsen et al. 2006), whereas *Elodea* fragments equally establish as long as temperatures are within growth limit (Haag 1979).

Propagule Development

Various abiotic conditions trigger vegetative propagule formation such as temperature changes. *Spirodela sp.* forms turions in response to low temperatures irrespective of light duration (Appenroth 2003; Appenroth 2010). The aquatic annual *Najas marina* also forms turions when temperatures drop below a particular threshold (Agami et al. 1986). This method of propagule initiation allows plants to remain photosynthetically active for the greatest amount of time while water temperatures are warm enough for enzymatic activity. Plants that utilize temperature as a propagule initiation signal must form structures quickly. Both turions of *N. marina* and *Spirodela sp.* require little morphological modification to form vegetative propagules.

Photoperiod regulates tuber and dormant bud formation in most *Potamogeton spp.* Flowering time of some aquatic plants is also regulated by photoperiod (Chambers et al.

1985; Jian et al. 2003; Spencer 1987; Spencer and Anderson 1987) and is linked to propagule formation (Winston and Gorham 1979; Woolf and Madsen 2003) or fragmentation (Nichols and Shaw 1986). Short days in autumn induce some *Potamogeton spp.* to form structures to overwinter. Due to the unique life cycle in *P. crispus,* in temperate regions, long days in spring induce formation of turions that over summer and sprout in the autumn (Chambers et al. 1985; Jian et al. 2003). Photoperiod remains constant allowing these plants to evolve highly modified turions (i.e. *P. crispus*). Utilizing photoperiod as a dormancy signal may impact survival by vegetative propagules in years when temperatures are unseasonal cold. When temperature changes initiate plant senescence, (Anderson 1982; Getsinger and Dillon 1984) propagules may not be fully formed or matured, leading to reduced viability.

Myriophyllum verticillatum and *Hydrilla spp.* are examples of aquatic plants that utilize both photoperiod and temperature to regulate dormant propagule formation (Pieterse 1981; Weber and Nooden 1976a). This method prevents seasonal confusion that may cause propagule formation in early spring, while allowing maximum growth in autumn.

Within *Potamogeton crispus,* turion formation begins when axillary buds of the plant break dormancy and elongate (Wehrmeister and Stuckey 1992) (Figure 1.1), while the apical meristem forms an inflorescence (Woolf and Madsen 2003). Elongated axillary buds accumulate starch causing the stems and basal portion of the leaves to thicken (Figure 1.2). The thin leaf tips are retained and continue to be photosynthetically active. The buds on the lower part of the plant mature into turions at a faster rate than

those closer to the water surface. When axillary buds have completely thickened they begin maturation and are considered turions (Figure 1.3). Each turion contains 5 – 7 buds; before maturation is complete the unmodified leaf tips decay and disappear (Wehrmeister and Stuckey 1992). The attachment point between the turion and the parental plant weakens. Turions will either dehisce from the parental plant at the node or remain attached and fall to the substrate during senescence (Figure 1.4).

Data regarding the hormonal controls for aquatic vegetative propagule formation are limited. Abscisic acid (ABA) will induce turion formation of *Spirodela sp.* (Smart and Trewavas 1983), *Lemna minor* (Steward 1969), and *Myriophyllum verticillatum* (Weber and Nooden 1976a). ABA is known to promote tuber production in potatoes (Suttle 2004) and may also control *Hydrilla spp.* tuber production (Klaine and Ward 1984; MacDonald et al. 1993; MacDonald et al. 2008). We measured 3-fold increase of ABA coinciding with the initiation of turion formation in *P. crispus* (Chapter 3). Wang and coworkers (2012) also suggest that ABA may play a role in turion formation when they found that GA, an ABA antagonist, inhibits turion formation in *P. crispus*.

Cytokinin prevents (Klaine 1986; Klaine and Ward 1984; Weber and Nooden 1976a) or retards (Wang et al. 2012) turion formation depending on species. In *P. crispus* turions, cytokinin changes turion morphology by inhibiting storage of starch (Wang et al. 2012). Turion production is correlated with an increase in carbohydrate accumulation while growth is correlated with the breakdown of starch (McLaren and Smith 1976). The link between cytokinin and starch accumulation suggests that propagule formation requires starch storage and is regulated by hormonal control.

Dormancy

Dormancy can be broken up into three different stages, pre-dormancy, imposed, and innate dormancy. Each stage has unique hormonal properties. At this time it is unknown if propagules move from one type of dormancy to another sequentially or if dormancy type is predetermined at formation.

In pre-dormancy vegetative propagules are held dormant by outside factors such as parental control. This type of dormancy prevents vivipary. Pre-dormancy appears to be very short in *P. nodosus* when dormant buds dehisce from the plant (Spencer and Ksander 1994), however it can be extended in the laboratory by removing the buds prior to dehiscence (Spencer and Ksander 1992). Extended pre-dormancy appears to be the case for other *Potamogeton* species such as *P. crispus* (Chapter 3) and *P. gramineus* (Spencer and Ksander 1992) when propagules are removed prior to natural dehiscence.

Imposed dormancy, dormancy maintained by environmental factors unfavorable to growth, appears to be the primary type of dormancy in most aquatic vegetative propagules. Many plants rely on light or temperature changes to break dormancy (Agami et al. 1986; Best 1979; Madsen and Adams 1988; Sastroutomo 1981; Van Wijk and Trompenaars 1985; Weber and Nooden 1976b). These environmental conditions initiate a signal cascade that will eventually result in sprouting. This type of dormancy would also explain the difference in propagules found in the substrate compared to species growing in an area.

For the most part, the study of hormonal control of dormancy has been focused on imposed dormancy. Hormonal control of dormancy within aquatic vegetative propagules

appears to be linked to propagule type. However, the controls are not always analogous to terrestrial counterparts. Potato tuber dormancy is maintained by ABA and broken with an increase in cytokinin (Suttle 2004), while in *Hydrilla spp*. tubers dormancy is broken by both GA and auxin (Steward 1969). Turion dormancy can be broken with cytokinin (Weber and Nooden 1976b), high concentrations of GA (Sastroutomo 1981; Weber and Nooden 1976b), and auxin (Sastroutomo 1981), whereas in terrestrial stems the main dormancy control hormone is GA with limited to no involvement of ABA (Olsen 2010). When hormones were measured in relationship to *Ceratophyllum demersum* dormancy by Best and coworker (1976; 1979) an antagonistic relationship between ABA and GA was found. A slight an antagonist relationship also occurred with ABA and auxin, but only during the winter quiescence.

There are some species that can build up propagule banks similar to long term seed banks. The innate dormancy, dormancy controlled by factors within the propagules, has not been studied in any great depth. *P. crispus* can have a large portion of turions in a stage of innate dormancy (40%) (Chapter 2) allowing the population to maintain establishment even if adult plants are removed before turions are produced. The same innate dormancy occurs in a subsets of *Hydrilla spp*. tubers (Netherland 1997). The plants where innate dormancy is recorded are invasive in non-native regions.

The hormonal control of innate dormancy is unknown. However, in Chapter 3 we concluded that within *P. crispus* turions innate dormancy is not controlled by ABA, unlike imposed dormancy.

As propagules transition from one type of dormancy to another, carbohydrate and hormone levels shift (Anderson et al. 2005). Starch reduction and free sugar increases have been correlated with changes in environment corresponding to sprouting (Appenroth 2010; Harada and Ishizawa 2003; Ley et al. 1997). Control of the type of dormancy induced as well as the environmental cues that transition dormancy from one type to another is unknown. Propagules many need to move through the stages of dormancy sequentially or environmental cues during formation may determine dormancy stage.

General Conclusions

The current knowledge of vegetative propagule dormancy is summarized in Figure 1.5. Aquatic plants receive an abiotic signal of light and/ or temperature to initiate propagule formation. The signal cascade which includes an increase in ABA levels promotes formation. GA and cytokinin both inhibit the formation process. Once propagules are formed they may enter one or more forms of dormancy. Whether propagules need to experience one stage of dormancy in order to move to another is unknown. Pre-dormancy is hypothesized to be maintained by ABA based on the increased ABA levels found during formation. GA may induce sprouting, but no aquatic plant studies have investigated this hypothesis. Propagules move to imposed dormancy in which a light and/or temperature signal initiates a hormone cascade resulting in sprouting. ABA most likely maintains dormancy and an increase of cytokinin or GA will cause sprouting. The lack of data on propagules expressing innate dormancy leaves researchers to speculate on what abiotic signals and hormones are involved. Although

dormancy and sprouting require synthesis and action of many hormones, ABA and GA appear to be the primary regulating hormones within aquatic vegetative dormancy.

Potamogeton crispus L.

Potamogeton crispus L. is an aquatic angiosperm native to Eurasia. The plant arrived in the United States between 1841 and 1842 (Bennett 1880). The New England population of *P. crispus* is thought to have arrived with fish from Europe and spread by fish hatcheries, ducks, and planting by wildlife managers (Moore 1915). The first documented case in Minnesota was in Lake Minnetonka in 1929 (MIN, #298022).

Since its arrival, *P. crispus* has spread throughout the United States and has become a nuisance in most freshwater ecosystems. *P. crispus* populations can change the surrounding environment by reducing substrate temperatures, light availability, alkalinity, and available CO_2 (Engelhardt 2006). Plants can also increase water pH, O_2, and periphyton biomass (Engelhardt 2006). During the active growing season, *P. crispus* populations can impede navigation. An added nuisance occurs when *P. crispus* plants senesce. *P. crispus* plants senesce and decompose when water temperatures are above 25 °C, usually around the beginning of July in Minnesota. As decomposition occurs, a steady rate of nutrients is released (Rogers and Breen 1982). The excess nutrients facilitate large algae blooms which in turn feed aerobic bacteria causing a reduction in dissolved oxygen (Wolverton and McDonald 1979). Large algae blooms degrade water quality, and in some cases, toxins are released at levels high enough to close swimming areas and endanger small animals (Puschner et al. 2008).

The successful invasion of this plant to North American is linked to its unique characteristics and life cycle. *P. crispus* is a winter annual or, under certain conditions, a winter perennial (Catling and Dobson 1985); therefore direct competition from native plants is limited. Vegetative propagules sprout in the autumn and overwinter as plants under the ice. The plant requires very low light to actively grow, 99 – 1500 lx (Guo-cai and Ying 1998; Wehrmeister and Stuckey 1992). As the water temperature rises in the spring, plants rapidly grow and mature into two forms of adult plants: Type I –at maturity the plant's apical meristem terminates as a flower stalk, and Type II – at maturity the plant's apical meristem terminates as a large turion (Figure 1.6).

The two adult plant forms differ in leaf structure and turion formation. Leaves of Type I plants, become stiff and dark green in color. The edges curl similar to lasagna noodles with serrated edges and the red midrib becomes more pronounced. Axillary buds, which eventually develop into turions, are found at each node (Figure 1.6B). Type II leaves do not thicken and remain paper thin similar the leaves of very young plants (Figure 1.6A). These plants are the first to develop mature turions each season and do not have axillary buds, therefore the only form one turion per stem. In both types of plants, leaves contain large aerenchyma cells in the mesophyll (Figure 1.7) possibly for use in gas exchange and structural support. Leaves are located on only the upper 1.5 m of the plant (Chambers et al. 1985; Jian et al. 2003). This growth form is thought to be due to the light quality available at that depth (Jian et al. 2003). The stems are oblong and also contain large aerenchyma cells surrounding the central vascular tissue (Figure 1.8).

P. crispus reproduction is primarily conducted through modified dormant stems called turions. A square meter of plants can produce 0-100 seeds and 1000 – 3000 turions (Wehrmeister and Stuckey 1992; Woolf and Madsen 2003; Yeo 1966). Turions form at the same time as flowering (Champion and Tanner 2000; Kunii 1989; Wehrmeister and Stuckey 1992; Woolf and Madsen 2003). Flowers are wind pollenated (Zhang et al. 2009) (Figure 1.9). After fertilization the flowers retract back under the water surface and produce up to 4 nutlets (Zhang et al. 2009). The fruits either drop off the flower stalk into the substrate or break off the stem as the plant decomposes. No sprouting from seed has been recorded in the field in North America, but under lab conditions the seeds need to be desiccated for at least 40 days before rehydration in order to sprout (Brock and H. 1998; Brock and Rogers 1998) .

Turions form from the bottom up with the lower turions being larger than the ones closer to the apical tip (Figure 1.6B). The size difference may be linked to the time allotted for maturation. All turions are initially green. Some turions eventually change color as the summer progresses to either orange, or brown (Figure 1.10). Turions that are older than one year are usually brown or orange; however green turions have been collected (Figure 1.11). Turions vary is shape ranging from pinecone-like (Figure 1.10) to stick-like (Figure 1.12). No metabolic differences have been attributed to color or turion shape.

Currently invasive populations of *P. crispus* are managed with either mechanical or chemical treatments directed at the adult plant (Foley 1997). Woolf and Madsen (2003) found that *P. crispus* is most vulnerable to herbicide treatment before turion

formation. However, their study did not address the issue of viable propagule banks which are present in most long established populations of *P. crispus*.

An understanding of *P. crispus* dormancy and the production of the long term propagule banks would allow managers to address the issue of long term population control of *P. crispus*. The following research was conducted to determine *P. crispus* turion physiology and hormonal controls of turion dormancy.

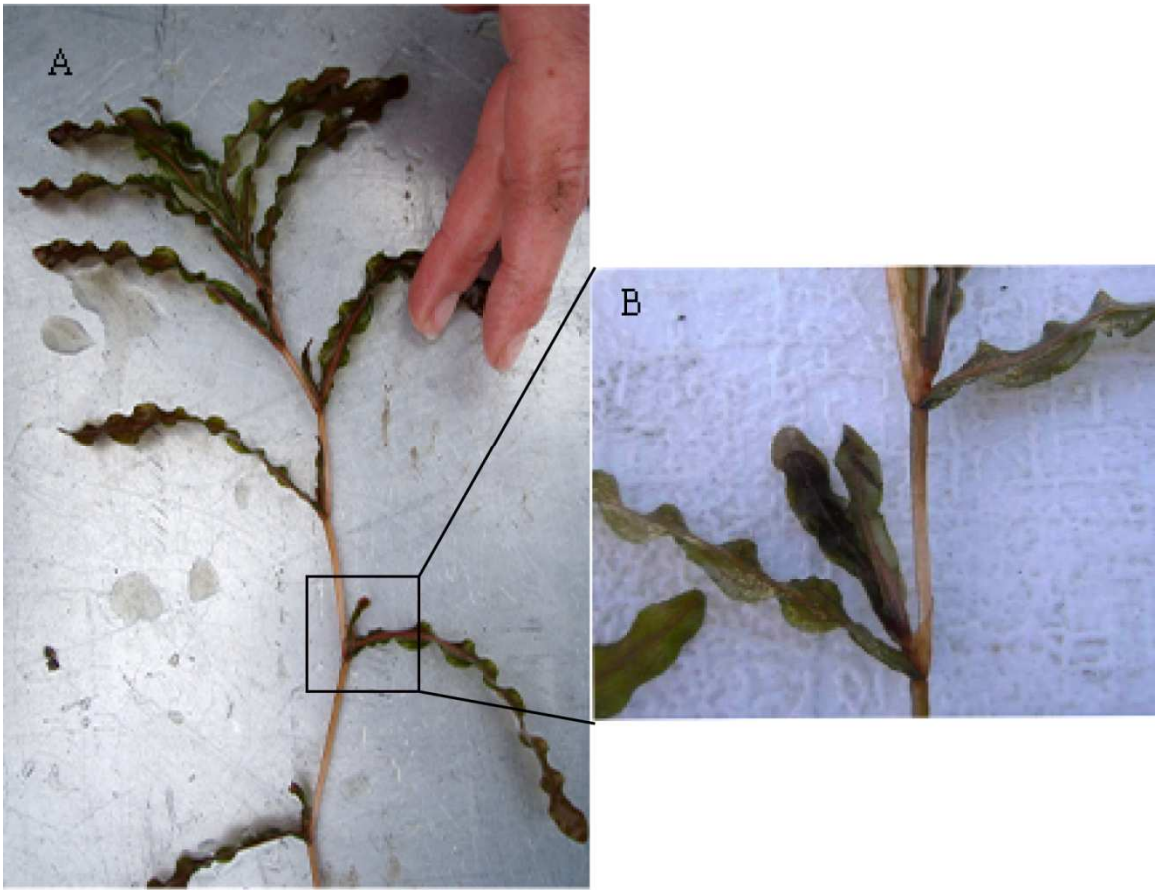


Figure 1.1. Initiation of *Potamogeton crispus* turion formation. Axillary buds begin to elongate and form visible leaves along a very short stem. A) Buds begin to elongate on the whole plant at approximately the same time. B) Close up of an elongating axillary bud.

Figure 1.2. Thickening of *Potamogeton crispus* axillary buds during turion formation. Turions thicken with stored starch while retaining thin leaf tips that are photosynthetically active. Apical buds at the base of the plant thicken faster than buds toward the apical tip.

Figure 1.3. Turion maturation of *Potamogeton crispus*. Axillary buds have thickened and are now called turions. Wing-like projections are still attached to turion and photosynthetically active. The attachment point between the turion and the parental plant weakens.

Figure 1.4. Mature turion of *Potamogeton crispus*. Turions will fall off the parental plant at the node. Some mature turions remain attached to the parental plant. These turions reach the substrate when the plant dies back as the water temperature increases.

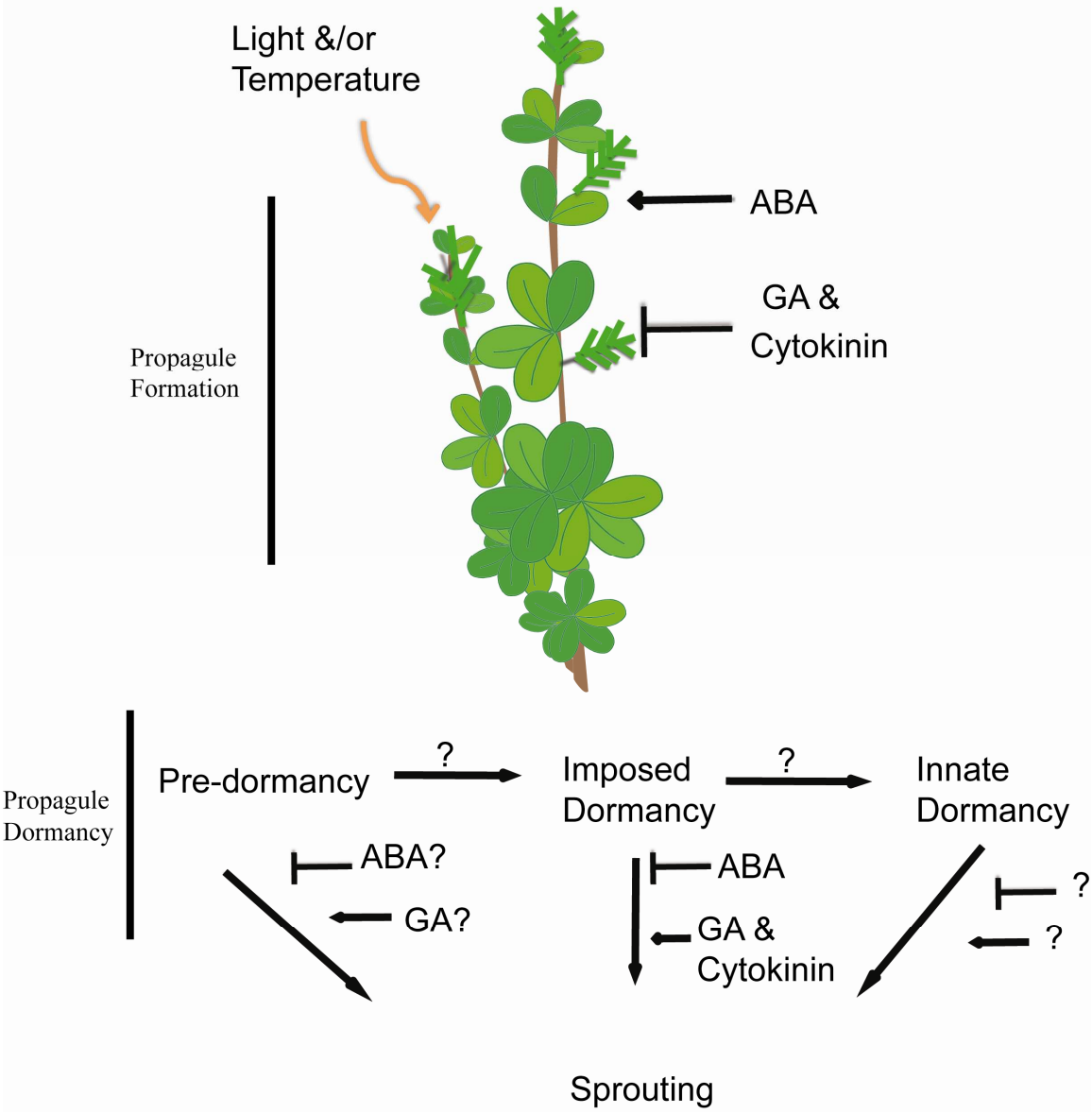


Figure 1.5. Proposed aquatic vegetative propagule dormancy model. ABA initiates propagule formation, while GA and cytokinin inhibit formation. Propagules enter into dormancy that maybe maintained by ABA. Sprouting is activated by the applications of GA or Cytokinin. Type of hormonal control on a specific types of dormancy is unknown.

Figure 1.6. Two forms of adult *Potamogeton crispus* plants. A) Type I *P. crispus.* No axillary buds are present and the apical tip forms one large turion. B) Type II *P. crispus* Turions form from axillary buds and the apical tip matures into an inflorescence.

Figure 1.7. Cross section of *Potamogeton crispus* leaf. Large aerenchyma cells are present in the mesophyll. No stomata are present. The majority of the chloroplasts are found in the thin palisade layer of the leaf.

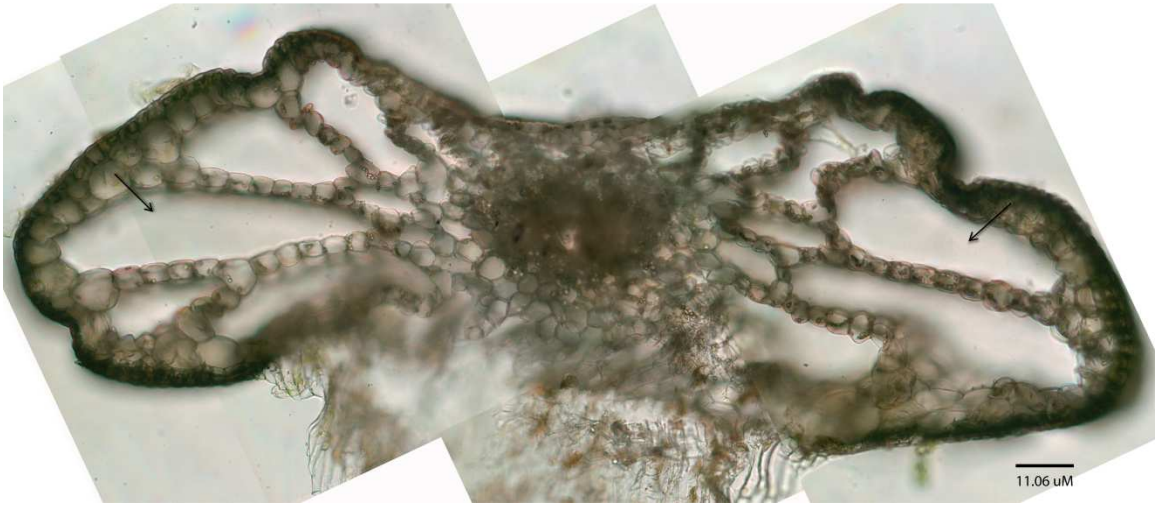


Figure 1.8. Cross section of adult *Potamogeton crispus* stem. Large aerenchyma cells surround a center vascular cylinder. Stems contain cells with chloroplasts on the inside lining of the epidermis.

Figure 1.9. Flowers of *Potamogeton crispus*. A) Flowers are raised above the water surface to be wind pollinated. B) After pollination flowers sink below the water surface and form fruits.

Figure 1.10. First year turions. Newly formed turions range in size and shape. All begin the summer season green and some eventually change color as time progresses. The most common shape of turions found is the “pine-cone” or “bur” shape.

Figure 1.11. Turions over one year old. Older turions generally range in color from solid orange to brown.

Figure 1.12. Newly formed stick-like turions. These type of turions form when turion formation is interrupted (See Appendix A).

Chapter 2 - Regulation of Turion Dormancy in Potamogeton crispus L.

Abstract

Vegetative buds (turions) are the major source of propagation for the aquatic invasive angiosperm, *Potamogeton crispus* L. (Potamogetonaceae). An understanding of the regulation of turion sprouting could lead to better methods of population control. Turions were divided into two different age groups, current season and overwintered. Sprouting was monitored in these different groups exposed to varying light durations and temperatures. Non-structural carbohydrates, photosynthesis, and aerobic respiration were measured to determine metabolic activity. Current season turions were found to sprout mainly in response to day length and were metabolically active over a 6 week period. Current season turions are in a quiescent state and 60-70% will sprout in the autumn. The remaining current season turions presumably go into a state of deep dormancy and remain dormant over the winter. Turions that have overwintered are not photosynthetically active, have stable carbohydrate levels, and can remain dormant but viable for several years. Under laboratory conditions, they sprouted mainly in response to an increase in water temperature. These different age groups correspond to different turion physiological states and can explain sprouting variability recorded by other researchers.

Introduction

The aquatic plant *Potamogeton crispus* L. (curly-leaf pondweed) is an invasive angiosperm in both lentic and lotic waters of temperate regions of North America. Its unique life cycle contributes to the plant's success. *P. crispus* has a very low light compensation point (Guo-cai and Ying 1998) which allows sprouts to remain photosynthetically active under the ice. Once water temperatures exceed 14^0C and day length increases, the plants resume growth, bolt, produce vegetative propagules (turions) and seeds before most native plants break winter dormancy (Chambers et al. 1985). During the summer, if water temperatures are higher than 25°C, *P. crispus* senesces and the roots remain dormant until autumn. In the autumn (September – October), the majority of turions and adult plants sprout to begin the next growth cycle(Chambers et al. 1985; Wehrmeister and Stuckey 1992).

Many aquatic plants use vegetative propagules as a reproduction strategy (Adamec 1999; Agami et al. 1986; Nichols and Shaw 1986; Sato et al. 2002; Van Wijk and Trompenaars 1985). Turions, modified stems produced by *P. crispus*, are the primary source of propagation (Catling and Dobson 1985; Wehrmeister and Stuckey 1992; Yeo 1966). Due to the large number of turions produced per season, turions collect in the lake substrate and form "turion beds" that can remain viable for many years (Johnson et al. 2012). This collection of dormant turions allows *P. crispus* populations to persist for several years even when intensive management of adult plants is applied (Johnson et al. 2012). To effectively control the growth of *P. crispus*, methods must be devised to target turion sprouting as well as adult plants. Current recommended

management strategies to control *P. crispus* focus on the control of the adult plant before turion formation with the use of many different types of herbicides (Foley 1997; Johnson et al. 2012). Control of *P. crispus* through its reproductive structures may be a viable management option if reproductive physiology is known.

Relatively little information exists regarding the physiology of *P. crispus* turions. The turions are believed to be summer dormant when they separate from the parent plant (Nichols and Shaw 1986; Sastroutomo 1981; Wehrmeister and Stuckey 1992). Turions retain their green color until autumn at which time they may change color to either orange or brown. Different colored turions have been reported to sprout at different rates (Sastroutomo 1981). The relationship between turion sprouting and sediment anoxia has also been investigated (Wu et al. 2009) indicating turions do not sprout in low oxygen environments.

Previous studies present a complex picture as to what abiotic signals cause 30%-100% of the *P. crispus* turions to sprout during autumn. Under field conditions, both photoperiod (Jian et al. 2003) and temperature (Wehrmeister and Stuckey 1992) were shown to influence sprouting. Combined interactions between temperature and photoperiod have been studied under controlled conditions (Sastroutomo 1981; Wu et al. 2009). Complex relationships between the influence of temperature and light on turion sprouting has been documented in other aquatic plants such as bladderworts (Adamec 1999), *Najas mariana* (Agami et al. 1986) and *Potamogeton trichoides* (Van Wijk and Trompenaars 1985). In other aquatic plants intracellular signals have been found to trigger vegetative bud sprouting; for example, *Lemna minor* turions respond to varying

nutrient levels (Dudley 1987), whereas *Spirodela polyrhiza* turions sense increased levels of sugars (Ley et al. 1997).

This study was undertaken to further investigate the abiotic signals to which *P. crispus* turions respond by breaking dormancy and sprouting. We also investigated how turions of different age classes, i.e., current season *versus* overwintered turions respond to these signals.

Materials and Methods

Turion Collection

Lake Sarah, Hennepin County, Minnesota, USA (T119N, R24W, Sec 34 & 35) was sampled for *Potamogeton crispus* turions. This 561 acre lake was chosen because it is heavily infested with *P. crispus* but it has been sporadically spot treated with herbicides. Samples were collected from the west end of the lake in May and June of 2009, 2010, 2011, and 2012 to coincide with turion development. Turions collected in May before development on the plant were known to be at least one year old and were designated the overwintered population. Turions collected in June were taken directly from the plants and were designated the current season population. Turions were also collected in September of 2008 to provide a field comparison of a mixed population. A ponar grab (Wildco, Wills Point, Texas) and dip-net were used to harvest the overwintered turions from the sediment in May and September. Turions were washed and submerged in distilled water (pH 6.5). A subset of each type, current season and overwintered, was frozen and stored at -20^0 C for initial measurements. Another subset

of each type was placed under experimental light and temperature conditions in the laboratory. Additional turions were stored at 4°C in the dark. The mixed population of turions collected in September was sorted by color. All green turions were assumed to be the current season's production. Orange and brown turions were the overwintered populations. Black turions were previously determined to be dead and were discarded.

Turion Sprouting Conditions

To determine the effects of light and temperature on turion sprouting, sets of 60 ± 15 turions were exposed to 16:8 light:dark cycles (L:D) and 10:14 L:D at 23^{o}C to simulate summer and autumn light durations, respectively. Four 34-watt Philips Econ-o-watt fluorescent bulbs were used for the 16:8 L:D cycles with a photosynthetically active radiation (PAR) value of 23 µmoles of photons m^{-2} s^{-1}. Two 40-watt Philips fluorescent bulbs with a PAR of 28 µmoles of photons m^{-2} s^{-1} were used for the autumn (10:14) light conditions. Additional sets of turions were placed in complete darkness, one at 4^{0}C and another at 23^{0}C. Turions were monitored for sprouting and the numbers were recorded. Sprouted turions were removed from each experimental set as soon as sprouts were visible. The sprouted shoots were removed from the turions and the turions were pooled by week of sprouting and frozen at -20^{0}C. In addition, five non-sprouted turions were removed from each experimental set each week and frozen for future chemical analysis.

To determine if exposure to two light durations would influence sprouting of current season turions, two different experiments were conducted. One set of current season turions (200 ± 50) was stored at 4^{0} C in dim light (PAR = 2.7 µmoles of photons

m^{-2} sec^{-1}; 16:8 L:D). After seven weeks, turions were placed in the four experimental light and temperature conditions used previously. In the second experiment, sets of turions (30 ± 5) were pretreated under 16:8 L:D conditions for one to five weeks before placing in 10:14 L:D. After each week of pretreatment, a subset of turions was moved to the autumn light conditions for an additional four weeks. The sprouting percentages of the pretreated turions was compared to a control population under continuous 10:14 L:D.

Glucose and Starch Analyses

Pooled turion samples were analyzed for glucose using a colorimetric Trinder assay. This method was originally developed for measuring glucose in blood samples (Trinder 1969) and was adapted for our plant extracts. The Trinder assay uses glucose oxidase to produce equal amounts of gluconic acid and hydrogen peroxide. The peroxide, catalyzed by peroxidase, reacts with 4-aminoantipyrine and p-hydroxybenzene sulfonate to form a quinoneimine dye. The absorbance of the dye is proportional to the amount of glucose in the sample. Trinder reagent was mixed daily and contained 0.5 mM 4-aminoantipyrine, 20 mM p-hydroxybenzene sulfonate, 15 U/ml glucose oxidase (*Aspergillus niger*), and 10 U/ml horseradish peroxidase in 100 mM Tris-HCl buffer, pH 7.0. All chemicals were purchase from Sigma Diagnostics (St. Louid, MO, USA).

Turions were lyophilized to dryness and ground to a fine powder. Dried samples were weighed (20 ±5 mg) and suspended in 0.5 ml distilled water. To remove tannins, which interfered with the analysis, a slurry of 30% diethylaminoethyl (DEAE) cellulose (GE Healthcare, Piscataway, NY) in water was added to the turion samples. This mixture

was incubated for 10 min at 23^0C and centrifuged at 8,500 g for 6 min. Aliquots of the supernatant were added to 0.5 ml Trinder reagent in 100 mM Tris-HCl buffer (pH 7.5) for a total volume of 0.6 ml. The reaction tubes were incubated at 37^0 C for 30 min and then placed in ice to stop the reaction. The absorbance at 506 nm was determined in a 8450A Diode Array spectrophotometer (Hewlett-Packard, CA, USA). The glucose concentration was determined from a standard curve.

Starch content was determined by suspending 20 ±5 mg of ground turion sample in 0.5 ml acetate buffer, pH 4.8. DEAE slurry (0.5 ml) was added and the mixture was incubated for 10 min at 23^0 C. After incubation, 50 µl of amyloglucosidase (10 mg/ml; from *Aspergillus niger)* were added and the mixture was further incubated at 60^0 C for 20 min. The mixture was placed on ice to stop the reaction and centrifuged at 8,500 g for 10 min. Aliquots of the supernatant were analyzed using the Trinder protocol. The concentration of starch in the sample was calculated by subtracting the amount of free glucose from the total glucose after amyloglucosidase digestion. Corn starch was used as an internal standard to verify the amount of digestion.

Chlorophyll and Tannin Analyses

Turions were lyophilized and ground to a fine powder. For chlorophyll analysis, the powder (20 ± 5 mg) was suspended in 1 ml 95% ethanol and incubated at 4^0 C for approximately 16 h in the dark. The suspensions were centrifuged at 8,500 g for 10 min and the visible spectrum (400-700 nm) of the supernatant was determined. The spectra for chlorophyll a and b was confirmed and the concentration of chlorophyll a was

calculated using the extinction coefficient ϵ = 82.04 mg/ml at 662 nm (Arnon et al. 1974).

For tannin analysis, the turion powder was suspended in water and stirred for approximately 16 h at 4^0 C. The suspension was centrifuged as above and the water-soluble phenolic content of the supernatant was determined with the Folin-Ciocalteau reagent (Gross et al. 1996). Tannic acid was used as a standard.

Photosynthesis and Respiration Rates

To measure photosynthesis and respiration rates, changes in the oxygen concentration of water containing turions was monitored using a Vernier Labpro (Beaverton, OR) oxygen probe. Samples of both current season and overwintered turions (15 ± 5) were placed in flasks with approximately 150 ml of 0.1 M Tris-HCl, pH 7.0. After inserting the oxygen electrode, the flasks were stirred gently under 10:14 L:D conditions and changes in oxygen concentrations were monitored for various periods of time ranging from 5-35 days. Light was provided by 3 cool white fluorescent lamps at a PAR of 65 µ moles of photons m^{-2} s^{-1}. Sprouted turions caused an noticeable change in oxygen concentration and were replaced by non-sprouted turions exposed to similar conditions.

Statistics

Experimental values are expressed as averages of three or more determinations with standard deviations. Values were compared for significance using the Student's t-test.

Results

Turion Sprouting

Approximately 60-70% of overwintered turions had sprouted after three weeks under both the 16:8 and 10:14 L:D conditions (Figure 2.1). The majority of overwintered turions sprouted during the first 72h of light exposure and no additional sprouting occurred after week three. Turions did not sprout at 4^0 C in the dark, but 40% sprouted in the dark at 23° C.

Less than 10% current season turions sprouted under any light conditions (Figure 2.2). When current season turions were stored for a year at 4^0 C in the dark and then place in experimental conditions, sprouting rates were similar to current season initially placed under experimental conditions. In contrast, current season turions that were exposed to 4^0 C in dim light for seven weeks, sprouted at 25-70% (Figure 2.3). Autumn light duration was the most effective in stimulating sprouting in all experiments. In conditions where sprouting occurred, the majority of turions sprouted within four days of light exposure with little sprouting after four weeks. Current season turions collected from the field in September and exposed to different light regimes under laboratory conditions sprouted at 60-75% (Figure 2.4), similar to the dim light experiment. No turions sprouted at 4^0C in the dark during any experiment.

Current season turions pretreated for one week under summer light before being placed in autumn light conditions had increased sprouting (1.5 fold) compared to untreated turions (Figure 2.5). Sprouting levels remained comparable to controls until

week three. After week three turions began to show signs of photo bleaching coupled with low sprouting levels.

Glucose and Starch Content

The total free glucose content initially determined in both types of turions was relatively low (approximately 2.5 mg/g dry weight; Table 2.1). In the overwintered turions, free glucose increased in the dark after 6 weeks. When final glucose concentrations were pooled by light vs dark exposure, a 2-3 fold difference was found (P-value: < 0.0001). In current season turions, glucose concentration increased slightly after 6 weeks in summer light (P-value: 0.004).

The total free glucose plus starch content in overwintered turions was approximately 32 mg/g dry weight and did not change significantly under any experimental conditions (Table 2.2). After sprouting, the total carbohydrate content declined by 90%.

Initial values show that the current season turions had approximately half the glucose plus starch content of their overwintered counterparts. When placed in either summer or autumn light conditions for six weeks, these turions accumulated carbohydrates to a level similar to that of the overwintered turions (Table 2.2). Current season turions collected in September have similar carbohydrate levels as turions exposed to light conditions (Table 2.2). In contrast, the carbohydrate content of turions placed in the dark at 23^0 declined. These data show that the current season turions are metabolically active, accumulating storage carbohydrates in the light and utilizing them

in the dark. No significant changes were seen in turion carbohydrate content in the dark at 4^0 C.

Chlorophyll a and Tannin Content

All turions regardless of age group or color contained chlorophyll a (Tables 2.3 and 2.4). Current season turions are mostly green and have approximately twice the chlorophyll a concentration of the overwintered turions which are a brown color (P-value: <0.0001). Chlorophyll levels declined by 23% in the current season turions after 4 weeks in the dark at 23^0 C. No significant change was noted in overwintered turions under any conditions.

Water-soluble tannin content was determined in current season turions after field collection. These turions have a variety of colors ranging from green, orange, and brown but are mostly green. Orange and brown turions had higher tannin content than the green turions which may account for some of the color differences (Table 2.4). Despite color differences, all these buds had chlorophyll a content similar to the values in Table 2.3. Overwintered turions were various shades of brown and contained tannins at levels similar to current season green turions.

Photosynthesis and Respiration Rates

Current season turions evolved oxygen in the light (10:14 L:D) at an average rate of 0.17 $\pm$ 0.034 mmoles O_2 h^{-1} mg Chl a^{-1} over approximately 5 days. Oxygen consumption in the dark was 0.07 $\pm$ 0.018 mmoles O_2 h^{-1} mg Chl a^{-1} indicating metabolic activity above the compensation point. Turions with sprouts were much more active;

0.73 ± 0.15 mmoles O_2 evolved in the light and 0.46 ± 0.14 mmoles O_2 consumed in the dark per hour per mg of chlorophyll a. Overwintered turions did not evolve or consume oxygen during 5 weeks of monitoring. Oxygen evolution was noted within 24 hours after sprouting in both types of turions.

Discussion

Control of turion viability and sprouting is essential to managing the growth and spread of *P. crispus,* and several research groups have studied the effects of abiotic parameters on sprouting. Sastroutomo (1981) collected turions from lake substrates in autumn and winter and divided them into new and old turions based on their color. Our data indicate that color can be a misleading criterion for estimating age of turions. Our overwintered turions, a year or older, were mostly brown, but some were bright orange. In contrast, current season turions, collected directly from plants, were usually green but a significant number turned orange or brown throughout the summer. Turion color is at least partially due to differences in soluble phenolic compounds and chlorophyll (Tables 2.3 and 2.4). We found no correlation between sprouting rates and turion color. By collecting old turions from the substrate in early spring and new turions directly from senescing plants, we have determined that we have two distinct stages in the life cycle of these vegetative buds.

Our data indicate either that overwinter turions are dormant and respond to changes in temperature signifying ideal sprouting conditions or dormancy has been broken before exposure to cold temperatures and sprout once the temperatures are warm

enough for enzymatic activity (Figure 2.1). Our results are consistent with studies where field sprouting was measured in the autumn (Rogers and Breen 1980; Wehrmeister and Stuckey 1992) and with autumn collected turions used in lab experiments (Sastroutomo 1981). The reduced sprouting of overwintered turions in the dark alludes to a minor role of light regulation (Figure 2.1).

Overwintered turions contain chlorophyll a. However, we found no measurable photosynthesis or respiration occurring in the turions. The Vernier O_2 probe is not very sensitive therefore low levels of activity would not be detected. However, no significant starch accumulation or degradation within turions was recorded over 6 weeks supporting the idea that overwinter turions are not photosynthesizing. After sprouting, overwintered turions show a 5 to 10 fold decrease in starch content compared to dormant turions (Table 2.2). Also, within 24 hours of root emergence, photosynthesis/ respiration can be measured before the first leaves have turned green. These data suggest that overwintered turions may utilize the free sugars digested from the starch for sprouting, possibly by using anaerobic metabolism as described for *Potamogeton distinctus* (Koizumi et al. 2011). Retention of low levels of chlorophyll during dormancy may facilitate rapid initiation of photosynthesis at sprouting to support growth.

In any collection year, 0 – 40% of overwintered turions do not sprout. These non-sprouted turions will sprout after re-chilling them at 4 °C for two weeks. However, in the field, the seasonal changes in water temperature do not explain why some turions remain dormant for more than one year and are still viable (Johnson 2010). These turions are in an extended dormancy. A mechanism that controls an extended deep dormancy would

allow for individual plant recruitment if the parent population does not produced turions and/or seed during multiple growing seasons.

Summer dormancy of *P. crispus* has been recorded under lab and field conditions through the lack of turion sprouting (Jian et al. 2003; Madsen et al. 2002; Rogers and Breen 1980; Sastroutomo 1981; Wehrmeister and Stuckey 1992). During this time, the turions are thought to undergo a metabolic maturation similar to seeds (Borisjuk et al. 2004). Our data confirm that current season turions have low sprouting rates (Figure 2.2) indicating summer dormancy. We also found current season turions to be metabolically active (Table 2.2) possibly leading to further turion maturation.

Current season turions contain higher levels of tannins and chlorophyll than the overwintered turions (Table 2.3 & 2.4). The tannin levels increase over the first season and were found to correspond with the orange color change (Table 2.4). The high levels of tannins maybe used to deter insect herbivory and inhibit microbial decay (Ostrofsky and Zettler 1986) contributing to the longevity during deep dormancy. Tannins also have antioxidant properties and may protect turions from intense UV light in shallow water (Rice-Evans et al. 1996).

Chlorophyll levels did not change throughout the summer for current season turions unless the turions were placed in complete darkness. The levels appear to drop in turions during the autumn based on September collected turion levels (Table 2.3). Unlike the overwintered turions, current season turions photosynthesize and respire. Turions are able to accumulate starch to levels similar to initial overwinter turions (Table 2.2). The carbohydrate changes in current season turions over the summer appear to be leading to

an altered turion status, either sprouting in autumn or prolonged dormancy. The starch accumulation in current season turions could be an indicator for breaking dormancy, aiding in sprouting, or preparing turions for longer term dormancy. The decrease in chlorophyll content and increase tannin levels indicate dormancy preparation.

Summer dormancy of current season turions is thought to be broken in the autumn when majority of turions sprout (Jian et al. 2003; Rogers and Breen 1980; Wehrmeister and Stuckey 1992; Woolf and Madsen 2003). The actual timing or the mechanism in which summer dormancy is broken is unknown. Summer dormancy has been experimentally broken by both extreme cold and hot temperatures (Sastroutomo 1981). We were able to break summer dormancy to by chilling turions at 4°C in dim summer light. After chilling a large portion of these turions (approximately 68%) sprouted under short day: long night conditions corresponding to autumn light (Figure 2.3). These data correspond with field studies indicating that the majority of turions sprout in autumn (Jian et al. 2003; Rogers and Breen 1980; Wehrmeister and Stuckey 1992; Woolf and Madsen 2003), and is supported by increased sprouting under autumn light and starch accumulation data of September collected current season turions (Figure 2.4 and Table 2.2).

The natural break in summer dormancy cannot be explained by chilling alone because natural water temperatures do not drop below 17°C at the time of autumn sprouting (Woolf and Madsen 2003). Also, turions stored at 4°C in complete darkness did not change from a summer dormancy sprouting pattern to a non-dormant sprouting pattern for up to one year after collection. Therefore, the added light component of the

cold storage could be breaking dormancy in the laboratory. The application of summer light before placing turion into autumn light increased sprouting (Figure 2.5), but not to autumn levels. These data indicate that current season turion sprouting is controlled by photoperiod (Figure 2.2). However, the breaking of summer dormancy may require a specific light intensity. *P. crispus* is a low light tolerant plant with negative reactions to high light intensity (Tobiessen and Snow 1984; Wehrmeister and Stuckey 1992). Turions that were placed in summer light for more than three weeks began to show signs of photo bleaching correlated with low sprouting levels.

In all dormancy breaking conditions, some turions did not sprout. The non-sprouted current season turions have transitioned from summer dormancy to an overwintering dormancy. These turions will sprout after chilling for more than two weeks. The mechanism that controls the decision to sprout or remain dormant is not known. Both temperature (Sastroutomo 1981; Wehrmeister and Stuckey 1992) and light signals (Jian et al., 2003) have been proposed to control the shift from summer to overwintering dormancy. Both types of dormancy may be controlled by an internal signal that promotes starch use for sprouting or starch retention for long term storage.

Conclusion

Different aged turions have distinct physiological characteristics that can be correlated with factors that affect sprouting. Current season turions are in a state of summer dormancy after formation. During summer dormancy, turions are metabolically active. In the autumn, current season turions either sprout or transition to overwintering

dormancy. Overwintered turions are in a secondary type of dormancy where turions are not metabolically active, but are primed for sprouting when the water temperature rises above 15 °C. Overwintered turions that do not sprout are in a deep dormancy where they can stay viable for up five years (Newman, personal communication). This extended dormancy can defeat current management strategies; these turions can sprout at any time and infest a lake that has been thought to be clear of a previous *P. crispus* infestation.

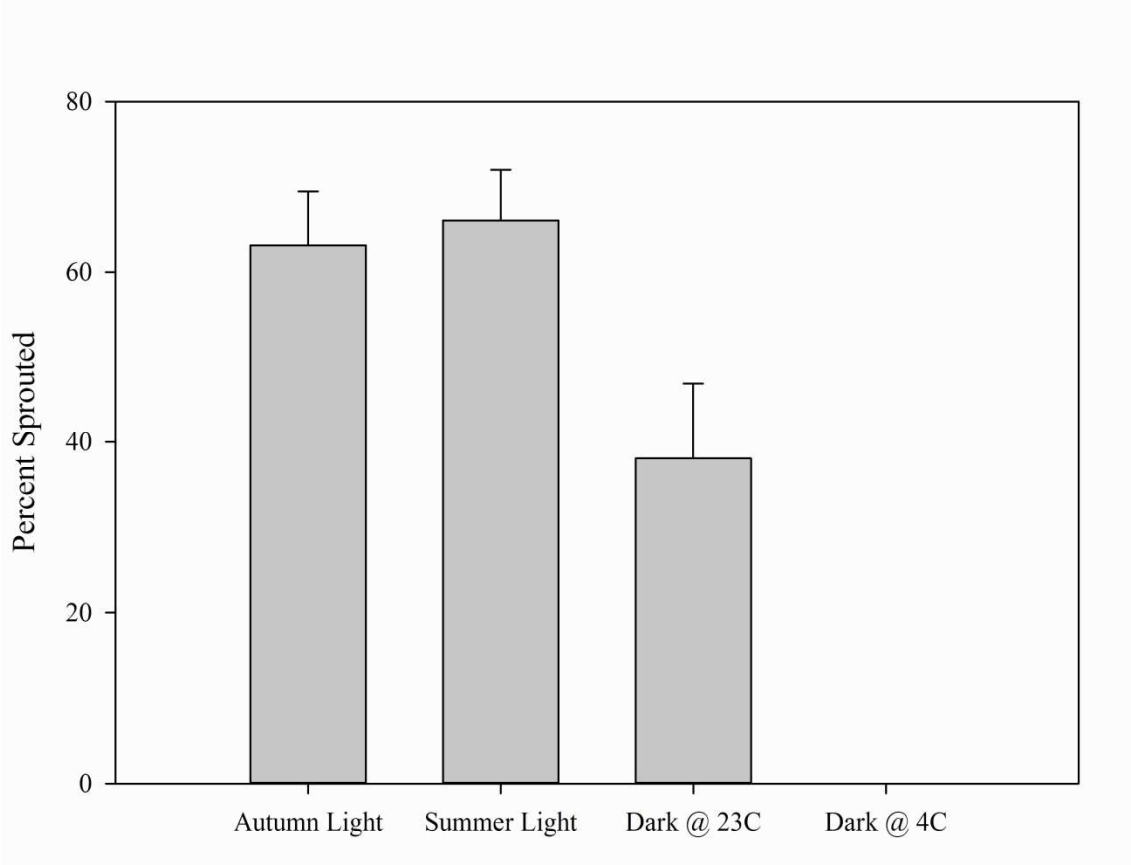


Figure 2.1. Overwintered turion sprouting percentages under different light durations. 50-75 turions were monitored for sprouting over a five week period. No turions sprouted in the dark at 4 ^{0}C. Experiments were conducted five times with turions collected in 2009, 2010, 2011.

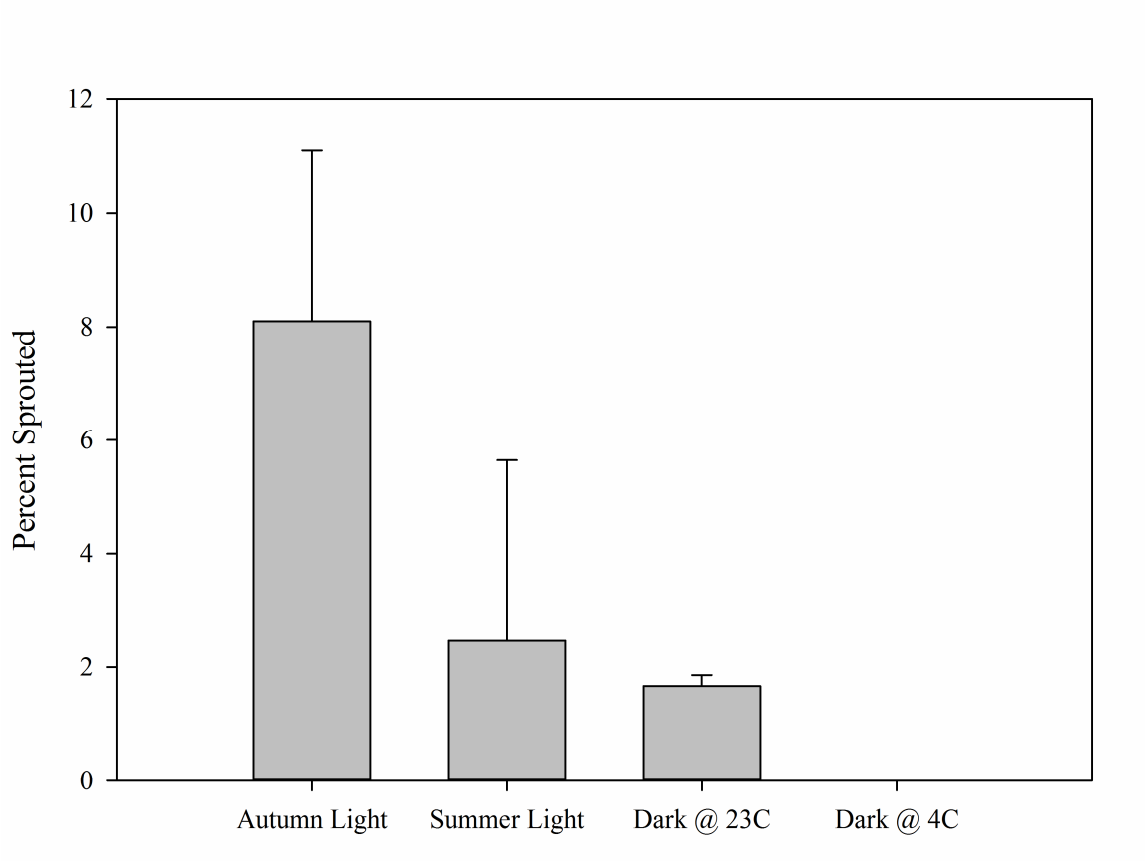


Figure 2.2. Current season turions sprouting percentages different light durations. 50-75 turions were monitored for sprouting over a six week period. No turions sprouted in the dark at 4 ^{0}C. Experiments were conducted four times with turions collected in 2011 and 2012.

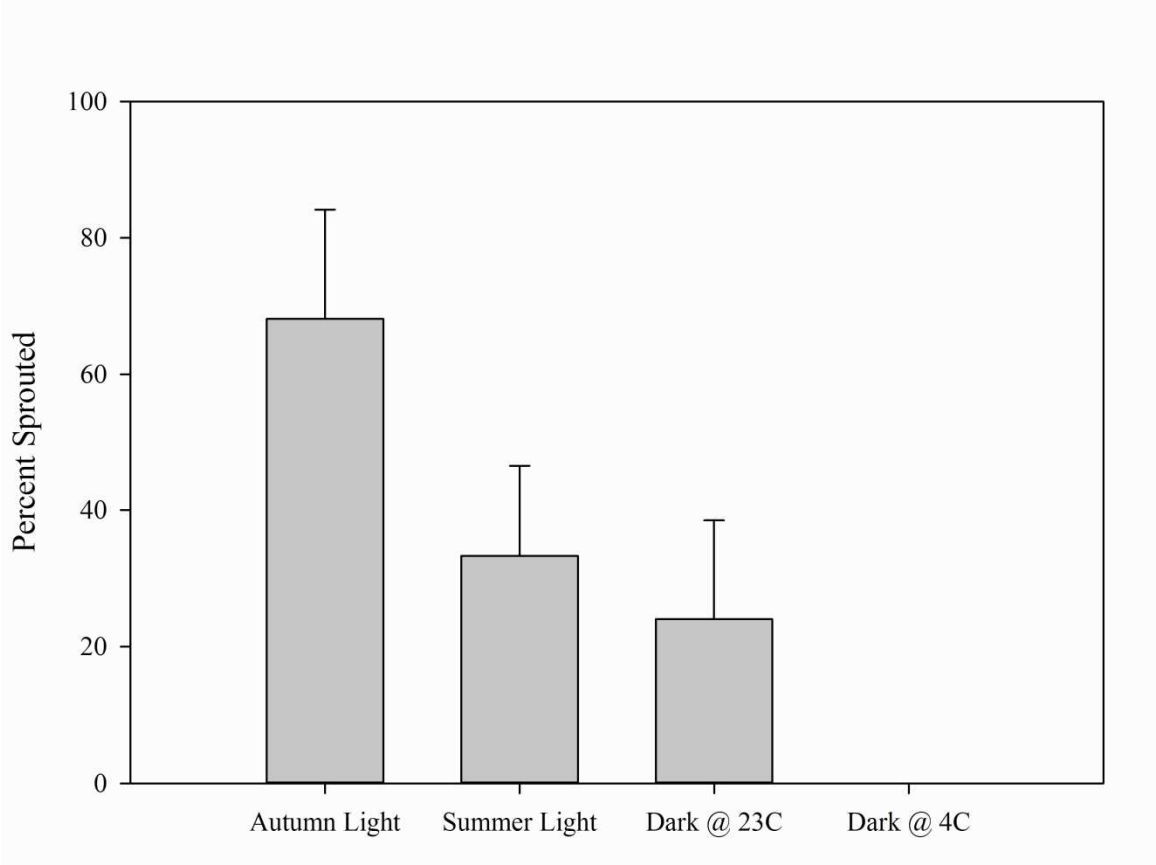


Figure 2.3. Sprouting percentages of current season turions exposed to dim light (PAR = 2.7 µmoles of photons m^{-2} sec^{-1}; 16:8 L:D) for seven weeks prior to experimentation. Turions were monitored for sprouting for six weeks. No turions sprouted in the dark at 4°C. The experiments were conducted four times with turions collected in 2010 and 2012.

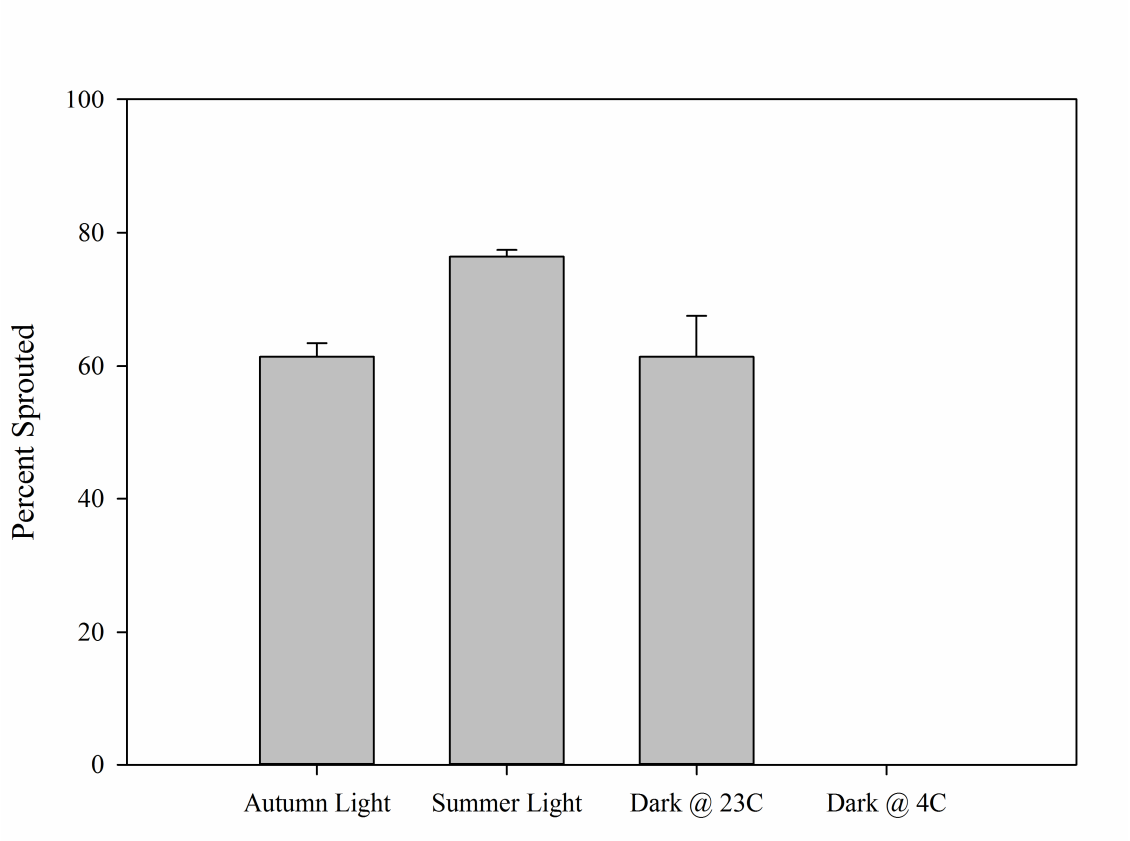


Figure 2.4. Sprouting percentages of current season turions collected from Lake Sarah, MN in September of 2008. Turions were monitored for sprouting for six weeks. No turions sprouted in the dark at 4°C. The experiment was conducted three times with turions collected in September of 2008.

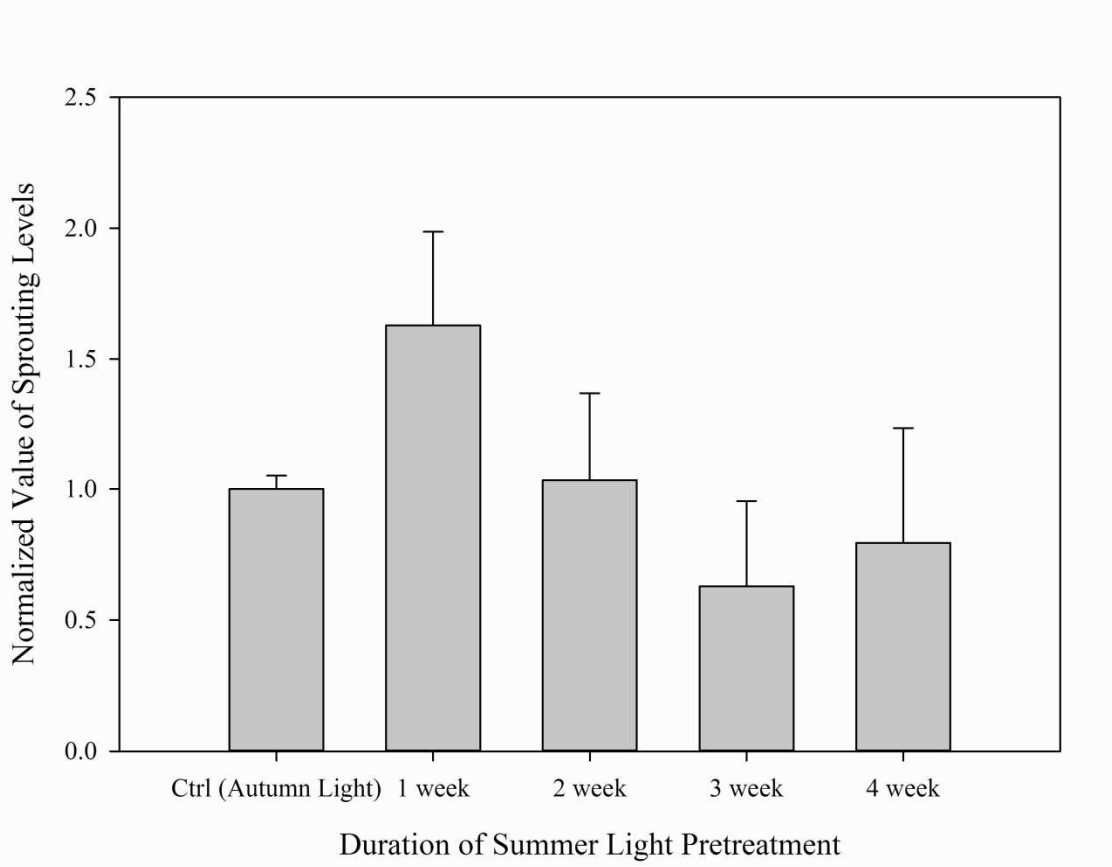


Figure 2.5. Normalized sprouting percentages of current season turions pretreated with summer light. Data was normalized compared to the control group placed in autumn light for the duration of the experiment. Photo-bleaching was visible after week 3 and proceeded to become more dramatic with longer exposure in summer light**.**

Table 2.1. Total free glucose levels of Current Season and Overwintered turions place in different light and temperature regimes during a 6-week period.

Turion Type	Conditions	Initial Free Glucose (mg/g dry wt.)	Final Free Glucose Non-sprouted (mg/g dry wt.)	Final Free Glucose Sprouted (mg/g dry wt.)
Overwintered**	16:8	2.4 ± 0.9	2.6 ± 0.3	0.8 ± 0 .1
	10:14	2.4 ± 0.9	2.1 ± 0.2	1.2 ± 0.7
	Dark @ 23^0 C	2.4 ± 0.9	3.8 ± 0.8	0.7 ± 0.2
	Dark @ 4^0 C	2.4 ± 0.9	3.5 ± 0.6	NA
Current Season	16:8	2.7± 0.2	3.8 ± 0.1*	ND
	10:14	2.7 ± 0.2	3.0 ± 0.3	ND
	Dark @ 23^0 C	2.7 ± 0.2	2.2 ± 0.2	ND
	Dark @ 4^0 C	2.7 ± 0.2	3.1 ± 0.8	NA
	September Collected (10:14)	NA	2.73 ± 0.2	ND

* Current Season turions placed in 16:8 (L:D) had a significant difference (P-value: 0.004) between initial and final free glucose levels. ** No significant difference between overwintered initial and final free glucose concentrations or between each final free glucose concentration, however final free glucose pooled into light treated turions verses dark treated turions are different (P-value: < 0.0001). NA = Not applicable ND = Not determined

Table 2.2. Total glucose & starch concentrations of Current Season and Overwintered turions place in different light and temperature conditions over a 6-week period.

Turion Type	Conditions	Initial Glucose & Starch (mg/g dry wt.)	Final Glucose & Starch Non-sprouted (mg/g dry wt.)	Final Glucose & Starch Sprouted (mg/g dry wt.)
Overwintered	16:8	31. 6 ± 2.7	24.43 ± 5.7	4.25 ± 1.10
	10:14	32.4 ± 11.1	44.7 ± 4.2	4.00 ± 2.46
	Dark @ 23^0 C	31. 6 ± 2.7	26.66, ± 13.52	2.52 ± 0.73
	Dark @ 4^0 C	31. 6 ± 2.7	26.7 ± 8.9	NA
Current Season	16:8	17.0 ± 3.8	34.8 ± 9.1*	ND
	10:14	17.0 ± 3.8	31.1 ± 6.8*	ND
	Dark @ 23^0 C	17.0 ± 3.8	9.9 ± 2.5 **	ND
	Dark @ 4^0 C	17.0 ± 3.8	19.1 ± 5.9	NA
	September Collected (10:14)	NA	33.30 ± 3.8	ND

*****Current season turions placed in light conditions significantly increased in starch (P-value: 0.0091). **Current Season turions placed in dark at 23 ^{0}C had a significant difference (P-value: 0.0003) between initial and final total glucose & starch levels. NA = Not applicable ND = Not determined

Table 2.3. Chlorophyll "a" mg/g dry wt. present before and after 4-weeks.

Turion Type*	Experiment					
	Initial	16:8	10:14	Dark @ 23^0 C	Dark @ 4^0 C	September Collected
Overwintered	0.50 ± 0.16	0.45 ± 0.05	0.47 ± 0.10	0.45 ± 0.05	0.56 ± 0.15	NA
Current Season	0.97 ± 0.12	0.96 ± 0.01	0.96 ± 0.11	0.74 ± 0.01 **	0.98 ± 0.18	0.64 ± 0.12

* Chlorophyll content between turion type differs significantly (P-value: <0.0001). ** Current Season turions retained the same amount of chlorophyll over time except for turions placed in the dark at 23°C (P-value: 0.016).

Table 2.4. Water - soluble tannin content (mg/g dry weight).

Turion Type	**Turion Color**		
	Green	Orange	Brown
Overwintered	NA	NA	34.74 ± 3.29
*(chlorophyll a)			(0.46 ± 0.01)
Current Season	31.77 ± 3.24	51.70 ± 4.99	44.93 ± 1.41
*(chlorophyll a)	(1.11 ± 0.05)	(1.51 ± 0.21)	(1.05 ± 0.12)

*Chlorophyll a content (mg/g dry weight) is shown indicating that turion color was not solely dependent on this pigment. NA = Not applicable

Connection between Chapters

In Chapter 2, *Potamogeton crispus* was found to have two different physiologically types of turions. Current season, newly formed, turions are metabolically active throughout the summer, but do not sprout until exposed to autumn photoperiod. Overwintered turions, turions older than one year, are not measurably metabolically active, but are still alive and will sprout when exposed to warm/ cold cycles. Both types of turions are expressing dormancy, although the abiotic signals that release dormancy are different.

Hormonal control of dormancy is unique to the type of dormant structure. Seeds utilize an abscisic acid/ gibberellin relationship; potato tubers utilize an abscisic acid/ cytokinin relationship, while bud dormancy is maintained by strigolactones and auxin. Chapter 3 investigates which hormones are involved in dormancy of *Potamogeton crispus* turions. Are the hormonal controls of the two types of *P. crispus* turions the same because of structural similarity or are they different based on physiology?

Chapter 3 - Hormonal Control of Dormancy of *Potamogeton crispus* L. Turions

Abstract

Hormonal control of dormancy in aquatic plant vegetative propagules is not well understood. Dormancy of these structures has been compared to seeds and buds which have very different hormonal pathways that initiate and maintain dormancy. The majority of *Potamogeton crispus* turions, modified stems, remain dormant over the summer and sprout in autumn, while a small subset of turions remain dormant for an unspecified time. We measured abscisic acid concentrations and sprouting levels in turions that were exposed to various hormones, temperatures, and photoperiods to elucidate hormonal control of dormancy. Newly formed turions sprout in response to changes in ABA and GA levels. A reduction of ABA in new turions is correlated with the breaking of dormancy. Turions older than one year do not sprout in response to ABA or GA changes. The results indicate two different pathways utilized by *P. crispus* turions to maintain and break dormancy.

Introduction

Dormancy in plants functions as a mechanism to extended survival of an individual or a species. Plants may induce both long and short term dormancy for different survival outcomes. Seeds are primarily used for long term dormancy and survival of a species. The duration of dormancy depends on plant type and environmental conditions. Some seeds can remain viable for over 100 years (Telewski and Zeevaart 2002). Aquatic plants are known to have exceptionally long seed dormancy (Shen-Miller et al. 1995). Vegetative propagules such as tubers, rhizomes, and stolons can become dormant in order to overcome seasonal changes that are not conducive to growth, i.e. cold or drought. This short term dormancy is regulated by environmental changes and is induced for an individual's genetic survival.

Seed dormancy is regulated by an antagonistic relationship between gibberellins (GA) and abscisic acid (ABA) that is well documented (Kucera et al. 2005). Within seeds, ABA initiates and maintains dormancy, while activation of the GA pathway results in both testa and embryo rupture causing germination (Kucera et al. 2005). The hormonal relationship in other dormant structures becomes more complex. Potato tubers utilize ABA for dormancy induction and maintenance. However, GA does not affect dormancy. Within tubers, GA is needed for cell elongation after dormancy is broken (Suttle 2004). The antagonistic relationship between ABA and GA is not found in perennial bud dormancy or lateral branching. ABA is needed for cell differentiation,

while GA initiates bud set. However, neither hormone initiates, maintains, or breaks dormancy (Olsen 2010).

Other hormones have been found to impact dormancy. Lettuce seeds will sprout in response to cytokinin after ABA applications (Khan and Downing 1968). Cytokinin will also promote potato tuber sprouting (Suttle 2004). Lateral bud dormancy is controlled by a cytokinin/ auxin relationship (Müller and Leyser 2011). Auxin controls production of strigolactones (Brewer et al. 2009), which have been found to maintain bud dormancy (Gomez-Roldan et al. 2008; Umehara et al. 2008).

Aquatic plants are highly specialized; they have specific mechanisms to overcome problems associated with living under water such as anoxia (Sato et al. 2002), CO_2 acquisition (Engelhardt 2006; Kadono 1980), and reproduction (Best 1979; Catling and Dobson 1985; Smart et al. 1995). The majority of aquatic plants form specialized vegetative structures, such as turions and tubers, for long term dormancy, together with seeds. Both tubers and turions are modified stems with multiple buds; turions form above ground while tubers form under the substrate. Turions can also function in short term seasonal dormancy along with winter buds and rhizomes.

Information regarding hormonal control of dormancy in aquatic vegetative propagules is limited. ABA will induce formation of winter buds of *Myriophyllum* (Weber and Nooden 1976a), *Hydrilla* tubers (Klaine and Ward 1984) and *Spirodela* turions (Smart et al. 1995). GA can retard turion and winter bud formation, while cytokinin will completely block the formation of the dormancy structures (Wang et al. 2012; Weber and Nooden 1976a). Abiotic signals initiating dormancy have been

extensively studied in aquatic plants (Adamec 1999; Agami et al. 1986; Jian et al. 2003; Smart et al. 1995; Van Wijk and Trompenaars 1985). However, the hormone signaling pathway that is triggered by the abiotic signal has not been thoroughly investigated. Dormancy of winter buds of *Myriophyllum verticillatum* was broken upon application of cytokinin, while ABA suppressed sprouting (Weber and Nooden 1976b). *Potamogeton nodosus* winter bud sprouting could be inhibited by large concentrations of ABA (Anderson 1982). These studies lead us to investigate an ABA/GA relationship that may control dormancy in turions of *Potamogeton crispus*. We propose that dormancy in *P. crispus* newly formed turions is controlled by an ABA/GA relationship, while another group of hormones are involved in maintaining dormancy of turions older than one year.

Material and Methods

Adult *Potamogeton crispus*

Lake Sarah, Hennepin County, Minnesota (USA), (T119N, R24W, Sec. 34 & 35) was sampled for *P. crispus* plants and turions. The west end of the lake was sampled in May and June 2012 to coincide with *P. crispus* turion development. This portion of the lake has not been treated with herbicides for *P. crispus* on a large scale. Adult plants were raked from the substrate of the lake. Plants were washed with distilled water (pH 6.5) to remove any epiphytes and insects. They were then categorized by turion development: pre-turion (axillary buds are small and dormant), phase I (axillary buds begin to elongate), phase II (axillary buds begin to thicken and harden), and phase III (axillary buds are mature turions). Each plant was frozen, lyophilized, and analyzed

separately. Before hormone determinations were conducted, plants and axillary buds were separated from each other.

Turions

A ponar grab (Wildco, Wills Point, Texas) and dip-net were used to harvest over-wintered turions in May of 2011and 2012. Turions collected in September of 2008 were also collected using a ponar grab. Turions collected in September of 2008 were sorted by color; green turions were regarded as current season, while brown and orange turions were most likely overwintered. Black turions were found to be dead. Current season turions were collected directly from senesced plants in June of 2011 and 2012 by raking the plants up from the lake and shaking the turions loose. The turions were washed and submerged in distilled water adjusted to pH 7.0 with 0.1 M Tris-HCl. A set of current season (40 ± 10) and overwintered turions (20 ± 5) were immediately frozen after washing to be used for initial measurements. Turions were either stored at 4°C in the dark or placed under experimental conditions. A subset of current season turions (200 ± 50) was stored at 4°C under 16:8 (light : dark) dim light (2.7 µmoles of photons m^{-2} s^{-1}) for six weeks before being placed under experimental conditions. Water was changed each week for experiments and every other week for stored sets, to prevent epiphyte growth, using distilled water adjusted to pH 7.0 with 0.1 M Tris-HCl. Dark stored turions were washed under green light (0.41 µmoles of photons m^{-2} s^{-1}).

Sprouting Assays

To determine the role hormones play in *P. crispus* dormancy, various sprouting assays were conducted. Turions were exposed to different light durations and various compounds that are known to have an impact on dormancy in plants.

Turions were exposed to autumn and summer light durations, 10:14 light: dark and 16:8 light dark, respectively. The 10:14 light box contained two 40-watt Philips fluorescent bulbs providing a photosynthetically active radiation (PAR) value of 28 µmoles of photons m^{-2} s^{-1}. Four 34-watt Philips Econ-o-watt fluorescent bulbs were used to provide 16:8 lighting with a PAR of 23 µmoles of photons m^{-2} s^{-1}. Overwintered turions placed at 15°C were exposed to 10:14 light duration *via* a 30-watt GE U-shaped fluorescent bulb producing a PAR of 30 µmoles of photons m^{-2} s^{-1}. Sprouted turions were counted and removed every 2 days during experiments.

Dark conditions were achieved by wrapping the glass quart jars with paper towel and tinfoil. Turions were placed in the dark at 23°C to mimic turions buried in substrate during the summer, while turions placed in the dark at 4°C mimicked turions during the winter under the ice and substrate. Any sprouted turions under dark conditions were counted and removed once a week under green light.

Various hormones and hormone inhibitors were added in a concentration series to determine roles, if any, on turion sprouting (Supplementary Table 3.1). All hormones and hormone inhibitors were research grade and purchased from Sigma- Aldrich Co. (USA).

The sprouting assays were conducted by filling glass quart jars with 250 ml of ddH_2O. The pH of the water was adjusted with 0.1 M Tris-HCl, pH 7.0 for a final pH of 7.0. Current season or overwintered turions (40 ± 10) were added to each experimental condition. The water was changed and new hormone or inhibitors were added once a week except for ABA, which was changed three times a week. The difference in water changes was based on chemical half-life and epiphyte growth. Current season turions were exposed to experimental conditions for six weeks. Overwintered turions were exposed to experimental conditions for five weeks. All recovery experiments were extended for an additional two weeks after the original experimentation. For recovery experiments, GA_3 and fluridone final concentrations were 10 µM, and 1 µM, respectively.

Abscisic Acid Measurements

Abscisic acid concentrations were determined in turions from light duration sprouting assays and hormone sprouting assays to determine the role ABA may have on maintaining dormancy. Turions were collected before and after exposure to experimental conditions, rinsed with ddH_2O, and frozen. A subset of turions was collected every two days for two weeks during exposure to autumn light. Frozen tissue was then lyophilized. Dry turions were pooled (5 ± 1) together and ground to a fine powder and stored at -20°C until assayed.

ABA was extracted using a modified version of the protocol of Chen and coworkers (1988). All reagents were research grade (Sigma-Aldrich Co., USA or Fisher

Scientific, USA). Ground turion tissue (0.02g ± 0.005) was incubated with 150 µl of homogenization buffer (65:35, propanol: 0.2 M imidazole, pH 7.0) in siliconized tubes. Tubes were mixed for 30 seconds and a known concentration of $^{2}H_4$-ABA was added. Samples were incubated overnight at 4°C. Samples were centrifuged for 10 minutes at 4°C (25 000 x g; 5417R Eppendorf Co., USA). Supernatant was removed and diluted with ddH_2O for a final volume of 1.5 ml.

An amine column (50mg VersaPlate tubes, Agilent Technologies, USA) was conditioned with following series: 600 µl of hexane, acetonitrile, and 0.2 M imidazole pH 7.0, then washed with 1.2 ml of ddH_2O three times. Diluted samples were loaded onto the column by gravity. If samples did not pass through the column within 10 minutes, a vacuum was applied by a manifold (Baker 10 Extraction System, J.T. Baker Chemical Co., NJ, USA). The column was washed with 600 µl each of the following series: hexane, ethyl acetate, and acetonitrile. Samples were eluted using 600 µl of 0.5 M acetic acid. Elute was confirmed to have a pH of 2.5 and then partitioned using 600 µl of ethyl acetate. Crystals of anhydrous sodium nitrate were added to the ethyl acetate fraction to remove any additional water. The aqueous portion was removed from the solid anhydrous sodium nitrate and 400 µl of methanol was added. Samples were then methylated using diazomethane ether as per Barkawi and coworkers (2010).

Methylated samples were suspended in 25 µl of ethyl acetate and injected into a GC-MS triple quad (+EI, Thermo Scientific Trace GC Ultra – TSQ Quantum GC).

Synthesis of ^{2}H – ABA

Deuterated ABA was synthesized as per the protocol outlined by Netting and Lidgard (1999). 10 mg of ABA was dissolved in l M $NaO^{2}H$ and deuterated in D_2O (10 ml) and stirred overnight at room temperature. The reaction was acidified with oxalic acid. ABA was extracted with methyl-t-butyl-ether. The ether supernatant was removed and dried under nitrogen gas. Deuterated ABA was suspended in methanol and the concentration was determined using spectrophotometry.

Results

Adult Plants

P. crispus adult plants and axillary buds (location of turion formation) were analyzed for ABA content as turions developed. ABA levels of phase I plants and elongating axillary buds are approximately 3 times higher than phase III plants and buds (Figure 3.1). The ABA concentration within mature turions (phase III buds) did not change after removal from the adult plant.

Current Season Turions

Light Conditions

ABA concentrations in turions were measured under differing environmental conditions to determine if any changes occurred. When current season turions were placed directly into different light conditions, ABA levels of non-sprouted turions remained unchanged at the end of the six week treatment. ABA concentrations within

the turions dropped significantly after sprouting was visible (Figure 3.2). No changes in ABA concentrations were found in turions stored in the dark at 4°C for up to 14 weeks compared to controls.

In the autumn photoperiod the majority of current season turions sprout during the first two weeks of experimentation. ABA levels were analyzed to determine if a change occurred during a shorter period. Turions were pooled (5 ± 1) to measure ABA concentrations every two days for two weeks under 10:14 (L:D) conditions. A 50% reduction in ABA levels began on the second day after being exposed to autumn light. ABA concentrations continued to drop until day eight. By day ten, ABA concentrations had returned to initial levels (Figure 3.3).

Sprouting levels were previously found to increase by pretreating current season turions with summer light before placing them under autumn light (Chapter 2). Therefore, turions were pretreated with dim summer light for six weeks at 4°C to simulate transition from summer to winter. The pretreated turions were then placed under experimental conditions. An increase in the rate and overall sprouting percentage occurred with pretreated turions compared to non-pretreated turions (Figure 3. 4). The pretreated sprouting rate was similar to current season turions that were collected in autumn (Figure 3.4A). Current season turions that were exposed to 4°C in the dark did not have a change in sprouting compared to field collected turions (Figure 3.4B). After 7 weeks of pretreatment at 4°C in dim light, a drop in ABA concentration was recorded in the non-sprouted pretreated turions compared to field collected turions (Figure 3.5). These lower levels were similar to sprouted turion ABA concentrations (Figure 3.5).

After an additional six weeks in various photoperiods and temperatures, ABA concentrations in non-sprouted pretreated turions did not change significantly (Figure 3.5).

Additions

Exogenous application of hormones and inhibitors was used to determine possible hormone roles in regulation of current season turion sprouting. Various solvents were compared utilizing the sprouting assay to determine if a solvent effect occurred. Methanol was chosen as the general solvent due the least effect on sprouting compared to water. While not significant, methanol (~3 µM) did increase control sprouting slightly (10-20%) in specific replications of the experiment (Supplementary Figure 3.1). ABA additions to current season turions reduced sprouting approximately four-fold (Figure 3.6). The non-sprouted turions took up and sequestered large amounts of ABA; 100 times more ABA was found within the treated turions at the end of the six weeks (Table 3.1). The addition of the ABA syntheses inhibitor, fluridone, increased sprouting by approximately 20 % (Figure 3.6). The ABA concentrations associated with the fluridone addition in the light were reduced to minute levels in contrast to ABA concentrations of fluridone added and incubated in the dark (Table 3.1).

Additions of GA_3 to current season turions increased sprouting both under summer and autumn photoperiods (Figure 3.7). Turion sprouting increased by approximately 32% after an application of GA_3 in the dark at 23°C, in contrast to dark controls (1% sprouting) (data not shown). The ABA concentrations recorded after the end of the six week period for both light and dark GA_3 exposed turions were reduced by

approximately 4-fold compared to the control (Table 3.1). Paclobutrazol, a GA synthesis inhibitor, did not reduce sprouting significantly in autumn light at the end of 6-weeks compared to controls (Figure 3.7). However, when the autumn light experiment was analyzed in time segments, paclobutrazol inhibited sprouting significantly ($P = 0.041$) during the first three weeks of a six week experiment. Sprouting rates of the turions exposed to paclobutrazol then increased during weeks 4 to 6 while control turions sprouted minimally. This delay in sprouting caused overall sprouting levels to be similar to the 6-week control ($P = 0.17$) (Figure 3.7). Turions exposed to paclobutrazol in autumn light had a 6-fold decrease in ABA concentration (Table 3.1). Sprouting levels increased to GA_3 sprouting levels when GA_3 was added to turions exposed to paclobutrazol (Figure 3.7).

The addition of hormones and inhibitors to pretreated turions did not change the overall sprouting patterns. Pre-treated turion sprouting increased with the applications of GA_3 and fluridone, while sprouting decreased with ABA additions (data not shown).

Overwintered Turions

Light Conditions

ABA concentrations in overwintered turions were also analyzed during differing environmental conditions to determine if any changes occurred during sprouting assays. ABA levels found in overwintered turion initials had similar levels to current season turion initials (Table 3.1 vs. Table 3.2). After exposing the turions to differing environmental conditions for five weeks, turions did not show significant changes in

ABA concentrations (Figure 3.9). Similar to current season turions, recently sprouted overwintered turions had reduced ABA levels. No change occurred in ABA concentration of overwintered turions stored at 4°C in the dark for 14 weeks.

Additions

Sprouting percentages of overwintered turion were very high in 2011 (80% - 100%). These high percentages made it difficult to determine if hormone additions enhanced sprouting. Temperature was lowered to determine if alternative sprouting conditions could reduce control sprouting rates without affecting the overwintered turion sprouting pattern or ABA concentrations (Figure 3.10). Therefore, 2012 overwintered turion exogenous hormone sprouting assays were conducted under lower temperatures (15°C) (Figure 3.10).

Dormancy related hormones and hormone synthesis inhibitors were added to turions to determine the possible roles of hormones in the regulation of sprouting. When ABA was added to overwintered turions directly after collection, the experimental turions did not respond differently than the control turions (Figure 3.10). Overwintered turions were place in autumn light for one week before the addition of ABA to determine if turions might require an environmental signal before becoming receptive to ABA. This pre-treatment of the turions did not have an effect on sprouting levels compared to controls (data not shown). No change in spouting was recorded with the addition of fluridone, GA_3, or paclobutrazol to overwintered turions (Figure 3.10).

ABA concentrations in overwintered turions did not change during the 5-week period, nor did the ABA levels change with the addition of ABA (Table 3.2). However,

an approximately 10-fold decrease in ABA was found in overwintered turions treated with fluridone, GA_3, and paclobutrazol under fall light conditions at 15°C (Table 3.2).

Discussion

Turion Formation

ABA mediates many aquatic plant processes such as heterophylly (Anderson 1982; Gee and Anderson 1998; Kane and Albert 1987; Lin et al. 2005), leaf senescence, (Jana and Choudhuri 1982) and turion morphogenesis (Smart et al. 1995). In *P. crispus*, ABA appears to facilitate turion morphogenesis. ABA content changes in the whole plant and axillary buds as the plant matures in early summer. A two-fold increase occurs in the plant itself as axillary buds begin to elongate, while a three-fold increase is found in the buds (Figure 3.1). This increase in ABA during bud elongation may indicate that ABA is being produced to initiate turion formation. The ABA is likely transported from the parent plant into the turions. This transport is similar to the process in seed production where maternal ABA controls seed maturation (Lee et al. 2010). ABA concentrations in both plant and buds decrease as the buds mature and begin to accumulate starch. As turion formation is completed, ABA levels in the buds remain constant while levels in the plants continue to decline (Figure 3.1). ABA within the bud is may now be functioning in turion maturation. Wang and coworkers (2012) reported that application of gibberellin to the plant will delay turion formation. Furthermore, GA application after turion formation has been initiated will prevent starch accumulation and

change turion morphology. These observations indicate an antagonistic relationship between the two hormones in *P. crispus*.

Individual turion formation occurs at varying rates depending on the location of the axillary bud on the parent plant. Turion maturation starts at the bottom of the plant and moves upward (Chapter 1). This variation in location is similar to that observed in *Solanum* tuber formation where individual tuber maturation depends on the distance from the mother tuber (Vreugdenhil and Struik 1989). These variations in formation demonstrate a hormonal continuum in the parent plant rather than a distinct switch between developmental states. A similar hormonal continuum is also found in *Potamogeton nodosus*, where individual leaf age and not plant age determine when heterophylly occurs (Gee and Anderson 1998).

Turions remain metabolically active after abscission (Chapter 2). Any changes in turion ABA concentrations are due to synthesis and/or catabolism within the turion itself. In this respect, turions are similar to seeds where ABA produced in the embryo initiates and maintains dormancy (Kucera et al. 2005), but differ from *Solanum* tubers where both maternal and tuber produced ABA are active in dormancy (Suttle 1995).

Current Season Turions

Turion maturation is required for sprouting. ABA is essential for the maturation of many reproductive structures, such as tubers and seeds (Strunik et al. 1999; Wobus and Weber 1999). *Solanum* tubers are unable to initiate dormancy until they have stored a specific level of starch (Strunik et al. 1999). The turions are not fully mature when they

abscise. Current season turions continue to synthesize and store starch throughout the summer until levels are similar to those found in overwintered turions (Chapter2). The turion maturation process may require ABA. Treating turions with ABA for 6 weeks prior to the addition of $GA_{3,}$ increases sprouting by 20% compared to GA_3 alone (Figure 3.7). Exposure to ABA prior to fluridone additions also results in a similar increase in sprouting (Figure 3.6). These results show that turions may need exposure to ABA for an allotted time period before becoming sensitive to ABA or GA changes. GA sensitivity in *Arabidopsis* seeds in the field does not occur until after ABA levels have remained at an active level for six months and then drop below a particular threshold (Footitt et al. 2011). The small percentage of turions that sprout in the first ten days after abscising from the parental plant may have been the first turions to form, and been exposed to ABA for the allotted time in order to reach maturity while still on the parental plant.

In the northern hemisphere, current season turions are dormant throughout the summer (Catling and Dobson 1985; Wehrmeister and Stuckey 1992); however turions are metabolically active (Chapter 2). We propose that a light signal is required to break summer dormancy. This light signal indicating autumn initiates a hormone cascade that promotes sprouting under specific environmental conditions similar to seeds (Kucera et al. 2005). The artificial breaking of summer dormancy in the laboratory via low light and temperature (4°C) mimics the sprouting response that occurs in autumn (Figure 3.4). A reduction in ABA concentrations also occurs after the pretreatment with dim light and cold temperature (4°C) (Figure 3.5). A decrease in temperature (4°C) in the dark alone does not further affect sprouting or ABA concentration compared to turions field initials

(Figure 3.4 & Table 3.1). These data indicate that both abiotic signals of light and low temperatures are required to induce high levels in sprouting and the reduction in ABA (Figure 3.4 & 3.5) that occurs naturally in autumn.

The primary signal is light. Light pretreatment conditions do not fully rescue sprouting to field levels, 30-40 % verses 60-70% respectively (Chapter 2), suggesting that a specific type of light exposure may be needed to induce sprouting or a secondary signal is needed. A possible secondary signal is temperature. Chilling is a common method used by many types of seeds to break dormancy. However in nature, turions are not exposed to cold water temperatures before the breaking of summer dormancy. Therefore, the vernalization requirement in some seeds to break dormancy does not apply to turions. Nonetheless, water temperatures do change from warm to cold. The signal cascade could occur once the water temperature drops past a particular threshold, triggering sprouting. In *Ceratophyllum*, stem dormancy is broken by a decrease in water temperature (Best 1979). In the laboratory, turions experience this change from warm to cold at the beginning of the cold treatment, but it is a dramatic shift from 23°C to 4°C, not the gradual one found in nature. When the trigger begins a signal cascade, the metabolic processes are too slow at 4°C to induce sprouting until water temperatures are greater than 11°C (Catling and Dobson 1985). The order of the signals cannot be switched. When turions were chilled and then exposed to light there was no change in turion response compared to current season turions directly from the field. Therefore, current season turions are responding primarily to light changes and secondarily to temperature changes.

ABA concentrations decrease only when turions sprout (Figure 3.2). Furthermore, a reduction of ABA concentration is correlated with breaking dormancy during autumn light exposure. A small percentage (10 - 15 %) of turions break summer dormancy in the first two weeks after abscission from the parental plant. Within current season turions ABA concentrations begin to decrease by day two of exposure to autumn light conditions to levels found in sprouted turions. After day 10, ABA concentrations in the non-sprouted turions return to dormancy levels (Figure 3.3). Differing light and changing temperatures do not induce further changes in ABA concentration between week two and week six (Figure 3.2) nor do any large turion sprouting events occur suggesting that ABA is responsible for maintaining summer dormancy

ABA as a component of summer dormancy is further substantiated by turion ability to take up exogenous ABA (Table 3.1). Turions respond to ABA additions with a reduction in sprouting (Figure 3.6). Therefore, turions are sensitive to an ABA signal. In a lake, exogenous ABA maybe available in the substrate where it leaches slowly out of decomposing plants. Seeds that imbibe ABA from plant litter maintain longer dormancy (Krock et al. 2002). Exogenous ABA may aid in long term dormancy of some turions. In pretreated current season turions there is an increase in ABA in the dark at 23°C compared to the turions exposed to light (Figure 3.5). This increase may indicate a reduction of ABA catabolism in the dark. This mechanism would be important for continued dormancy of current season turions that were exposed to a summer light signal and then buried in the substrate.

ABA turnover occurs in current season turions. The addition of fluridone to turions under autumn light conditions causes a decrease in ABA concentration (Table 3.1) and stimulates sprouting (Figure 3.6). Fluridone, a commonly used aquatic herbicide, (Madsen et al. 2002; Pedlow et al. 2006; West et al. 1979) competitively inhibits phytoene desaturase which catalyzes the formation of zeaxanthin, a precursor for carotenoid and ABA synthesis. Fluridone activity is clearly light dependent (Bartels and Watson 1978) as no changes in either ABA concentration or sprouting occurred in turions in the dark (Table 3.1). Zeaxanthin is also a precursor to other hormones known to have impacts on dormancy (López-Ráez et al. 2008; Matusova et al. 2005). Consequently there may be other hormones regulating dormancy in conjunction with ABA within the current season turions such as strigolactones (López-Ráez et al. 2010).

An increase in sprouting occurs with the addition of GA_3 in both summer and autumn light (Figure 3.7). GA_3 applications also result in a reduction of ABA levels both in light and dark conditions (Table 3.1). The reduction of ABA levels after application of GA_3 to turions is similar to the reduced ABA concentrations found in sprouted turions (Table 3.1). Gibberellins can promote a reduction in ABA concentrations by up-regulating gene expression of ABA 8'-hydroxylase, a catabolic enzyme (Sawada et al. 2008). This correlation suggests that gibberellins act to reduce ABA levels. Therefore, we hypothesize GA synthesis in turions is required to break dormancy and stimulate sprouting.

The effect of paclobutrazol on turion dormancy is complex, but supports the requirement of GA for sprouting. Paclobutrazol under autumn light significantly reduces

sprouting ($p=0.03$) during the first three weeks of experimentation compared to controls (Figure 3.8). However, sprouting rates during the second half of the experiment accelerate causing overall sprouting to be similar to control levels after six weeks (Figure 3.8). Turions also have a reduction in ABA concentration after 6-weeks with the application of paclobutrazol (Table 3.1). Paclobutrazol, a commonly used gibberellin inhibitor, inhibits gibberellin synthesis via *ent*-kaurene oxidase, a cytochrome P450 monooxygenase. The inhibitor is nonspecific triazole (Fletcher et al. 2000). Triazoles are known to inhibit ABA 8'-hydroxylase causing elevated ABA levels (Fletcher et al. 2000; Kushiro et al. 2004). The initial delay in sprouting and the inability of decreased ABA concentrations to break dormancy alone suggests that GA synthesis is required for sprouting. The accelerated sprouting during the second half of the experiment could indicate the activation of a secondary sprouting mechanism. As modified stems, turions may respond to various apical bud sprouting signaling pathways such as ethylene and sugar (Ruttink et al. 2007) after the primary sprouting mechanism is inhibited. The change in sprouting rate could also occur because *P. crispus* turions may either metabolize paclobutrazol or produce more *ent*-kaurene oxidase to counter act inhibition of active GA synthesis. Paclobutrazol does increase chloroplast production in *Solanum* and *Zea* (Sopher et al. 1999; Tsegaw et al. 2005) where *ent*-kaurene oxidase is located. The shift in sprouting rate is not due to paclobutrazol degradation because the half-life of paclobutrazol in water is 24 days (Chand and Lembi 1994) and paclobutrazol was refreshed every week during experiments.

The delayed sprouting behavior with paclobutrazol could also be explained by an increase in turion sensitivity to GA. If this is the case, the shift in sensitivity occurs at week three leading to increased sprouting and the decline in ABA content found in non-sprouted turions. Sensitivity and response strength to GA varies in barley based on cell location and cell type (Ritchie and Gilroy 2000). In *Salix* stems, light duration has been shown to impact GA sensitivity (Junttila and Jensen 1988). GA sensitivity has also been shown to cycle over time within dormant seeds correlated with external signals (Footitt et al. 2011). Changes in GA sensitivity have been linked to environmental differences that occur during seed formation (Debeaujon and Koornneef 2000).

We propose that current season turions require both maturation and abiotic signals to break dormancy in the autumn. Current season turions abscise from the parental plant with an inhibitory level of ABA. ABA concentrations remain constant while other biological processes occur such as starch production. When turions have reached maturity, they become sensitive to light and temperature signals. Summer photoperiod may initiate GA synthesis. GA slowly begins to up regulate ABA catabolism. As the rate of catabolism exceeds the rate of production, turions are primed to sprout. Then autumn photoperiod and a reduction in temperature starts a signal cascade that results in synthesis of a higher active level of GA, which initiates sprouting. Both a decrease in ABA and an increase in GA levels are required for this process to occur. Other hormones besides ABA and GA may be involved in the primary signal cascade or may comprise a secondary signaling pathway. Hormones such as cytokinin (Appendix D) and auxin (Sastroutomo 1981) have been found to positively impact turion sprouting. Both

hormones are involved in tuber sprouting (Hartmann et al. 2011; Suttle 2004), and seed germination (Liu et al. 2007; Riefler et al. 2006). Cytokinin also impacts *P. crispus* turion formation (Wang et al. 2012). The remaining un-sprouted turions (40%) overwinter and have a different physiology and sprouting mechanism.

Overwintered Turions

Overwintered turions are in deep dormancy. The turions (40-80%) sprout in response to an increase in temperature (15°C- 23°C) after being under the ice (4°C) (Chapter 2). ABA is not the main factor in maintaining dormancy of overwintered turions. Endogenous ABA in non-sprouted turions does not change in response to the changes light and temperature (Figure 3.9). Also, the overwintered turions do not sprout when ABA concentrations decrease (Figure 3.10 & Table 3.2), nor do they sequester exogenous ABA, like current season turions (Table 3.1 & 3.2). After sprouting, a decrease in ABA is detected (Figure 3.9 & Table 3.2). With these results it is not possible tell if the decrease in ABA causes sprouting or if the sprouting is activating ABA catabolism. ABA turnover appears to be occurring because fluridone applications lead to a reduction in ABA levels (Table 3.2). Therefore, ABA may be functioning in turions similar to *Populus* buds where ABA is utilized in bud formation but not dormancy initiation or maintenance (Olsen 2010).

GA_3 applications give similar results to fluridone with a reduction of ABA concentration without the increase in sprouting compared to controls (Figure 3.10 & Table 3.2). Gibberellins may still trigger ABA catabolism without breaking dormancy

unlike current season turions. Approximately a quarter of cold response genes have been found to be GA regulated. Also cold temperatures (4°C) have been found to up regulate GA synthesis genes (Yamaguchi 2008). The ABA/GA ratio, not the total concentrations of the hormone, regulates plant functions. The uptake of exogenous hormones varies depending on timing and endogenous levels (Kucera et al. 2005), which may account for why exogenous ABA does not appear to be taken up, but exogenous GA leads to a decrease in ABA concentration.

Paclobutrazol applications also show that turions are insensitive to ABA; ABA concentrations decrease significantly upon application without an increase in sprouting (Figure 3.10 & Table 3.2). Paclobutrazol may cause an increase in ABA concentration by inhibiting ABA catabolism (Fletcher et al. 2000). However, a reduction in ABA concentration has been found within some species upon application of paclobutrazol. This reduction in ABA has been attributed to different growth conditions, application methods, and differing species (Fletcher et al. 2000). In *Solanum* and *Zea* plants, paclobutrazol applications have also been found to increase carotenoid accumulation and cytokinin production (Sopher et al. 1999; Tsegaw et al. 2005). An increase of cytokinin has been found to correlate with a decrease in ABA activity (Khan and Downing 1968). These results may indicate paclobutrazol is either inhibiting carotenoid breakdown which is used to form ABA precursors or cytokinin is involved in turion ABA regulation.

Deep dormancy of overwintered turions is determined by the first autumn after formation. These turions reach and remain at a steady concentration of ABA until dormancy breaks unlike *Solanum* tubers which slowly catabolize ABA over time (Suttle

1995). Non-sprouted overwintered turions maintain ABA levels similar to non-sprouted current season turions (Table 3.1 & 3.2). Carbohydrates of overwintered turions are also maintained at a steady state similar to current season turion levels produced by the first autumn (Chapter 2). Within these turions, neither changes in light and temperature, nor exogenous applications of ABA or GA, impact dormancy. However, these turions can still perceive a temperature signal (Chapter 2), which may activate a secondary sprouting pathway different from current season turions. A portion of the population of overwintered turions sprout (60 -80%) once water temperature rises high enough for enzyme activity (11 – 15°C) irrespective of light duration. The non-sprouted turions are known to be alive because multiple cold/warm cycles will eventually cause sprouting (data not shown). Overwintered turions have been observed to sprout in the field after cycling through warm/cold temperatures over several years (Newman personal communication). The varying degrees of dormancy preprograming in seeds has been linked to the environment of the maternal plant, such as cold temperatures during seed development causing deeper dormancy (Roach and Wulff 1987). The differences between years and microenvironments during growth and development of turions could account for the large variation found in turion dormancy. This variation would aid the plant in maintaining establishment during stressful conditions such as herbicide treatments and drawdowns.

Conclusion

P. crispus turions have at least two possible dormancy syndromes. Current season turions are in shallow dormancy. This dormancy is characterized by turions that are metabolically active and receptive to hormone signaling. ABA and GA play a significant role in shallow dormancy. These turions quickly sprout in response to environmental stimuli compared to overwintered turions. It is unclear if non-spouted current season turions transition from shallow dormancy to deep dormancy or if deep dormancy is predetermined at formation. Overwintered turions conversely are in deep dormancy. These turions are not metabolically active and do not sprout in response to exogenous hormones. Overwinter turion dormancy is not primarily controlled by ABA or GA. The dormancy state can be maintained for many years.

Having turions in different states of dormancy is advantageous to *P. crispus*. Turions are clones of the parent plant and lack genetic diversity to aid in species plasticity. However the different syndromes in dormancy allow the species to sprout under varied conditions and still invade new habitat. If conditions are harsh and kill a large portion of the population, there are still turions dormant and ready to sprout once environmental conditions improve.

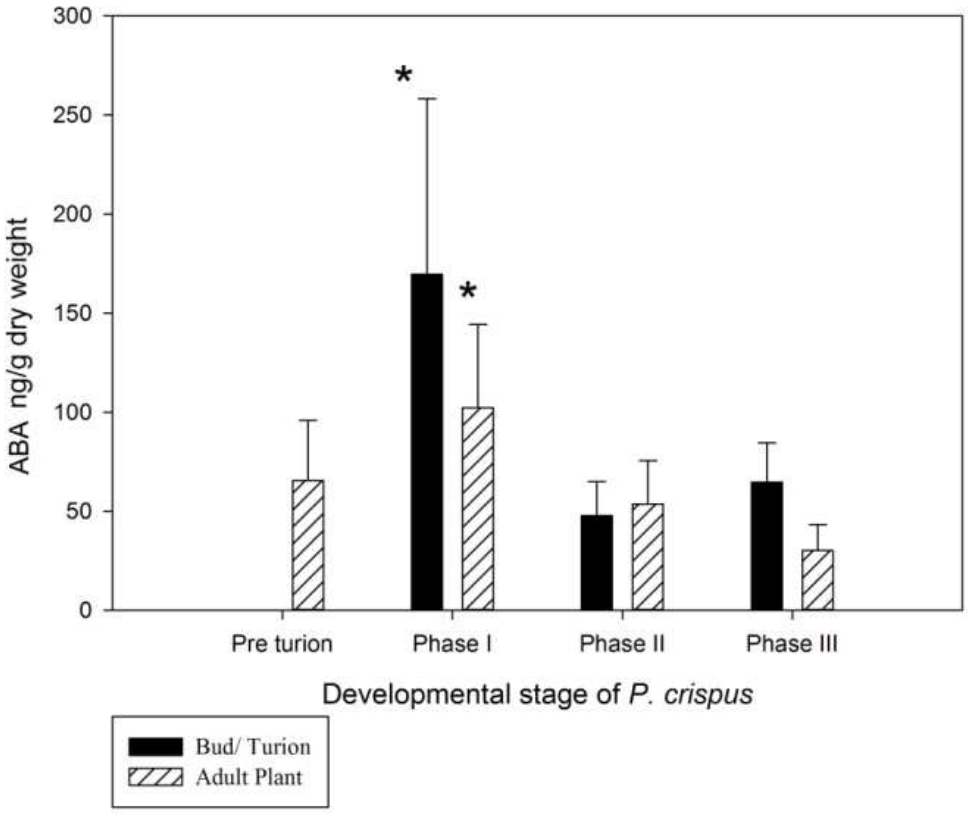


Figure 3.1. ABA concentrations (ng/g dry weight) in *P. crispus* plants and buds at different developmental stages of turion formation. Plants are categorized by turion development: pre-turion (axillary buds are dormant), phase I (axillary buds begin to elongate), phase II (axillary buds begin to thicken and harden), and phase III (axillary buds are mature turions). *Concentration of ABA in Phase I buds and plants significantly differed from all other growth stages ($p<0.01$ and $p<0.04$ respectively) except pre-turion development. Experiment repetitions = 3.

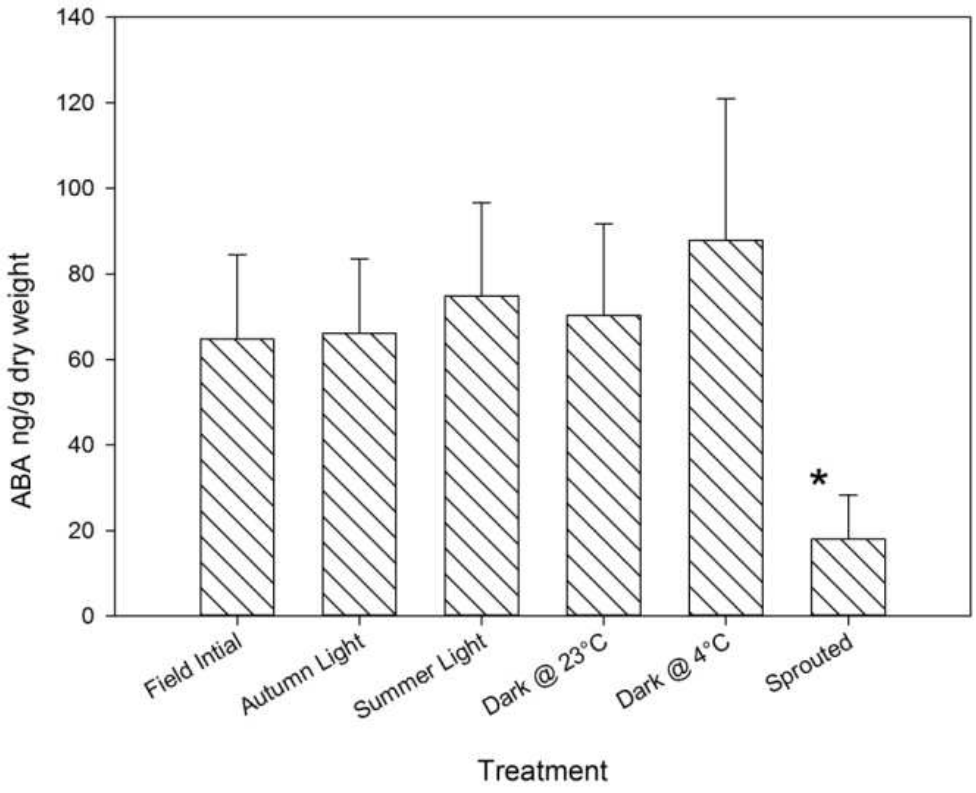


Figure 3.2. ABA concentrations (ng/g dry weight) of current season turions after exposure to differing environmental treatments. Current season turions were exposed to various light and temperatures for 6-weeks. The autumn light duration was 10:14 (L:D) and summer light duration was 16:8 (L:D). *Only sprouted turions had a significant difference in ABA levels (p=0.002) compared to controls after 6-weeks. Experiment repetitions = 3.

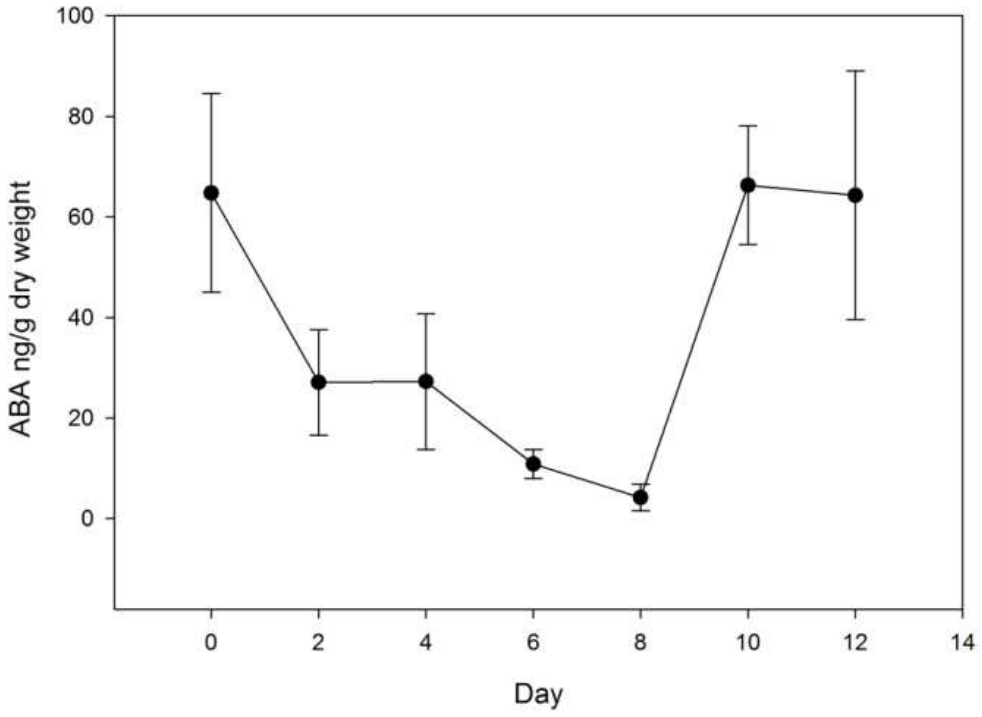


Figure 3.3. ABA concentration (ng/g dry weight) of current season turions during the first 14 days of sprouting. Turions were placed under autumn light conditions for 14 days and analyzed every two days. Non-sprouted turions were pooled for the assay (5 ± 1). ABA levels of the pool dropped significantly by day two and remained low until day 8. Between day 8 and 10, pooled ABA concentrations returned to intial levels. Experiment repetitions = 3.

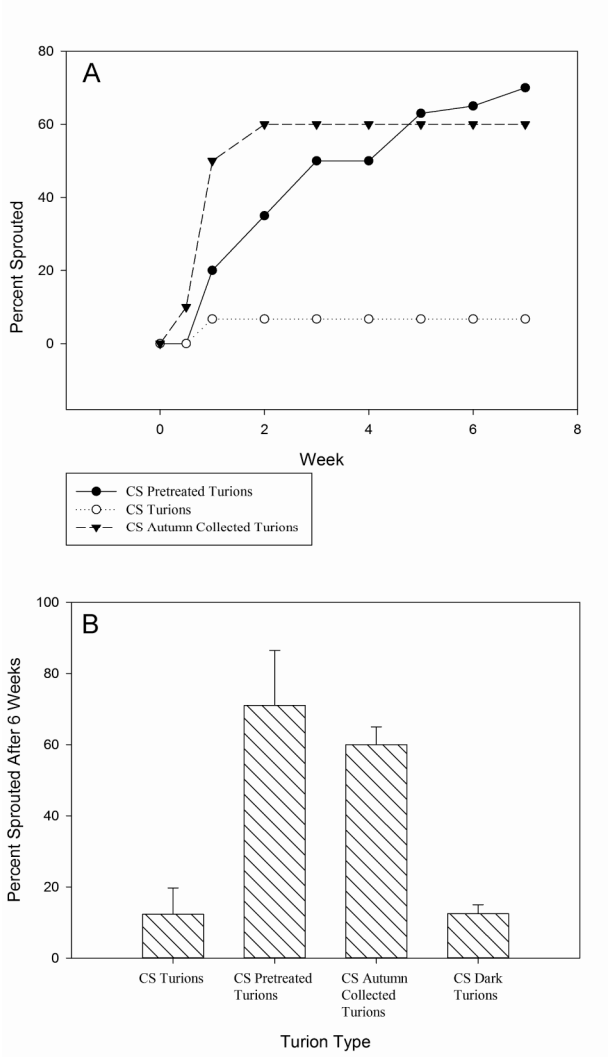


Figure 3.4. Rate comparison and overall sprouting of differing types of current season turions (CS). All sprouting was conducted under autumn light conditions (10:14; L:D). A) Sprouting rate comparison (% sprouted/week) between different types of current season turions. Pretreating turions returned sprouting rates to CS Autumn Collected levels. B) Overall percent sprouting of differing types of current season turions after 6-weeks. CS Pretreated Turions were exposed to dim light and 4°C for 7-weeks prior to exposure to autumn light. CS Autumn Collected Turions were collected in September. CS Dark Turions were stored at 4°C in the dark for one year prior to sprouting. Experiment repetitions = 3

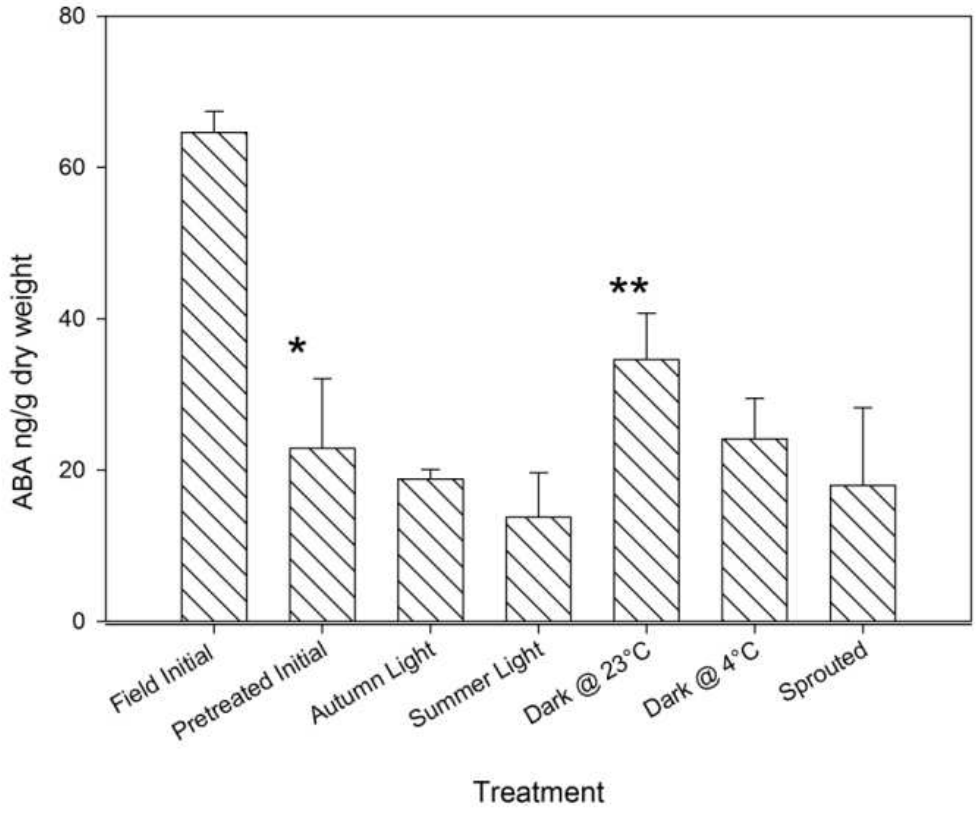


Figure 3.5. ABA concentrations (ng/g dry weight) in pretreated current season turions after exposure to differing experimental conditions. A subset of current season turions, Field Initial, was analyzed after collection in June. Another subset of current season turions was pretreated with dim light at 4°C for 7-weeks. Pretreated Initial turions were analyzed after the pretreatment. Pretreated turions were exposed to various light and temperature conditions for an additional 6-week period. The autumn light duration was 10:14 (L:D) and summer light duration was 16:8 (L:D). *A significant decrease ($p < 0.0001$) in ABA levels was found in turions after pretreatment. **ABA levels of dark at 23C turions was significantly different than light exposed (Autumn and Summer) turions. No significant change in ABA concentration occurred in different environmental conditions compared to pretreatment controls.

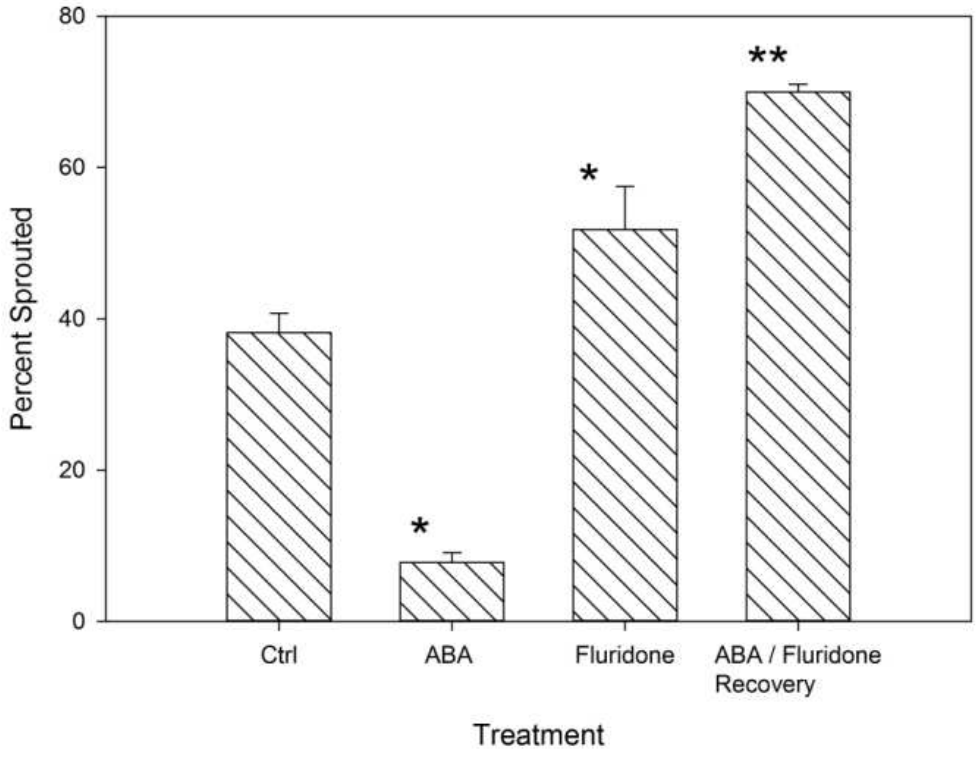


Figure 3.6. Sprouting effect of ABA and fluridone additions to current season turions. All compounds were dissolved in 100% methanol. Methanol (~3 µM) was added to control turions to account for any solvent effect. The final concentrations of ABA and fluridone were 10 µM and 1 µM respectively. *Exogenous ABA decreased sprouting significantly (p= 0.004) while fluridone increased sprouting (p= 0.075). **A increase in sprouting occurred when turions were exposed to both ABA and fluridone after a 6-week exposure to ABA alone compared to just a fluridone addition. Experiment repetitions = 3

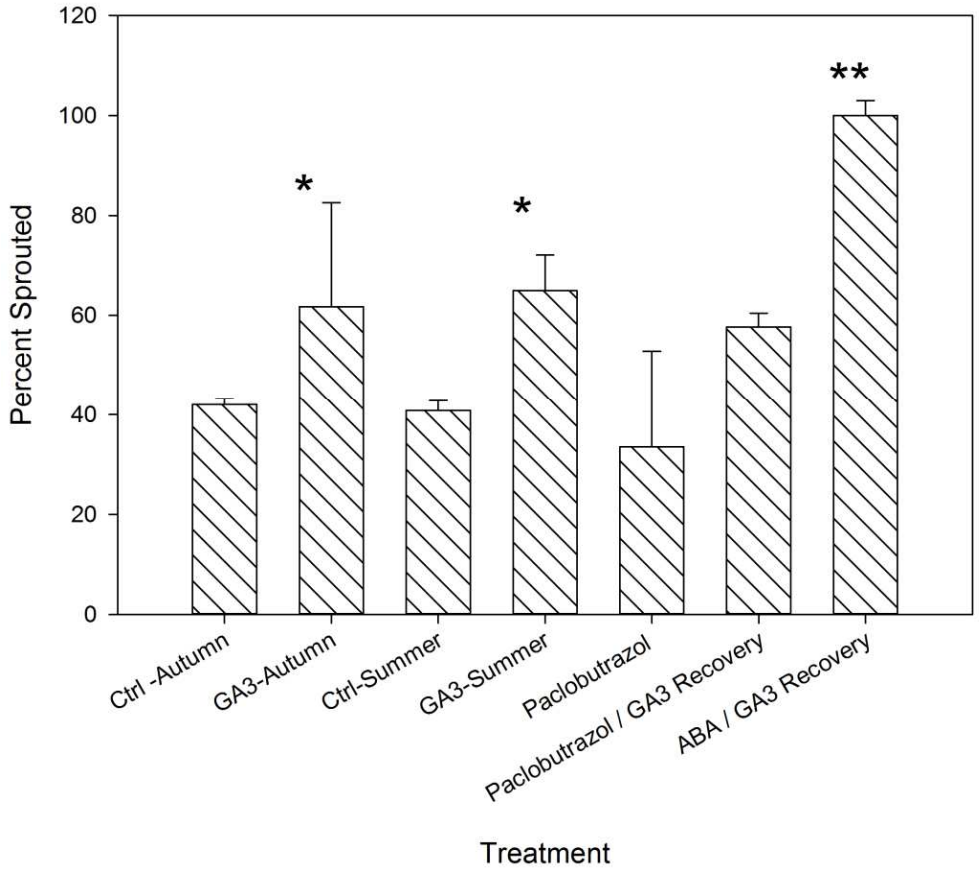


Figure 3.7. Sprouting effect of GA_3 and paclobutrazol additions to current season turions. All compounds were dissolved in 100% methanol. Methanol (~3 µM) was added to control turions to account for any solvent effect. The final concentrations of GA_3 and paclobutrazol were 10 µM and 50 µM, respectively. *Sprouting increased significantly when turions were exposed to GA_3 compared to controls in both summer (16:8, L:D) and autumn light (10:14, L:D). **An increase in sprouting occurred when turions were exposed to both ABA and GA_3 after a 6-week exposure to ABA compared to GA_3 applications. No overall change in sprouting occurred after 6-weeks in turions exposed to paclobutrazol. An increase in sprouting occurred when GA_3 was added to turions after the 6-week exposure to paclobutrazol.

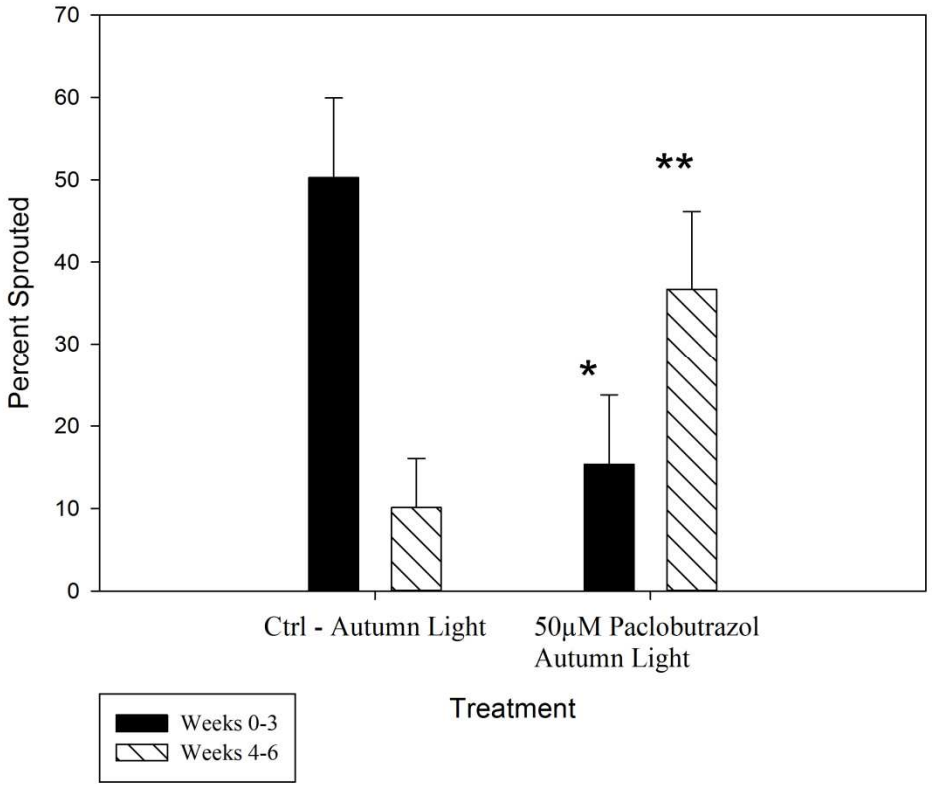


Figure 3.8. Current season sprouting percentages by week of exposure to paclobutrazol. Paclobutrazol was dissolved in 100% methanol. Methanol (~3 µM) was added to control turions to account for any solvent effect. Turions were exposed to 50 µM of paclobutrazol for 6-weeks. Sprouting percentages were separated into two time categories, 0-3 weeks and 4-6 weeks. *Paclobutrazol exposure significantly reduced (p= 0.030) spouting in the first 3 weeks compared to controls. **During weeks 4-6, sprouting significantly increased compared to controls (p= 0.040). Therefore, the overall sprouting at the end of a 6-week period was similar between control turions and paclobutrazol exposed turions. Experiment repetitions = 3

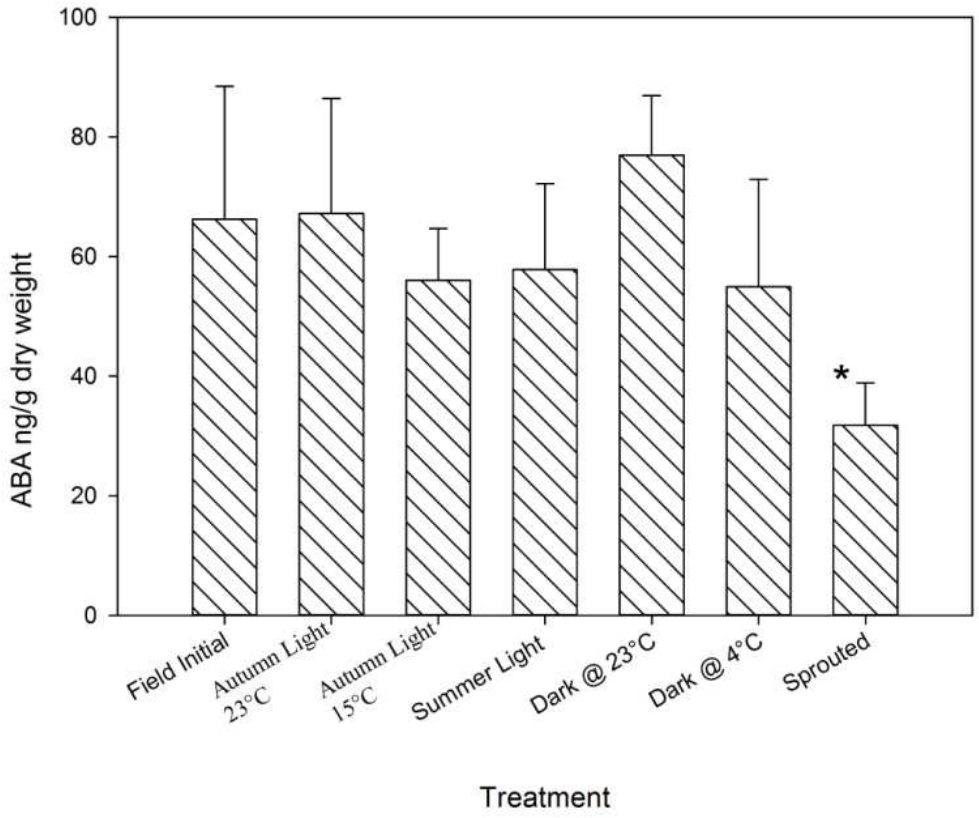


Figure 3.9. ABA concentrations (ng/g dry weight) of overwintered turions after exposure to differing environmental treatments. Overwintered turions were exposed to various light and temperatures for 5-weeks. The autumn light duration was 10:14 (L:D) and summer light duration was 16:8 (L:D). *Only sprouted turions had a significant difference in ABA levels ($p=0.0013$) compared to controls. Experiment repetitions = 3

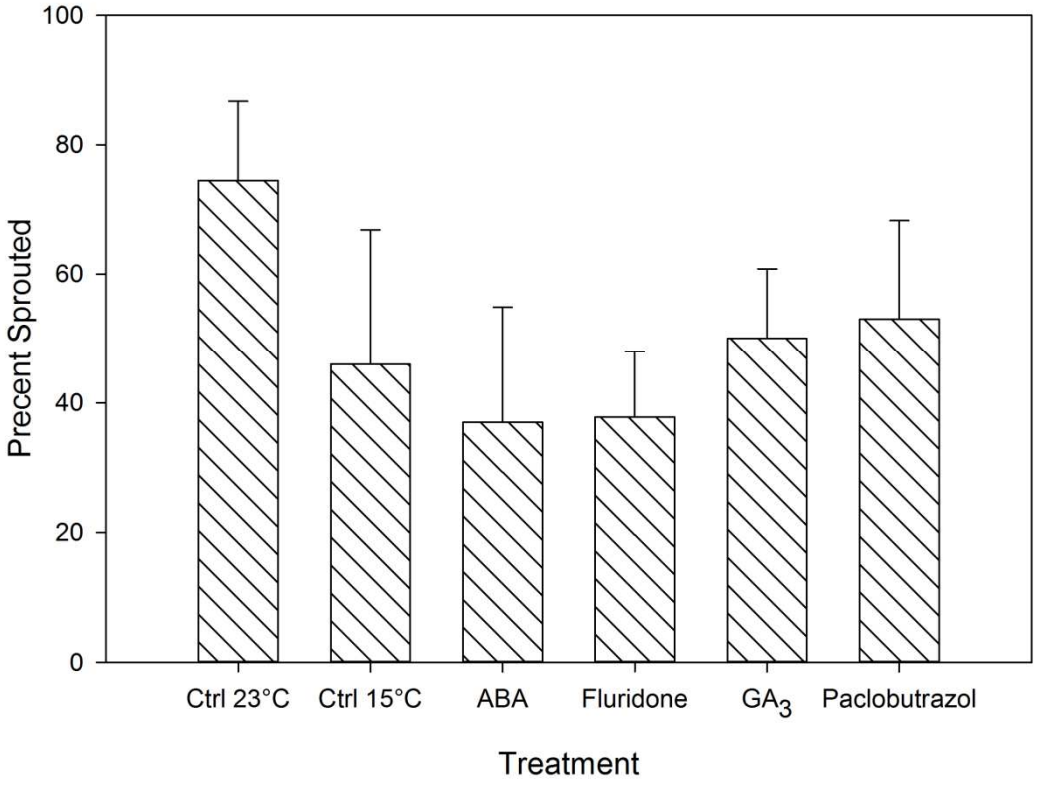
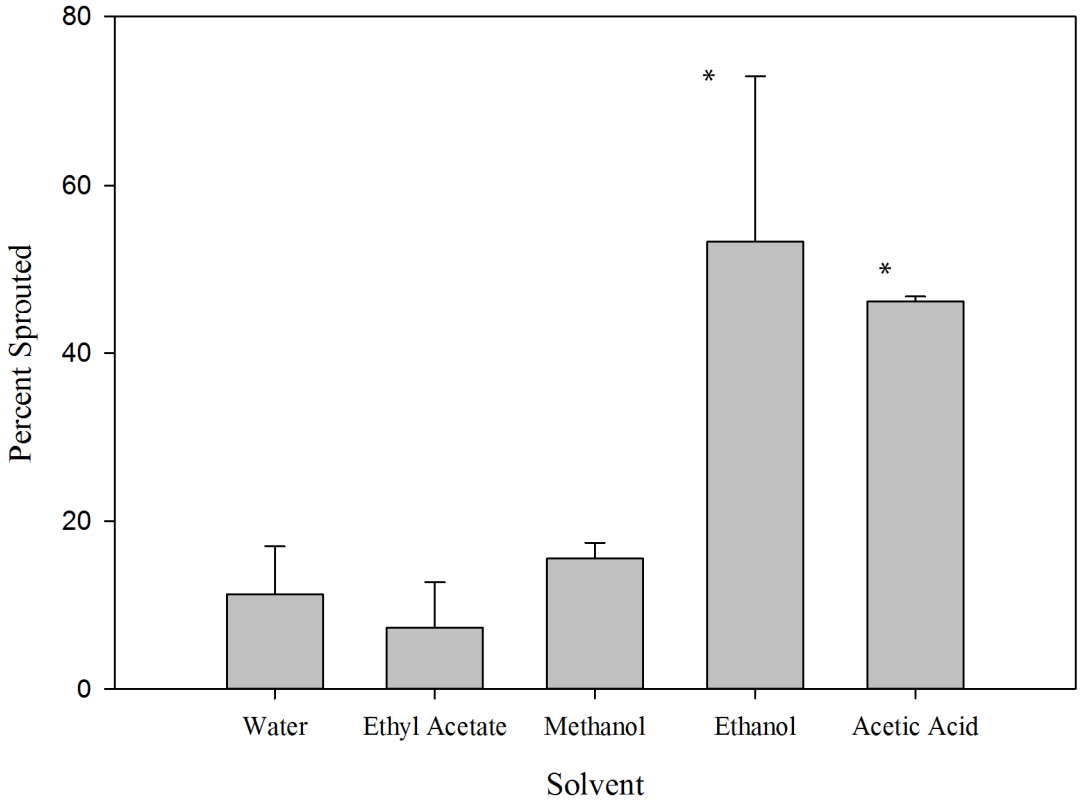


Figure 3.10. Overwintered turion sprouting associated with hormone and inhibitor additions. There was no significant difference in sprouting between 23°C and 15°C; however there was a slight reduction in sprouting with 15°C. All hormone additions were performed at 15°C. Methanol (~3 µM) was added to control turions to account for any solvent effect. The final concentration of ABA and GA_3 was 10 µM; paclobutrazol and fluridone concentrations were 50 µM and 1 µM, respectively. Hormones and inhibitors had no significant impact on sprouting. Experiment repetitions = 3



Supplementary Figure 3.1. Current season turion sprouting upon application of different solvents. No significant increase in sprouting occurred with turions exposed to methanol or ethyl acetate. However, samples containing ethyl acetate were variable. *Ethanol and acetic acid increased sprouting significantly (p=0.003). Methanol was chosen because of the similar sprouting profile to water and small range of sprouting variability.

Table 3.1. ABA concentrations in current season turions. Different hormones and inhibitors were added to current season turions. A significant increase in ABA concentration occurred in turions with the application of ABA ($p<0.00001$). Significant decreases in ABA concentration were found after the application of fluridone in the light, GA_3, and paclobutrazol ($p<0.001$). Experiment repetitions = 3

Addition	**Initial (ng/g dry wt.)**	**Final (after 6 weeks) (ng/g dry wt.)**
None (Autumn Light)	64.79 ± 19.72	66.11 ± 17.37
Sprouted (Autumn Light)	NA	17.98 ± 10.27
ABA (10 µM)	64.79 ± 19.72	703.67 ± 212.59
Fluridone (1 µM – Light exposed)	64.79 ± 19.72	4.09 ± 1.64
Fluridone (1 µM – Dark exposed)	64.79 ± 19.72	83.68 ± 29.11
GA_3 (10 µM – Light exposed)	64.79 ± 19.72	17.08 ± 12.93
GA_3 (10 µM – Dark exposed)	64.79 ± 19.72	17.85 ± 12.85
Paclobutrazol (50 µM)	64.79 ± 19.72	10.30 ± 1.68

Table 3.2. ABA concentrations of overwintered turions. Different hormones and inhibitors were added to overwintered turions. Significant decreases in ABA concentration were found after the application of fluridone in the light, GA_3 or paclobutrazol ($p<0.001$). Sprouted turions also had a significant decrease in ABA concentrations compared to non-sprouted turions ($p< 0.01$). Experiment repetitions = 3

Addition	Initial (ng/g dry wt.)	Final (after 5 weeks) (ng/g dry wt.)
None (Autumn Light)	66.25 ± 22.2	56.06 ± 8.64
Sprouted (Autumn Light)	NA	31.75 ± 7.08
ABA (10 µM)	66.25 ± 22.2	59.20 ± 18.08
Fluridone (1 µM – Light exposed)	66.25 ± 22.2	5.48 ± 2.62
GA_3 (10 µM – Light exposed)	66.25 ± 22.2	9.77 ± 5.97
Paclobutrazol (50 µM)	66.25 ± 22.2	7.21 ± 4.17

Supplementary Table 3.1. Hormone additions to turions. Final serial concentrations of solvents added to turions.

Hormone/Inhibitor	Stock	Final Concentrations
GA_3	0.1 M in MeOH	1µM, 10µM
ABA	0.1 M in MeOH	1 µM, 10 µM
Fluridone	0.01 M in MeOH	1 µM, 0.5 µM, 0.25 µM
Paclobutrazol	0.013 M in MeOH	5 µM, 10 µM, 50 µM

Chapter 4 - Concluding Remarks

Summary

Invasive species, both terrestrial and aquatic, have large economic and environmental impacts (Knight and Hauxwell 2009). Management of invasive plants in freshwater ecosystems focuses on the control of adult plants (Foley 1997; Johnson et al. 2012; Madsen et al. 2002). The mechanisms that control vegetative reproduction have largely been ignored as possible population controls. Our understanding of aquatic vegetative propagule dormancy is limited compared to terrestrial seed and tuber dormancy. Not all propagule information is transferable from terrestrial to aquatic environments because of the unique challenges that occur in aquatic environments. This lack of knowledge impedes aquatic managers from manipulating desirable and undesirable plant populations effectively. This work was undertaken to better understand the vegetative reproductive physiology and dormancy of *Potamogeton crispus* to be able to target the reproductive structures of this and other invasive species without affecting the native populations.

Potamogeton crispus develops two different types of turions, each with its own physiology. Current season turions are metabolically active, confirmed by the production and storage of additional starch throughout the dormancy period (Chapter 2). The plant hormone, abscisic acid (ABA) most likely maintains dormancy of these turions and may aid in turion maturation (Chapter 3). Current season turions sprout in response to photoperiod (Figure 2.2 & 2.5). There may be a secondary sprouting pathway that is initiated when the primary mechanism is inhibited (Figure 3.8).

The second type of *P. crispus* turion, overwintered, is categorized as turions over one year of age. These turions have no measurable metabolic activity (Chapter 2). ABA does not influence dormancy regulation (Chapter 3). Turions sprout in response to temperature changes (Chapter 2). The hormonal mechanisms that maintain overwintered or innate dormant turions remain unclear and should be explored further.

We found that increases in ABA concentration correlate with turion formation in *P. crispus* (Figure 3.1) similar to *Spirodela polyrhiza* turions (Appenroth 2010). ABA is also known to initiate formation of potato tubers (Suttle 2004) and seed dormancy and maintenance (Kucera et al. 2005). If ABA initiated maturation of turions is occurring on the parental plant, like potato tubers, or after dehiscence is unknown.

Management Implications

Management of *P. crispus* infestations must take into account that turion beds contain two physiological different populations of turions. Woolf and Madsen (2003) recommend early season herbicide treatments to prevent turion accumulation, but only address the current season turion population and not the overwintered turions still present in the substrate.

During early season fluridone herbicide treatments of *Myriophyllum spicatum*, managers have noticed *P. crispus* population explosions during the autumn (Madsen et al. 2002). Our data suggest that fluridone is impeding ABA maintenance within current season turions and increasing autumn sprouting rates, thus the population explosions. *P. crispus* turion response to fluridone could be exploited by treating lakes with fluridone in early spring, then in the autumn, treat adult plants with traditional herbicides. This dual

herbicide treatment schedule would result in sprouting of any current season turions that formed and prevent any new turions from entering the pool of dormant turions, thus reducing the amount of turions that potentially sprout in subsequent years.

Lake managers may also regulate turion production in early spring with ABA inhibitors such as fluridone and abamine. Abamine, a more recent ABA synthesis inhibitor, inhibits 9'-cis-neooxanthin dioxygenase specifically targeting ABA synthesis (Han et al. 2004) and should be investigated further as a possible dormancy specific herbicide.

Future Directions

With the finding of two physiologically different populations of turions in *Potamogeton crispus* several questions arise concerning the relationship between the two populations and more detailed questions concerning each population.

The first question is, when do the two turion populations differentiate from one another and the second, what triggers that differentiation. *P. crispus* current season turions are observed to have a population physiology split during the first autumn after development; approximately 60% of turions sprout while the other 40% remain dormant (Chapter 2). Whether sequential stages of dormancy are expressed by any aquatic vegetative propagule is unknown. The majority of aquatic vegetative propagules studied express imposed dormancy, while environmental and hormonal controls of pre-dormancy and innate dormancy remain relatively unstudied. Aquatic vegetative propagules may need to move from pre-dormancy to imposed dormancy and then to innate dormancy.

However dormancy type may be dictated at formation by the parental plant's environmental exposure.

Turion formation was observed to move from the bottom of the plant upward. An increase in ABA concentration was correlated with initiation of turion development (Chapter 3). A link between the location of turion formation and dormancy may occur. To determine if turion formation plays a role in dormancy type, turions should be separated based on location and subjected to the experimental conditions detailed in Chapters 2 and 3.

More detailed questions regarding current season turion dormancy arose with our finding that turions broke summer dormancy in response to photoperiod (Chapter 2). Light quality required for current season sprouting remains unknown. The light quality reaching a soil substrate is different and complex in aquatic environments compared to terrestrial environments. *P. crispus* is commonly found in hypereutrophic waters where most native aquatic plants cannot grow due to the reduced light quality. During the turion summer dormancy period, the amount and quality of light reaching the substrate is reduced due to increases in phytoplankton populations. The classical two-state photo-equilibrium model for phytochromes (i.e. red: far red dependence for seed germination) could play a part in sprouting, although, blue light may also play a role since it is able to reach greater depths than red light (Ragni and Ribera D'Alcalà 2004; Smith 1982). Light spectra and PAR measurements should be surveyed at the substrate in a lake over two – three years and compared to physiological changes in turion dormancy within that environment.

A decrease in ABA concentrations was correlated with current season sprouting events in the laboratory (Chapter 3). A reduction of ABA levels in turions that have sprouted compared to non-sprouted was recorded in both current season and overwintered turions (Chapter 3). Our studies were unable to determine if the drop in ABA concentration is causing sprouting or if sprouting causes the reduction in ABA. Therefore, ABA concentrations should be monitored in the field and then further experimentation based on those results should be explored to determine causation.

My studies concentrated on current season turions while only conducting basic experiments on overwintered turions. Overwintered turions sprouted in response to increase in temperature (Chapter 2). It is unclear if this phenomenon is due to the breaking of dormancy when temperatures rise or if dormancy was broken at an earlier unspecific time and the sprouting mechanisms are inhibited by low temperatures. Within lakes in Minnesota, water temperatures have two periods of rapid temperature change, spring and autumn. The timing of these temperature changes correspond with *P. crispus* growth and turion sprouting. Whether overwintered turions sprout in the spring under field conditions is unknown. Further investigation should be conducted into the correlation between lake temperature changes and dormancy in both current season and overwintered turions.

No changes in ABA concentrations occurred in overwintered turions over a 5-week period. The same was found for current season turions until ABA measurements were taken during a sprouting event. ABA concentrations should be measured during the

large sprouting event that occurs two –three days after exposure to increased temperatures.

Overwintered turions sprouted in response to cytokinin (Appendix D) and a possible secondary sprouting mechanism was suggested in current season turions (Chapter 3). Hormones involved in apical dominance may be the key to understanding the overwintered turion dormancy mechanism. This direction of research should be investigated if field and laboratory experiments lack a correlation between ABA concentration and sprouting events.

Additional questions arose during the experimentation conducted for this dissertation. During the flooding of the 2012 growing season, *P. crispus* was observed to halt turion formation and the apical stems elongated. When the water receded to normal levels, the aborted turions reformed with a more stick-like appearance (Appendix A). There were no changes in ABA concentrations during the aborted formation or reformation (Table A.1). Ethylene triggers cell elongation in aquatic plants, which is the opposite of the effects that are observed in terrestrial plants. Ethylene also causes reorientation of microtubules within the cells (Osborne 1984). The morphological changes in *P. crispus* turions may have been regulated by ethylene production and should be further investigated.

Turions with a "stick-like" form have been observed to maintain dormancy in the substrate longer than "bur" shaped turions (Johnson, personal communication). The link between shape and dormancy is unknown. Turion dormancy based on form may also be linked to hormonal conditions present at the time of formation. Environmental

conditions of the parental plants have been known to impact seed dormancy (Debeaujon and Koornneef 2000), while epigenetic expression of certain physiological characteristics in offspring is activated by environmental conditions of parents (Richards et al. 2010). The surrounding environment may direct genetic shifts in dormancy. These relationships should be investigated further.

While culturing *P. crispus* turions in the laboratory, epiphyte growth was a concern. Each year different types of epiphytes (microorganisms) were seen growing on the turions. As of now, the type of relationship between *P. crispus* and microorganisms remains undefined. The microorganisms could be detrimental to the propagule by promoting decomposition. This relationship might explain the high tannin content found in turions that do not sprout in the first year (Table 2.5). However, some microbes might be beneficial by either having a commensal or mutualistic relationship. The microbial community might even promote innate dormancy and/or protection based on the type of dormancy the turions present. The relationship between turions and microorganisms is of interest since many plant species have been shown to require symbiotic relationships for successful growth. If microbial communities are specific to a dormancy type, then the communities could be used to define specific stages of dormancy in aquatic vegetative propagules.

There are many questions in the aquatic vegetative propagule story (Chapter 1). The abiotic and hormonal switches between types of dormancy remain a mystery. Currently early season chemical treatments are used in Minnesota and Wisconsin because most of the native species are still dormant (Johnson 2010). A more in depth

understanding of vegetative propagule dormancy of one species (i.e. *Potamogeton crispus*) might create understanding of another invasive plant's dormancy that could be exploited to create new management techniques.

A more targeted approach for invasive plant control can only be reached with precise physiological data of the target species. This dissertation was undertaken to advance the knowledge of *P. crispus* turion physiology and dormancy controls to aid future researchers and lake managers in creating more species specific management plans.

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Appendix A - Turions Formation During Flooding

Purpose

Potamogeton crispus plants developed similarly independent of seasonal climate variation year to year. However, turion shape was observed to change with water level fluctuations. The following images are a pictorial observations and descriptions of turion formation with water level variations.

Materials and Methods

Potamogeton crispus growth and turion formation was observed between May and June of 2009 – 2012. Water levels fluctuated during turion development in 2012. Plants, turions, and elongating stems were collected and analyzed for ABA content according to Chapter 3 protocols.

Results

During the 2012 *P. crispus* spring growing season Lake Sarah (Dakota County, MN) experienced record flooding. On May 16th 2012 many *P. crispus* plants were at the water surface (Figure A.1). Plant beds were in the process of flowering. Type I plants ranged between pre turion formation and stage II. Many of the type II plants were at stage III with large terminal turions.

Four very large thunderstorms moved through the area between May 20th and May 27th. By May 30th the lake water level had risen to the lake's 100 year flood mark. Only Eurasian water milfoil (*Myriophyllum spicatum*) reached the water's surface; the

tops of *P. crispus* plants were below 2 meters of water (Figure A.2). At this time, axillary buds at pre-turion formation to stage II of turion formation began to elongate and form lateral branches (Figure A.3). ABA levels of the lateral branches were comparable to pre-turion and stage II ABA formation levels. (Table A.1). Fully formed turions toward the base of the plant did not elongate unlike the upper buds of the plant (Figure A.3).

By June 8th, 2012 the lake levels had returned to normal levels. The expansion of new lateral branches halted. Turion formation continued through stages I-III on the newly formed lateral branches. ABA levels remained constant. The turions that resulted were not compact, but elongated and stick like (Figure A.4).

Discussion

Turion formation has been studied in both lentic and lotic environments. Sauvageau (1894) noted two different forms of *Potamogeton crispus* turions, bur-like and elongated. However, no change in turion structure has been attributed to water fluctuations. *Potamogeton crispus* turion development is dependent light (Jian et al. 2003; Sastroutomo 1981). However, formation of turions has not been found to occur in water temperatures less than 13 °C even if light conditions are favorable (Sastroutomo 1981), this phenomenon is possibly due to ideal temperatures for specific enzymatic activities.

Under flooded conditions photosynthetic light conditions change, and the diffusion rates of CO_2 and O_2 become less (Jackson 2008). Plants recognize the change

in depth by the change in the ratio of red: far red light with differing photoreceptors (Ragni and Ribera D'Alcalà 2004). This change most likely signals a halt of turion formation, and then stems elongation of apical buds.

Based on the levels of ABA found in both in the plants and the apical buds, ABA does not play a main role halting of formation or the re-initiation of turion formation. However, ethylene is known to promote stem elongation under flooded conditions (Jackson 2008). Under these conditions it has been found that the cells are GA dependent for the ethylene response. Therefore, ethylene may play an important role in the interruption of turion formation.

The ethylene produced suppresses the turion formation mechanism until either the plant reaches the surface or the water levels return to original levels. At which point, ethylene levels are reduce and the inhibition is removed reinstating turion formation in the original stem location. Because the stems have been elongated, the resulting turions are thinner and longer (Figure A.4).

Further investigation of the flooding response should be investigated. The physiological response might be exploited for management use.

Table A.1. ABA concentrations (ng/g dry wt.) of adult plants and axillary buds at differing stages of turion development. NA = Not applicable

Stage of Turion Formation	Plant ABA Concentration (ng/g dry wt.)	Axillary Bud ABA Concentration (ng/g dry wt.)
Pre-turion	65.56, ± 30.27	NA
Stage I	102.18, ± 42.2	169.65, ± 88.56
Stage II	53.69, ± 21.84	47.73, ± 17.19
Stage III	30.11, ± 13.1	64.79, ± 19.72
Lateral Branch Formation	71.00, ± 26.58	NA

Figure A.1. *Potamogeton crispus* at the water surface May 16, 2012. Many of the plants were developing flowers and forming turions. Plants ranged from pre turion formation to Stage III turion formation. A) Healthy *P. crispus* bed at water surface. B) Flower tops breaking the surface of the water in a large *P. crispus* bed.

Figure A.2. *Potamogeton crispus* on May 30, 2012. The tops of *Potamogeton crispus* that were at the surface of the water two weeks earlier were now two meters under water. Only *Myriophyllum spicatum* could reach the water surface.

Figure A.3. ***Potamogeton crispus* collected on May 30th, 2012.** A) Fully formed turions near the base of the plant remained dormant. B) Axillary buds that had not completed turion formation elongated forming lateral branches.

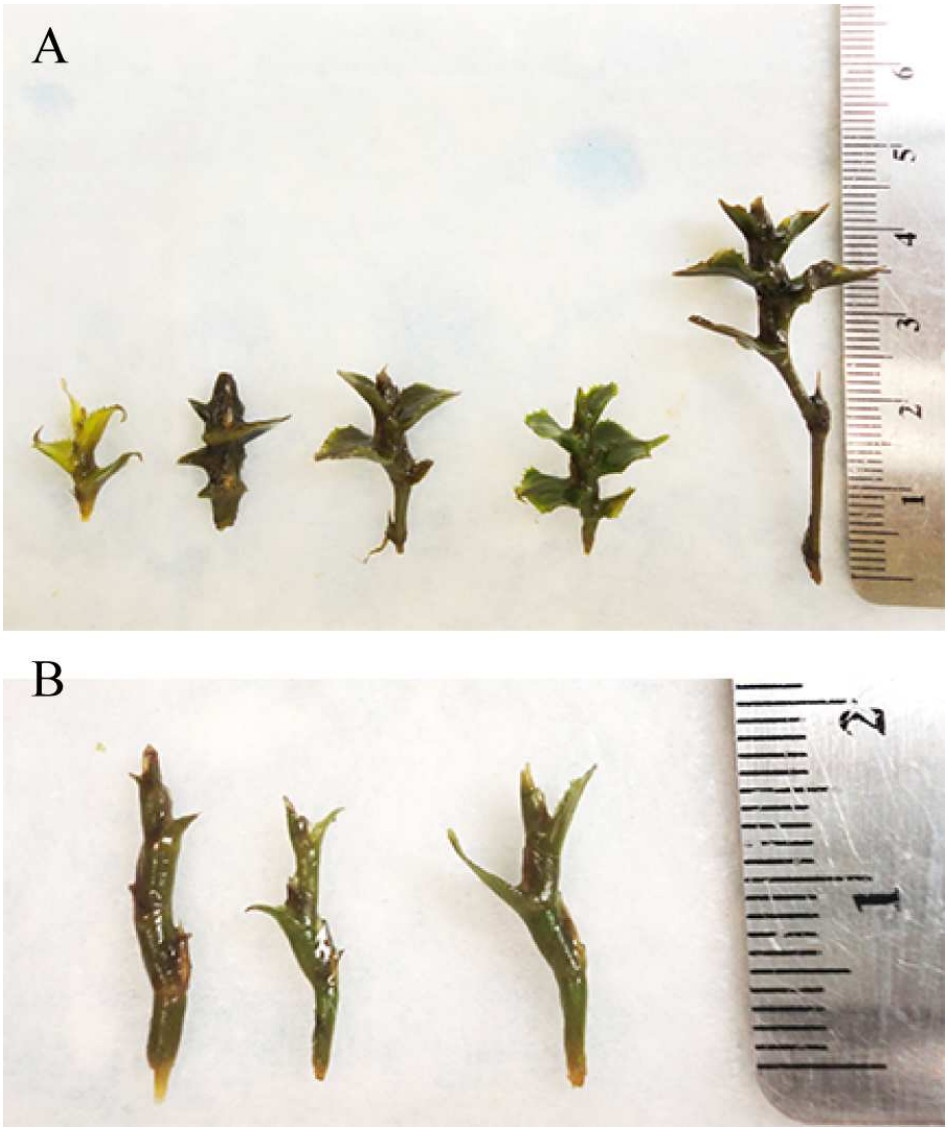


Figure A.4. Current season turions formed in 2012. A) Current season turions formed before flooding (May 16th, 2012). B) Current season turions formed from elongated stems after flooding (June 8th, 2012).

Appendix B - Turion Storage Verification

Purpose

Verify that storage methods for both overwintered and current season turions have minimum effects on turion physiology compared to field collected turions.

Materials and Methods

Overwintered and current season turions collected as per Chapter 2 and 3. Once in the laboratory, overwintered turions were washed with distilled water under green light (0.41 µmoles of photons m^{-2} sec^{-1}). Current season turions were triple washed with distilled water under normal light conditions. Both types of turions were either placed under experimental conditions or stored at 4 °C in complete dark until experimentation. Water was changed for all stored turions every two weeks under green light. Turions were placed under various environmental conditions and sprouting levels were compared. Carbohydrates, chlorophyll, and ABA levels were also measured and compared.

Results

No sprouts were seen in overwintered turions stored for up to 14 weeks. After a year of storage less than 10% sprouted in 2010, and 0% in 2011, and 2012. ABA levels were measured in 2012 overwintered turions stored (Table B.1). While ABA levels did not change significantly over time, 75 - 100% of overwintered turions sprouted when they were placed into 10:14 light conditions at 23 °C.

Current season turion ABA concentrations did not change significantly while in storage a 4 °C in the dark for up to eight weeks (Table B.2). No current season turions were seen to sprout for up to one year in storage. When a sub-set of the turions stored for one year was place under various light durations the sprouting levels were similar to turions that sprouted directly from the field (Figure B.1)

Discussion

Overwinter turions sprout in response to increase temperature with a small response to photoperiod, while current season turions sprout in response to photoperiod (See chapter 2). No changes ABA concentrations were found in any turions stored at 4 °C in complete darkness. Therefore, both overwintered and current season turions were stored at 4 °C in complete dark to prevent or minimize possible metabolism in the event additional experimentation was needed at a later date.

Table B.1. ABA concentrations (ng/g. dry wt.) of stored overwintered turions.

Time in storage	ABA Concentration (ng/g dry wt.)
Initial	66.25, ± 22.05
4 weeks	59.20, ± 18.07
8 weeks	70.26, ± 1.23
14 weeks	63.62, ± 28.90

Table B.2. ABA concentrations (ng/g dry wt.) of stored current season turions.

Time in storage	ABA Concentration (ng/g dry wt.)
Initial	64.79, ± 19.72
4 weeks	64.56, ± 16.59
8 weeks	66.06, ± 1.03

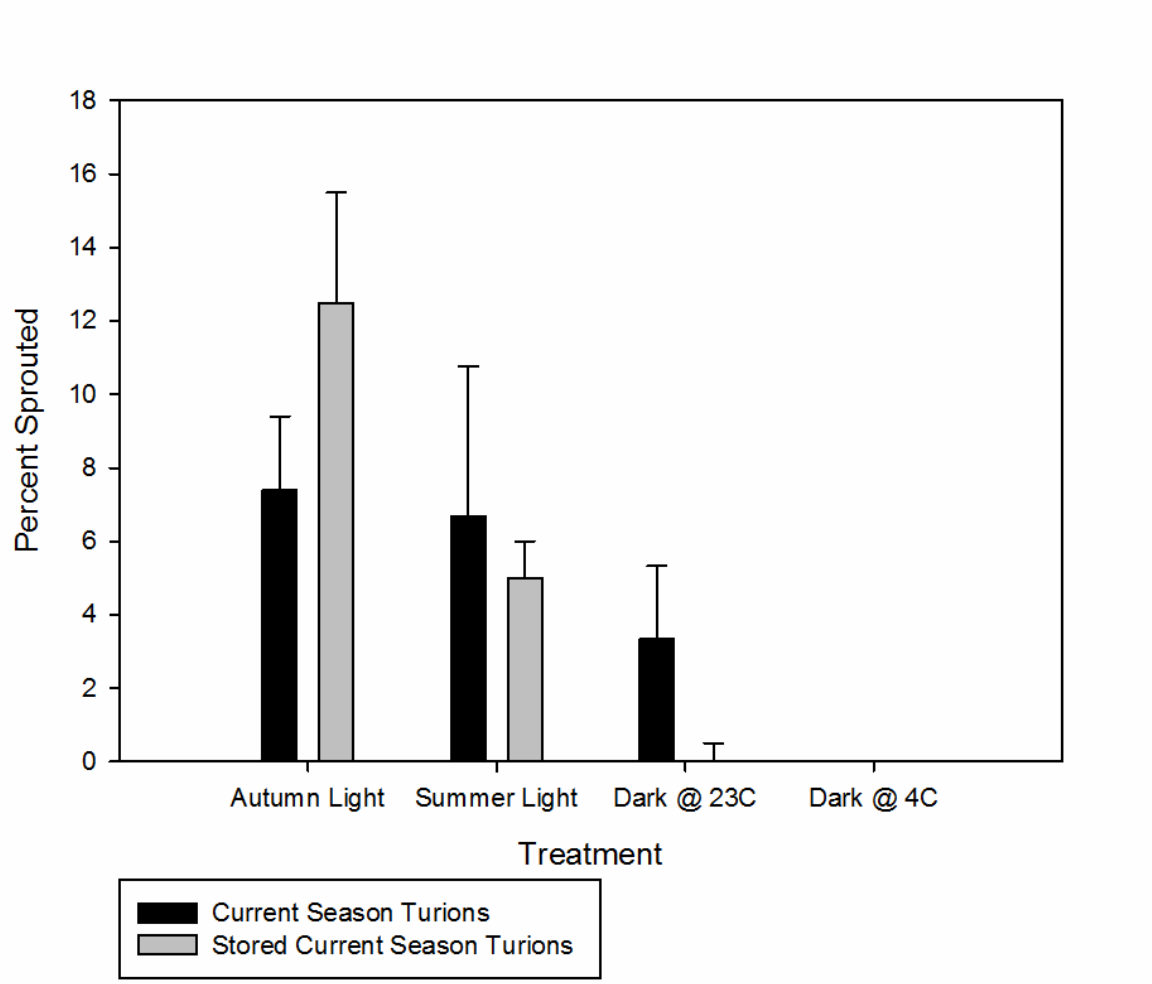


Figure B.1. Current season turion sprouting of field collected turions verses current season turions stored at 4 °C in the dark for one year. No significant difference in sprouting was recorded.

Appendix C - ABA Extraction Protocol Verification

Purpose

Verify ABA extraction protocol for efficiency.

Methods

ABA was extracted using a modified version of Chen and coworkers (1988). 150µl of homogenization buffer (65:35, propanol: 0.2M imidazole, pH 7.0) was diluted with ddH_2O for a final volume of 1.5 ml. 12 ng of ABA standard was added to the liquid, and then loaded onto a conditioned NH_2 column (50mg VersaPlate tubes, Agilent Technologies, USA) as per the protocol listed in Chapter 3 methods. Flow-through was collected at sample loading and each wash step of the protocol. Aqueous flow-through fractions were adjusted to pH 2.5 with 0.1M HCl, then liquid partitioned with ethyl acetate to remove ABA from aqueous solution. The ethyl acetate supernatant was dried down with N_2 before adding methanol. The hexane, acetonitrile wash fractions, and 50% methanol: 1% acetic acid test elution was also dried down. 600µl of methanol was added to each dried sample before methylation.

Ground turion tissue (0.02g ± 0.005) was incubated with 150µl of homogenization buffer (65:35, propanol: 0.2M imidazole, pH 7.0). Samples were incubated overnight at 4°C with 12 ng of ABA standard. Samples were centrifuged for 10 minutes at 4°C (25,000 rcf; 5417R Eppendorf Co., USA). Supernatant was removed and diluted with ddH_2O for a final volume of 1.5 ml. Samples were loaded onto

condition NH2 columns as per the protocol listed in Chapter 3. Only elute and non-aqueous washes were collected, methylated, and measured.

Plant test samples were found to have excess plant metabolites contributing to a large amount of background noise to the original 50% methanol: 1% acetic acid test elution. Therefore, 0.5 M acetic acid elute of the NH_2 column with liquid partitioning and a C18 column (50mg VersaPlate tubes, Agilent Technologies, USA) were tested to further purify samples. The C18 column was conditioned with 600 µl of methanol then loaded, using gravity, with a 0.5M acetic acid elute fraction from the NH_2 column. If any sample remained unloaded after 10 minutes a vacuum was applied (Baker 10 Extraction System, NJ, USA). ABA was eluted off with 100% methanol.

Each collected fraction was spiked with 3.3 ng of $2H_4$-ABA before the addition of diazomethane. The fractions were methylated and measured on a single quad GC-MS (Agilent 5973/6890).

Using an isotope dilution formula (Figure C.1) the amount ABA standard collected was determined and percent sample recovery was measured.

Results/Discussion

No ABA was found in the loading or hexane wash flow-through. Minimal amounts of ABA were released from the column when washed with ethyl acetate and acetonitrile, 2% & 3% respectively. Eluting the column with 100% methanol was not acidic enough to release ABA. Acidified methanol (50% methanol: 1% acetic acid) and 0.5M acetic acid were able to release large amounts of ABA from the amine column,

89% & 77% respectively. Additional ABA was lost when the 0.5M acetic acid fraction was applied to a C18 column. Less plant metabolite contamination was found in the plant test sample when using the 0.5 M acetic acid elute verses the 50% methanol elute or further purification through a C18 column. While 50% methanol: 1% acetic acid elution recovered the most ABA, removing the water from the sample proved time consuming prior to methylation. Therefore, 0.5 M acetic acid elute with ethyl acetate liquid partitioning was chosen for the final protocol (Table C.1).

$$y = (Co / C_f - 1)x / R$$

$C_f = Am/z194 / (Am/z190 + Am/z194)$; $C_o = 0.989$; x=concentration of $^2H_4$-ABA; R= $(Am/z190 / Am/z190 + Am/z191 + Am/z192 + Am/z193 + Am/z194 + Am/z195 + Am/z196) / (Am/z194 / Am/z191 + Am/z192 \ldots + Am/z196)$

Figure C.1. Isotope dilution formula adapted from Barkawi et al. (2010).

Table C.1. Recovery rates from each wash step in the ABA extraction protocol. N=3

WASH	% RECOVERED
Load	0%
Hexane	0%
Ethyl Acetate	2%
Acetonitrile	3%
100% Methanol	2%
50% Methanol: 1% Acetic Acid	89%
0.5 M Acetic Acid	77%
C18 100% Methanol	55%

Appendix D - Additional Hormone Responses

Additional experiments were conducted to address questions/ hypotheses that were not added to the main body of the dissertation.

GA_3 Additions/ Inhibitors-

Purpose

Determine if GA_3 is produced and/or receptive under different light durations.

Methods and Materials

Current season turions were collected as per Chapter 3. Turions were divided into four subsets. The first subset was placed into 10:14 (PAR: 28 µmoles of photons m^{-2} sec^{-1}), 16:8 light duration (PAR: 23 µmoles of photons m^{-2} sec^{-1}), dark at 23°C, and dark at 4°C with a final concentration of 10 µM GA_3. The second subset of turions was placed in 10:14 and 16:8 with a final concentration of 50 µM paclobutrazol. A third subset of current season turions was placed into 16:8 light for three weeks with a final concentration of 50 µM paclobutrazol. Turions were then wash free of the paclobutrazol and placed into 10:14 light for an additional four weeks. The fourth subset of turions was placed in 16:8 light for three weeks with just distilled water (pH adjusted with 0.1 M Tris-HCl pH 7.0 for a final pH of 7.0). After three weeks turions were washed and placed into 10:14 light with a final concentration of 50 µM paclobutrazol. Subsets one and two were conducted for six weeks, while subsets three and four were conducted for a total of seven weeks. For every subset solvent controls were conducted at each chemical

or light change. Each individual variable (jar) contained 20 ± 5 turions and was replicated three times.

Results

Turions in the first subset exposed to GA_3 increased in overall sprouting compared to controls. There was no difference between the increased response in summer light (16:8) verses autumn light (10:14). Current season turions even responded to GA_3 in the dark at 23 °C. No sprouting response was recorded for turions exposed to 4 °C in the dark (Figure D.1). Current season turions decreased in overall sprouting when exposed to paclobutrazol (subset two). However, the decrease was significantly greater in turions placed in the summer light duration (Figure D.2). The third subset of turions had an initial drop in sprouting during the first three weeks exposed to paclobutrazol. When the turions were washed and moved to autumn light conditions turions began to sprout within three days. The overall sprouting level of the treated turions became equal to the level of the control that sprouted within the first three weeks in the summer light duration (Figure D.3). Subset four the reverse experiment to subset three, had the same amount of sprouting occur during the summer light duration, but a drop in sprouting in the autumn light duration once the paclobutrazol was added (Figure D.4).

Discussion

Turions are receptive to GA_3 directly after senescence from the parental plant as long as the water temperature is conducive to sprouting (Figure D.1). Turions had a

greater sprouting response under lighted conditions. This phenomenon could be caused by the increased production of endogenous gibberellin under lighted conditions in concert with the addition of GA_3. If we assume that paclobutrazol is affecting the ent-kaurene oxidase in the GA biosynthesis, then current season turions produce more gibberellins under long day conditions compared to short day (Figure D.2). However, turions do have the ability to increase production of gibberellin to a set amount during short days if a gibberellin synthesis is inhibited during long days (Figure D.3 & D.4).

Cytokinin and Auxin Additions-

Purpose

Determine if turion dormancy was related to apical dominance dormancy hormones.

Methods

Current season turions were collected as per Chapter 3. Turions were divided into two subsets; one set of turions (30 ± 5) were exposed to kinetin, and the other to NAA. Kinetin an adenine type cytokinin **(**6-Furfurylaminopurine**)** was purchased from Sigma-Aldrich Co. (USA). Kinetin was dissolved in 0.1M acetic acid and was added at various final concentrations (10 µM, 1 µM, 0.1 µM) to the **t**urions. NAA (1-naphthaleneacetic acid), a synthetic auxin, was purchased from Sigma-Aldrich Co. (USA) and dissolved into 70% ethanol. The stock solution was added to the jars one day before turions were added to minimize ethanol's impact on turion physiology. The final concentrations for

NAA experiments were 0.01 µM, 0.1 µM, 1 µM. Waterand chemicals were changed three times a week for both kinetin and NAA based on the half-life of the chemical.

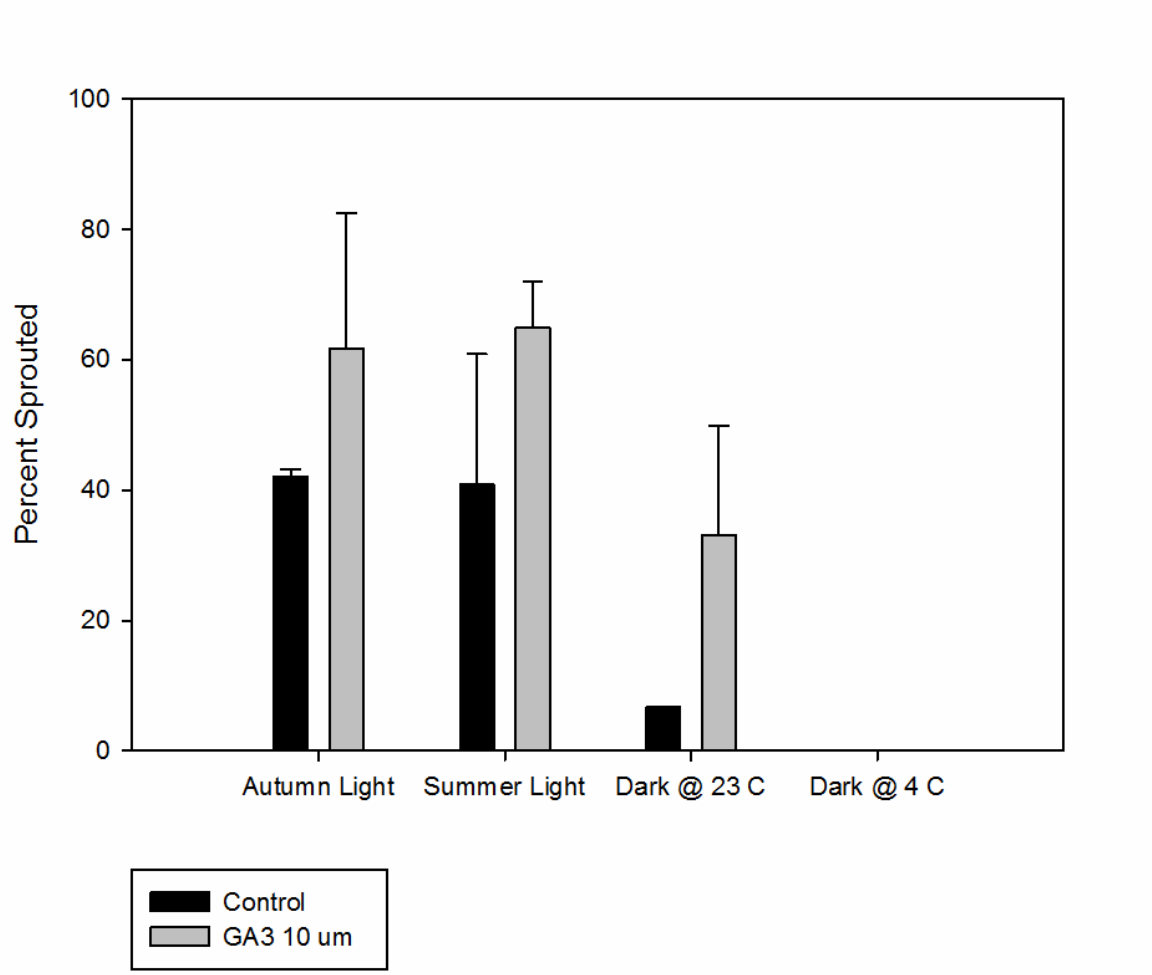
Results/Discussion

The addition to kinetin not only increased sprouting of the turions (Figure D.5), but caused sprouting of every bud on the turion to break dormancy (Figure D.6).

NAA additions were inconclusive. No sprouting changes were observed in response to increased concentrations of auxin (Figure D.7). However, the turions formed a mucus-like substance that coated the turions after three weeks of exposure to 70% ethanol. The mucus like substance did not contain fungi or bacterial cells.

When turions were cut in half to determine if there was a possible endogenous apical dominance relationship, turions did not increase in sprouting, nor was there a preference in which half of the cut turion sprouted (Figure D.8).

The balance between auxin and cytokinin controlling apical dominance in turions is inconclusive. The mucus like substance may be extruded by the plant as a protective mechanism, because of the lack of epiphyte cells found. Because turions are not surface sterilized, the substance may also be produced by epiphytes. Further investigation into the hormonal relationship between cytokinin and auxin with in turions should be conducted. As well as to what is the mucus like substance produce when turions are exposed to ethanol.



FigureD.1. Overall sprouting levels of current season turions exposed to 10µM GA_3 at different environmental conditions.

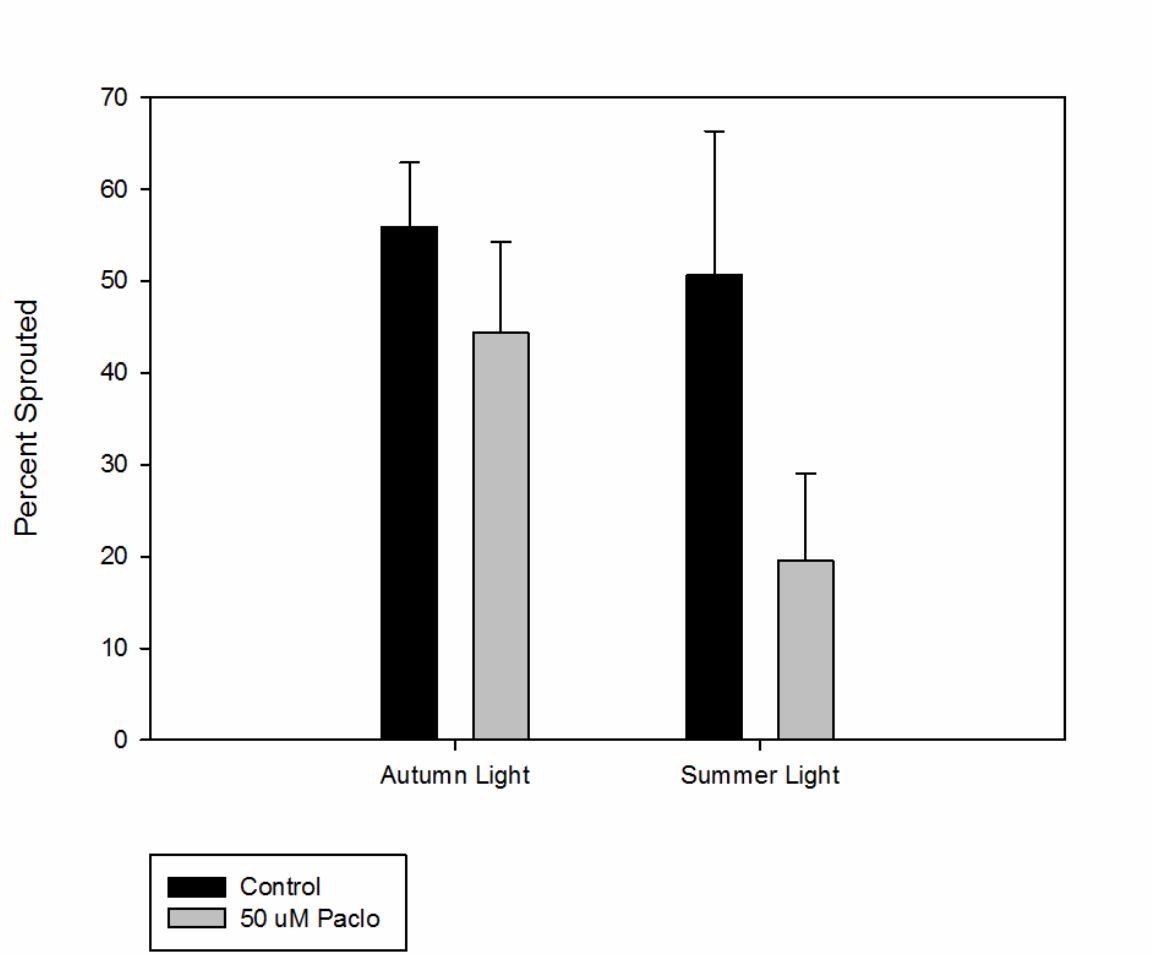


Figure D.2. Overall sprouting levels of current season turions exposed to 50µM of paclobutrazol under different light durations.

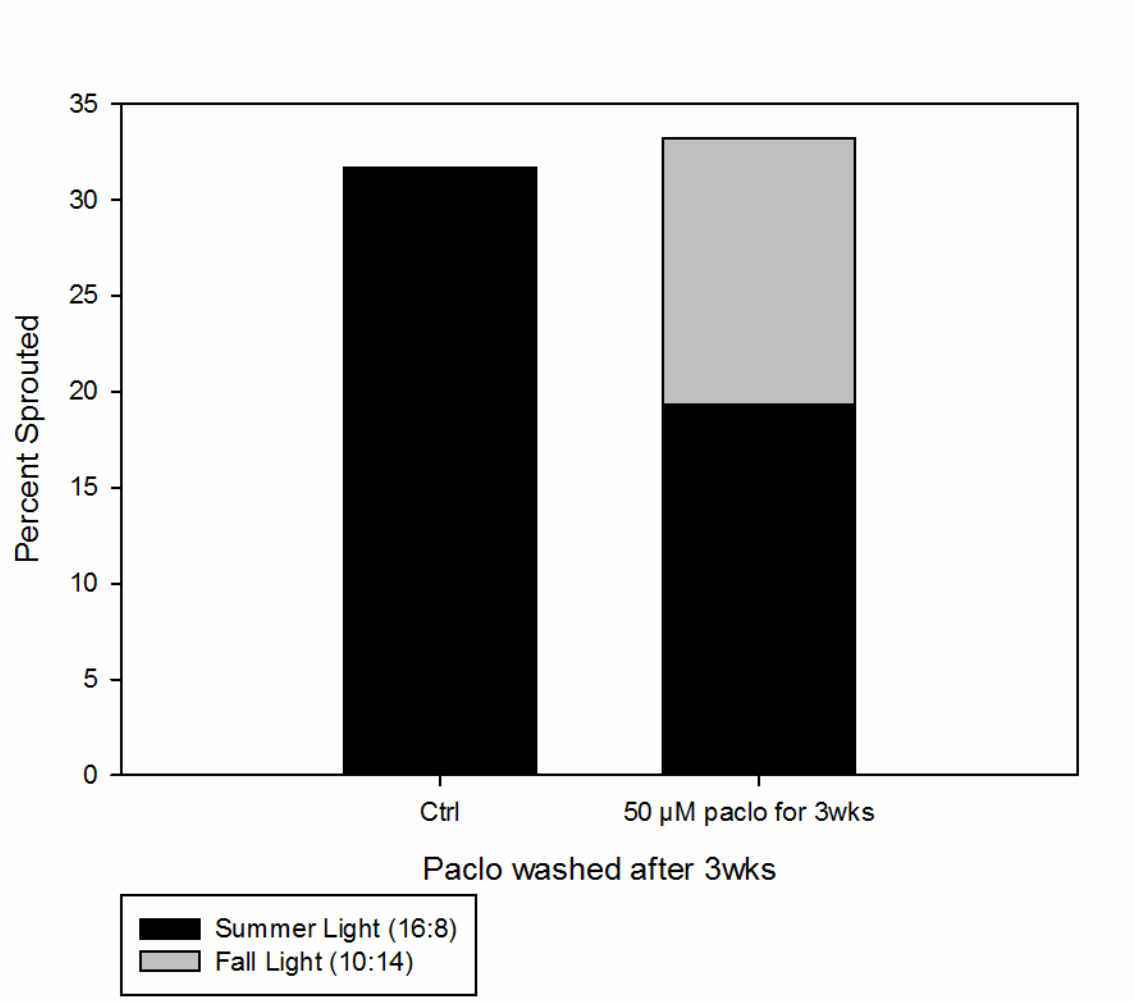


Figure D.3. Sprouting levels of current season turions exposed to 50µM of paclobutrozol for three weeks in 16:8 light then washed and placed in 10:14 light.

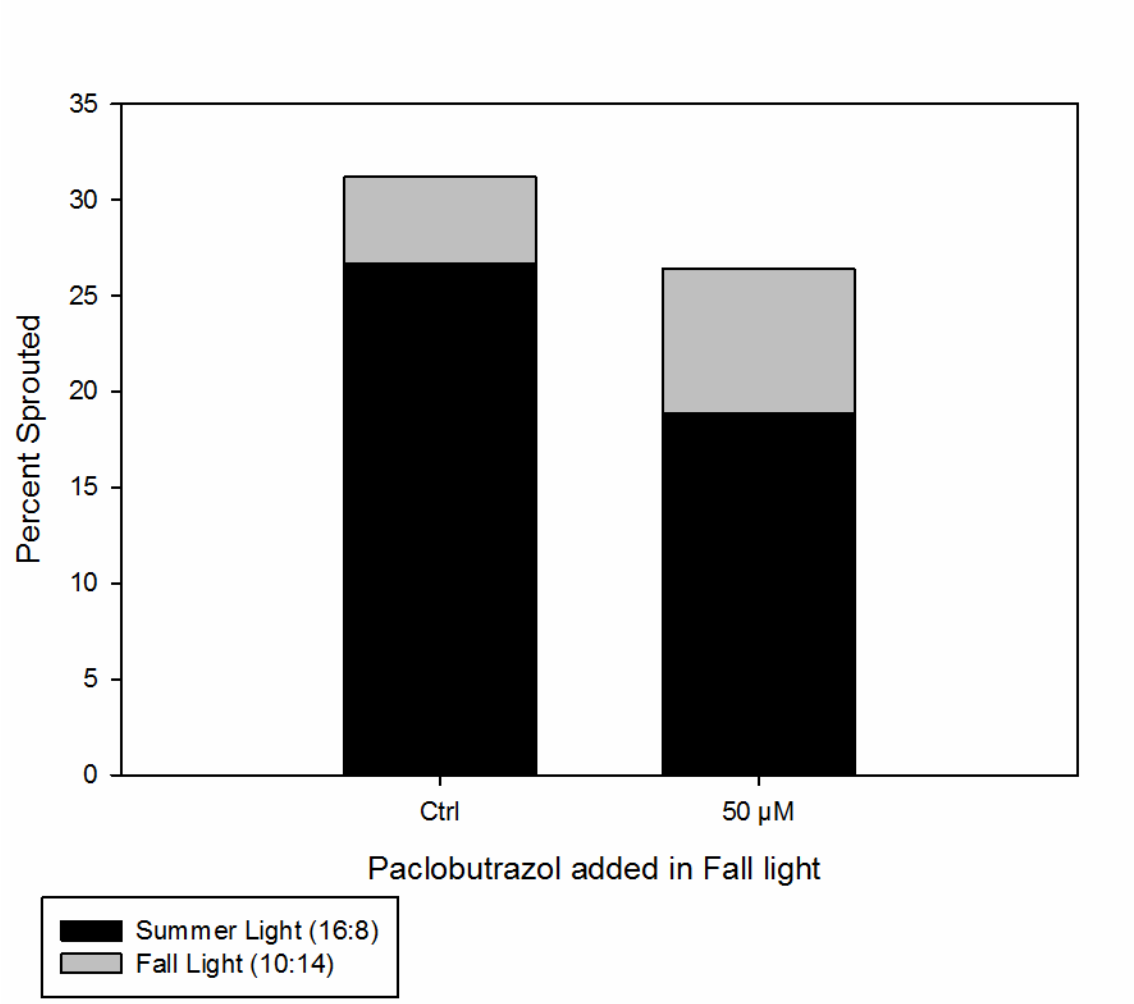


Figure D.4. Sprouting levels of current season turions exposed to 16:8 light then washed and placed in 10:14 light with 50µM of paclobutrozol.

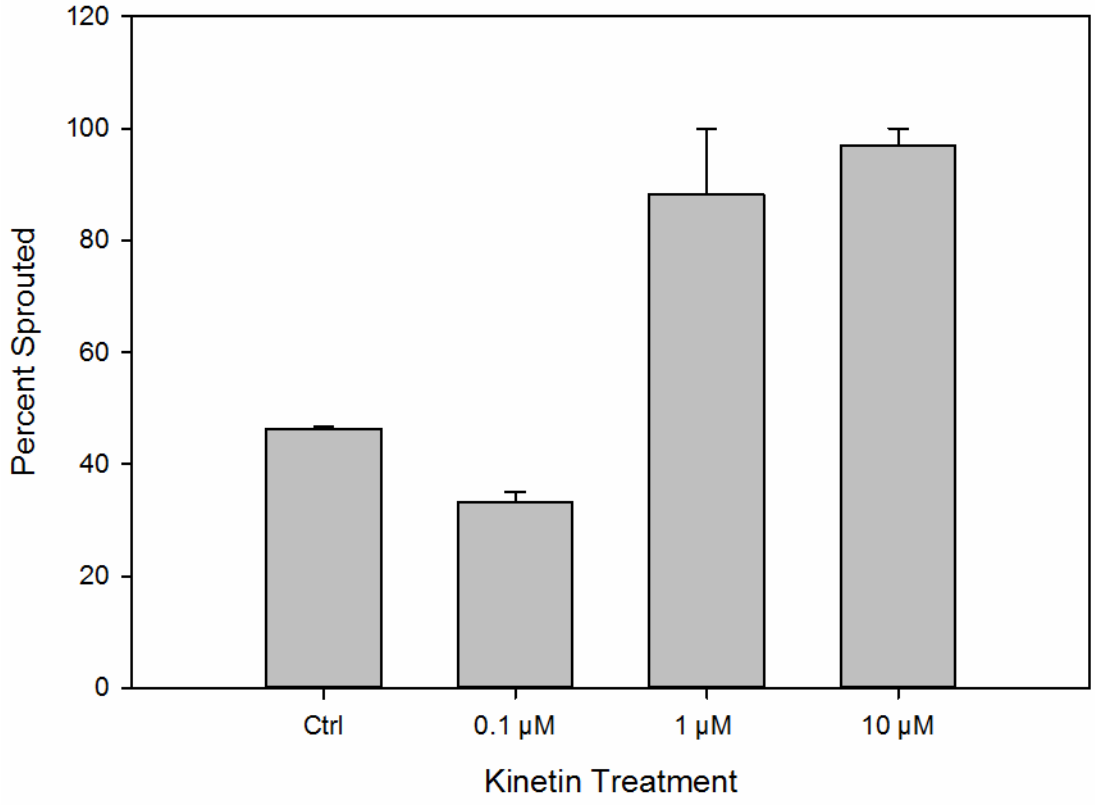


Figure D.5. Overall sprouting of current season turions exposed to kinetin.

Figure D.6. Kinetin response in turions. All buds broke dormancy.

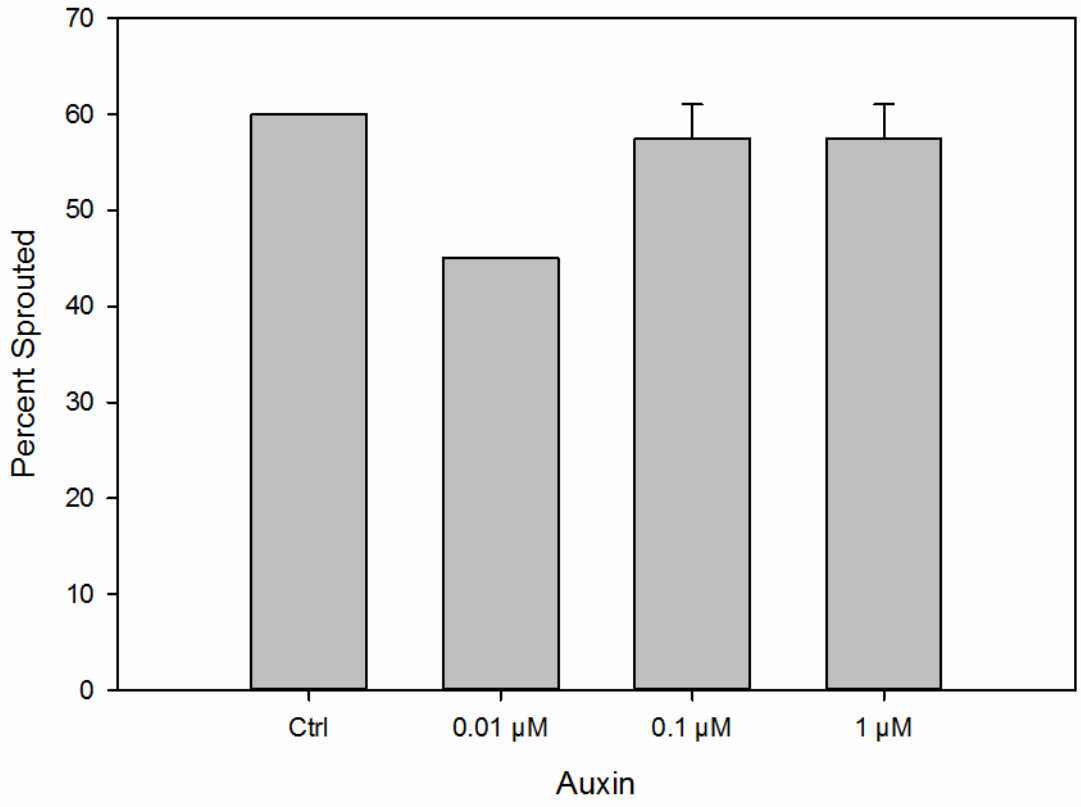


Figure D.7. Overall all sprouting of current season turions in response to NAA.

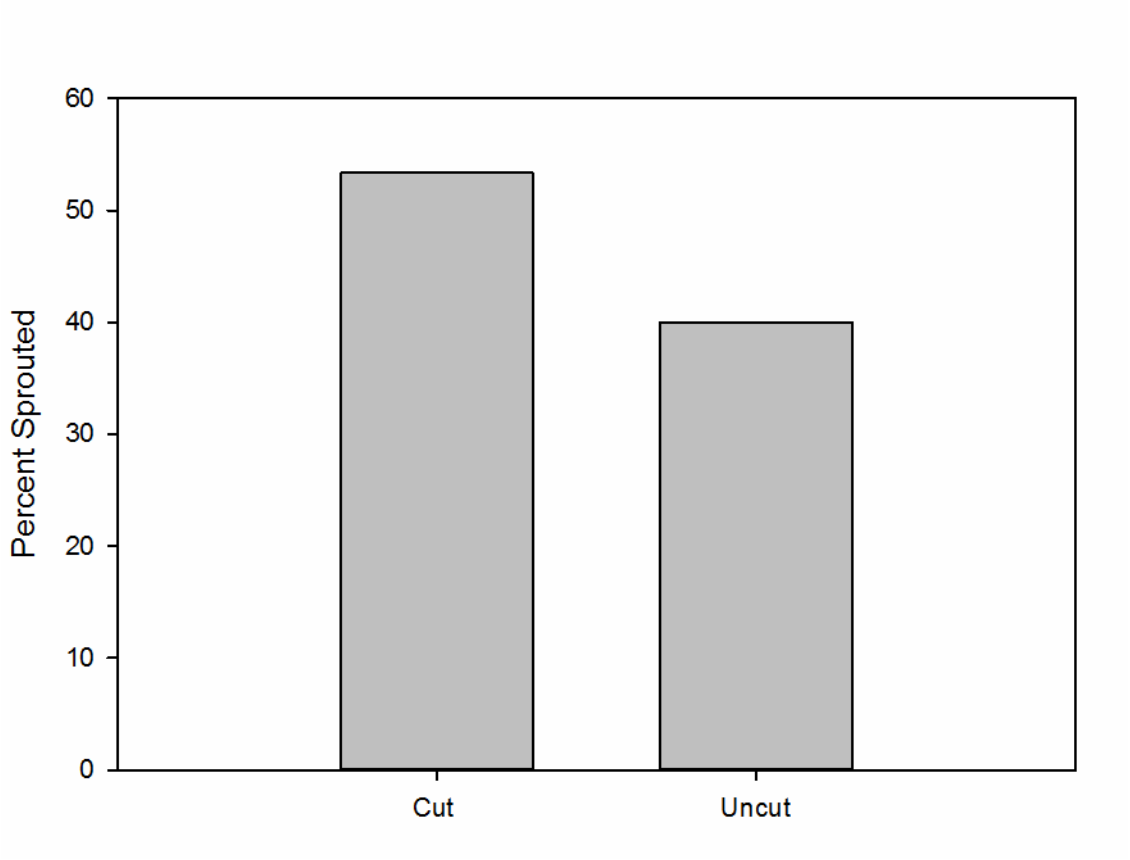


Figure D.8. Overall all sprouting of current season turions in response to cutting turions in half.