CARBOHYDRATE STATUS OF *IN VITRO* GROWN TRILLIUM RHIZOMES

by

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A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Master of Science in Plant and Soil Science

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ABSTRACT

Trilliums are herbaceous perennials and forest understory geophytes that grow from underground rhizomes commonly producing only a single new shoot each year. Reasonable protocols need to be further developed for the micropropagation of trilliums. Excision of apical shoots from tissue-cultured rhizomes caused the generation of more mini-rhizomes (MR) in *Trillium sulcatum* but had no affect on *T. decumbens.* The following studies focused on field establishment and maturation of tissue-cultured rhizomes of *T. decumbens, T. discolor,* and *T. maculatum* by analyzing for carbohydrate status using both qualitative (Lugol's iodine staining) and quantitative colorimetic starch assays, and by quantitating the soluble sugars glucose, fructose, ribose and sucrose via HPLC. To study the effect of carbohydrate changes on the successful reestablishment and growth of trillium, a series of both *in vitro* and *ex vitro* treatments were initiated on tissue-cultured rhizomes. Growth regulator experiments {maintenance medium augmented with auxin [1% solution of Dip 'N Grow (5 μM indole-3-butyric acid (IBA) and 2.7 μM 1-naphthaleneacetic acid (NAA)], cytokinin [benzyl adenine (BA) adjusted to 0, 4.4 (control) or 8.8 μM], or gibberellic acid [(GA) 5 μM]; followed by *ex vitro* coldroom (4°C) storage for 8 weeks} failed to induce a carbohydrate concentration increase in trillium rhizomes. Greenhouse and coldroom experiments to understand starch fluctuation confirmed the

depletion of starch after 9 weeks. When trillium rhizomes were placed in 4-, 6-, or 8-week offset cold-warm stratification periods starch was clearly detectable after 12 (6-week stratification) and 16 (4- and 8-week stratifications) weeks. Field-grown rhizomes were also examined to profile natural carbohydrate fluctuations associated with seasonality. Trillium rhizomes displayed a pattern similar to that of other geophytes whose seasonal carbohydrate fluctuations are known.

Chapter 1

INTRODUCTION & LITERATURE REVIEW

1.1 Trillium

1.1.1 Botany

Trillium species are herbaceous perennials and woodland under-story plants. There are approximately 48 species known throughout the world (Fig. 1). This thesis will focus on trilliums native to North America, particularly those of the eastern United States. A mature trillium plant will produce a tri-foliate leaf on its stem, with a terminally born flower consisting of three sepals and three petals, ranging in color from white, yellow, and maroon (Jacobs and Jacobs, 1997).

Trillium, a monocotyledonous spring ephemeral, develops its structures in sets of three, as is common to plants in the Liliaceae (*sensu lato*), though recently trilliums have been placed in the family Trilliaceae. Trillium has six anthers and three stamens which are fused at the base and separate at the stigma (Case and Case, 1997; Rudall et al., 2000).

Distinct morphological features divide trilliums into two categories. Plants in the subgenus *Trillium* have a stalk that extends the flower above the leaves and are therefore called pedicellate. This type tends to have more attractive flowers when compared to the sessile form in the subgenus *Phyllantherum*. Sessile trilliums produce

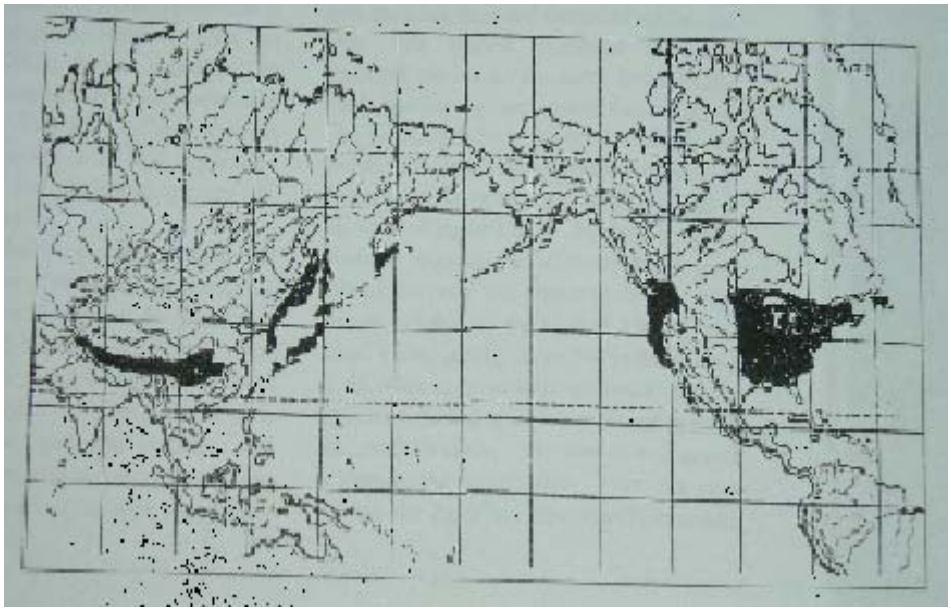


Figure 1. The worldwide distribution of Trillium (Jacobs and Jacobs, 1997)

a flower that sits directly on top of the leaves, and often the petals of the flower remain closed in an upright position, obscuring the reproductive structures. However, trilliums of this type can have very ornate leaves, with a mottled appearance of light and dark green. (Case and Case, 1997).

The fruit is a berry consisting of an outer fleshy pericarp and inner chambers bearing seeds, and is shed completely from the plant by abscission prior to seed dispersal. An elaiosome consisting of a white, oily substance is attached to the side of each seed. Equal in volume to the seed, the eliaosome attracts ants which transport the seeds, and after consuming the oil, abandon the seed, accounting for seedling growth relatively distant from the parent plant (Case and Case, 1997 or Berg, R.Y., 1958).

Growth of trillium from seed requires a period of dormancy prior to emergence. Commonly, species of trilliums require two chilling periods in order to break dormancy (Case and Case, 1997); however, in some species, only a single period of cold is enough to bring about seed germination (Solt, 1996).

Trillium produces an underground storage organ, considered a subterranean stem or rhizome. Most species of trillium require a cool soil of neutral pH (Case and Case, 1997) and prefer early spring sunlight upon emergence, but need shaded conditions once the plant has completed growth. Following fruit development and senescence in late summer and early fall, a new shoot tip develops at the terminal end of the rhizome (Henderson, 1998; Frett personal communication). Contractile roots then pull the rhizome down, helping to keep it covered by the soil and leaf litter throughout the winter.

Vegetative propagation from rhizome stock is slow and unreliable. When propagated from seed, under normal growing conditions, trillium takes at least 3 and up to 10 years before developing a rhizome large enough to support a flower (Case and Case, 1997). Since trillium requires such a lengthy period prior to flowering, there is a considerable investment of time and money required in the propagation of trillium plants for the nursery trade. Those that are sold by nurseries are often initially derived from wild-dug stock. Wild digging endangers the future of trillium in its native habitat.

1.1.2 Micropropagation for conservation and maintenance

Early work on the development of tissue culture methods for trillium was carried out by Pence and Soukup between 1986 and 1995. Immature tissue from leaf buds developed outgrowths which when removed and cultured separately formed rhizome-like organs which were termed mini-rhizomes (MR). The MR were induced to form shoots *in vitro*, which subsequently rooted on medium containing NAA. This work was instrumental in establishing optimal concentrations of sucrose, auxin, cytokinin and Murashige & Skoog (1962) salts in the medium used to initiate and proliferate trillium in culture. Working with five different species, they also concluded that each species of trillium varied in response to the *in vitro* environment and often required slight adjustments for a successful micropropagation scheme (Pence and Soukup, 1986, 1993, 1995).

Regeneration of trillium explants in tissue culture medium is being explored as an option for expediting rhizome growth associated with flower development. Tissue culture offers an opportunity to initiate flowering in less than 23 months (Foster, 2002). The regenerates from various plant tissues (ovary, leaves, etc.)

have been subcultured on Maintenance Medium (see Chapter 2 Materials and Methods for medium components) once every four weeks, resulting in the growth and development of MR (Pence and Soukup, 1995). The MR can then be used in experiments to develop strategies for field establishment.

Micropropagation, not only allows for growth that is clonal and, therefore, produces predictable characteristics, but also speeds growth in comparison to plants propagated from seed or by rhizome division. This allows the grower to have added confidence in a healthy product with known characteristics, and rapid production (Margherita and Pierre, 2004).

In a study comparing propagation by tissue culture and stem cuttings in *Vaccinium vitis-idaea* (lingonberry), the rate of survival was 15% higher for plants produced in tissue culture. The same tissue cultured specimens produced more rhizomes, formed healthier plants and had higher fruit production for both first and second year growth, when compared to those propagated by cuttings (Gustavsson and Stanys, 2000).

When *in vitro* regenerated plantlets of *Curcuma* were planted in soil and acclimated to the outside temperature in a nethouse, those that displayed 2-4 *in vitro*-generated roots for every shoot had a survival rate of 96-100% (Tyagi et al., 2004). Several references, including ones studying trillium, stated that *in vitro* rhizomes were rooted previous to acclimation under greenhouse conditions (Pence and Soukup, 1993; Pence and Soukup, 1995; Margherita et al., 1996). The available references for trillium do not give survival data indicating the success of MR field establishment. Previous data from our lab attempting to determine conditions for field establishment of MR (Foster, 2002), have been inconclusive.

1.1.2.1 Proliferation of plant material

1.1.2.1.1 Growth regulators

1.1.2.1.1.1 Auxin

Auxin is often used to induce adventitious roots in culture. Auxin is also known to expedite differentiation in a plant's vasculature (Raven et al., 2005) possibly providing more phloem tissue for the transport of metabolites. The synthetic auxins indole-3-butyric acid (IBA) and 1-naphthaleneacetic acid (NAA) are more stable in tissue culture medium than the naturally occurring indole-3-acetic acid (IAA) and the combination of auxin types in culture medium may have a synergistic effect on rooting (George, 1993). In a study of *in vitro* generated orchid rhizomes of *Geodorum densiflorum*, the addition of 2 μM of NAA promoted the growth of rhizomes. It was further shown that rhizome growth was negated by the addition of 5 μM of the cytokinin, benzyladenine (BA). Little is known about the affects of auxin on carbohydrate concentrations of *in vitro* grown tissues.

1.1.2.1.1.2 Cytokinin

Cytokinin has traditionally been used in tissue culture to promote shoot formation. The ratio of auxin to cytokinin is often manipulated to favor the induction of either roots or shoots (Raven et al., 2005). Cytokinins also induce cell division in plants, which can be measured by increases in ribose concentrations in actively dividing tissue. Chu et al. (2002) demonstrated the influence of benzylaminopurine (BAP or BA) on carbohydrate flux in cultured plantlets of three *Dioscorea* species.

Concentrations of both starch and total soluble carbohydrates increased with increasing concentrations (0.44, 2.2, 4.4, 11 and 22 μM) of BAP.

1.1.2.1.1.3 Gibberellic Acid

Gibberellic acid (GA) is generally known to induce shoot elongation and cell division in plants (Raven et al., 2005) and is often used to break dormancy in buds and seeds. GA has been shown to induce tuber formation from potato stem in tissue culture (Xu et al., 1998). It has also been shown that increasing concentrations of GA can be correlated with an increase in number of primary and secondary potato tubers (Escalante and Langille, 1995).

1.1.2.2 Re-establishment

Development of a reliable method for establishing tissue cultured trillium rhizomes *ex vitro* is needed. This will include determining cycles of chilling and warming necessary to obtain optimal field performance upon planting the tissue cultured material and the role of growth regulators in carbohydrate cycling.

A study of *in vitro* generated *Ranunculus asiaticus L*. plants placed in plastic pots with perlite and stored for 60 days at 15°C, iterated the importance of an acclimatization period prior to field growth. After an additional 50 days at 28°C and 28 days at 10°C, the plants were placed in the field to continue growth. After three months they displayed increased maturity in both shoot number, flowering date, and flower number when compared to those grown from seed (Margherita et al., 1996; Margherita and Pierre, 2004). It was also shown that the size of the rhizome prior to

planting in soil was a fairly reliable indicator of flower size, number of potential flowers, and the length of time to flowering, once planted in the field.

In a recent follow-up, the same group has published a complete protocol, detailing a beginning to end procedure for tissue culture and field establishment of *Ranunculus* rhizomes. The protocol included important steps to allow the tissue cultured plant to undergo elongation and rooting 1 to 2 months prior to planting in the field. The rooting procedure was initiated by the addition of 4.9 μM IBA (indole-3-butyric acid) to MS based subculture medium (Margherita and Pierre, 2004).

In experiments to determine the optimal temperature in order to suspend dormancy, rhizomes of *Podophyllum peltatum L.*(American mayapple) were placed at 4°C for varied lengths of time, followed by growth in a 21°C greenhouse. After senescence, the plants were returned to 4°C for 90 days before placing them in the greenhouse. The study showed that percent emergence and plant height were positively correlated to the amount of time each plant spent at 4°C, the optimal duration being 75 days (Maqbool et al., 2004). The mayapple study was used as a starting point for time and temperature experiments using trillium MR.

1.1.2.3 Acclimation and stratification

1.1.2.3.1 Field growth

In *Trillium erectum*, the carbohydrate required for fruiting is stored in the leaves and stem early in the growing season (mid-June for Zone 4). This suggests that the rhizome is not the main carbohydrate source for an actively growing plant (Lapointe, 1998). Lapointe (1998) has shown that starch is the most abundant carbohydrate in the *T. erectum* rhizomes throughout every season. Starch content of

field grown rhizomes of *T. erectum* was highest in spring and early summer and waned steadily in the early autumn, continuing to decrease throughout the dormant period in winter. In as little as three weeks, starch in the rhizome was replenished when the temperature began to warm in the spring (Lapointe, 1998).

An experiment using eight species of woodland, understory plants measured carbohydrate in rhizomes as total non-structural carbohydrate (TNC), in order to determine seasonal fluctuations. The most obvious similarity in the carbohydrate storage habits amongst these eight species was the peak of TNC in the autumn (Flinn et al., 1985). It is also interesting to note that *Maianthem canadence*, a member of the Liliaceae, had levels of fructosans that were several orders of magnitude higher than that of any of the other studied species.

Risser and Cottam (1968), showed that in the membranous corms of two species of spring ephemerals, *Erythronium albidum* and *Erythronium americanum*, accumulation of carbohydrate in the late summer and early autumn, was mainly starch. By late autumn, the roots and shoot for the following year had already begun to develop, and the amount of soluble sugar was increasing as the bulb prepared for dormancy.

1.1.3 Analysis of rhizome carbohydrate status

As a spring ephemeral, trillium accumulates the majority of the present-year carbohydrates through photosynthesis in both the leaves and stem, early in the growing season (Risser, 1968; Lapointe 1998). Once the plant senesces late in the summer, excess carbohydrates are stored in the rhizome and used in development of the shoot apex for the coming year.

The storage of carbohydrates, specifically starch, in the rhizome appears to be a crucial indicator for determining timely field establishment of MR. Starch content is found at a higher concentration in rhizomes undergoing vegetative growth compared to those that are dormant (Foster, 2002). Foster (2002) also showed that analysis of the specific carbohydrates present in the *in vitro* maintained MR as compared to mature rhizomes in the field, was a crucial component for enabling the development of a protocol for field establishment of MR.

Knowing which carbohydrates are stored, when they are stored, and their connection to field establishment and maturation, will provide a better understanding of the required biochemical status for MR to initiate early flowering. By measuring the concentration of carbohydrates following various rhizome treatments such as length of *ex vitro* chilling and warming periods, various greenhouse growth conditions, and growth of plants in the field, a more accurate picture of the carbohydrate (carbon source) requirements for successful field growth will be gained. Ultimately, this will allow for a more cost effective and non-environmentally destructive method for propagating trillium.

1.1.3.1 Starch stain method

Carbohydrate storage in plants is often accomplished through the formation of starch molecules of amylose and amylopectin. Amylose is a helical macromolecule made of repeating units of α(1,4)-linked D–glucose. Its helical conformation allows it to bind iodine (I2) molecules inside the helix, along the longitudinal axis (Zubay, 1998).

Amylopectin is also formed by repeating units of α(1,4)-linked D–glucose but with molecules in an α(1,6)-linked D–glucose conformation at regular intervals,

causing it to branch many times. This makes amylopectin a heavier and more bulky carbohydrate than amylose. Because of the helical formation of its branches, it too is able to bind molecules of iodine. A qualitative evaluation of starch concentration in the rhizomes was made by staining various fresh rhizome cross sections with a potassium iodine dye.

1.1.3.2 Starch quantitation method

Because the method of iodine staining gives only a subjective measurement of starch, an assay was necessary to quantify the starch per rhizome concentration. One way to assay a polymer like starch is by digesting it enzymatically and assaying the concentration of its units, in this case units of glucose. To denature *in vivo* cellular or extracellular enzymes in various cultivars of cotton, Viator et al.(2005) ground plant material and incubated at 100°C for 1hr. in 1mL of 0.2 N KOH. Following incubation the pH was neutralized by addition of 200 μL of 1N acetic acid and digestion of starch was begun by incubation of the solid plant material with alpha-amylase at 85°C for 30min. Amylo-glucosidase was added after the sample pH was lowered to five. The sample was transferred to a 55°C water bath for an additional hour. A colorimetric reagent was added and color development measured in a spectrophotometer. Starch concentration was reported in milligrams of glucose per gram of plant material dry weight (Viator et al., 2005). In Zinselmeir et al. (1999), starch was enzymatically digested after boiling the plant material, using a mixture of alpha-amylase (1 mg/mL) and amylo-glucosidase (2 mg/mL), and allowed to incubate at 55°C for 2 hours at a pH of 4.5. Once the starch is digested, the resulting glucose units can be measured in a variety of ways including HPLC analysis

and colorimetric absorbance assays utilizing p-hydroxy-benzoic-acid hydrazide (Schmidt, 1985) or Benedict's Reagent (Nelson, 1944).

1.1.3.3 HPLC method

High Performance Liquid Chromatography (HPLC) is a sensitive and proven method for quantitative analysis of many substances. There are many forms of HPLC, each one designed to work better with certain applications. Ion-exchange chromatography is applied for quantification of many organic and biochemical substances, and can be used for experiments to quantify carbohydrates in plant material.

In the 1970s, ion-exchange chromatography was established as a very useful method for separation of compounds in solution, according to the characteristics of their ionic charges. Ion-exchange resins can be made of either a co-polymer of styrene and divinylbenzene, or microscopic silica beads that are packed into a 10 to 30 cm stainless steel column. Charges are added to the beads by means of attaching functional groups, and in effect, coating the beads with ions. The functional groups can be de-protonated carboxyl, amine or sulfonyl groups (Skoog et al., 1998).

Samples are analyzed by injecting a specified volume of a sample solution into the HPLC column. The detector converts the measured ionic properties of the substance to a signal display of peaks, read in reference to time in minutes. After analysis, the addition of a strong acid or base washes off the resin by changing the pH and altering the ionic properties of the functional groups. The column can be reused until the beads become stripped of their functional groups and detection is no longer sensitive.

Chapter 2

IN VITRO CULTURE

2.1 Introduction

As a method for rapidly propagating trillium rhizomes, tissue culture methods are being developed to proliferate mini-rhizomes (MR) in culture. Experiments designed to improve proliferation of plant material *in vitro* included comparison of sterilization techniques and a shoot excision method for expediting the initiation of MR in culture.

To analyze carbohydrate concentrations in trillium rhizomes, methods for assaying starch and soluble sugar carbohydrates were developed.

Once planted in the greenhouse or field, a rhizome must form roots and then grow a new shoot and leaves in order to begin photosynthesis and become autonomous in its provision of carbon. By determination of an *in vitro* protocol designed to result in the accumulation of carbohydrates, specifically starch, a rhizome will be better prepared for successful growth while acclimating to the *ex vitro* environment. In order to accomplish this, auxin, cytokinin, and gibberellic acid growth regulators were studied for their influence on the accumulation of carbohydrates in rhizomes.

2.2 Materials and Methods

2.2.1 Plant Material

Trillium sulcatum was received from the Mount Cuba Center Inc. and initiated in culture on April 6th, 1999. *T. maculatum* and *T. decumbens* were received on April 25th, 2001. The plants were initially cultured on regeneration medium (Foster, 2002), and subsequently subcultured on maintenance medium every four weeks.

2.2.2 Tissue culture media

Maintenance medium (MM) consisted of 1.5%(w/v) sucrose, ½ Murashige Skoog (1962), 100 mg/L i-Inositol, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine•HCl, 0.4 mg/L thiamine•HCl, 2 mg/L glycine, 1.0 mg/L (4.4 μM) benzyl adenine, 0.3 mg/L 2,4 – dichlorophenoxyacetic acid, in water. The solution was stirred and pH adjusted to 5.7-5.8. Phytagar at 7 g/L was added and the solution heated to 120°C for 5min. It was mixed by swirling and dispensed into jars (55 mm x 72 mm) at 25 mL/jar. The jars were capped (15mm x 48mm) and autoclaved at 121°C and 124 kPa, and the medium allowed to cool for 24 hrs. before use.

For *in vitro* auxin experiments, a 1% solution of Dip 'N Grow (5 μM indole-3-butyric acid (IBA) and 2.7 μM 1-naphthaleneacetic acid (NAA)) from Dip 'N Grow, Inc. (Clackamas, OR) was added to MM. For experiments with gibberellic acid (GA), 5 μM of gibberellic acid was added to MM following sterilization through a 0.2 μm filter, once the MM had been autoclaved and cooled. Experiments with cytokinin used MM with concentrations benzyl adenine (BA) adjusted to 0, 4.4 (control) or 8.8 μM.

2.2.3 Re-establishment protocol

Rhizomes were removed from tissue culture jars using forceps and placed into warm water containing a drop of bleach. The agar remaining on the rhizome was washed off using a small, soft bottlebrush. The rhizomes were placed into a container and sprayed with water to keep them damp. Individual rhizomes were weighed and a flat tag created detailing information about their species, date of planting and weight. They were stored individually in plastic, zip-lock bags and stored at 4°C until planting. The rhizomes were normally planted the same day they were taken out of tissue culture.

Two types of planting containers were used in the experiments: a six-cell pack (cell dimension: 5 cm x 4 cm, Griffin, Tewksbury, MA) which fit 10 to a flat, and a pot (top diameter: 11 cm, Griffin, Tewsbury, MA) which fit into flats which held 15 pots. Containers were filled with MetroMix 510 (soilless medium) (SunGro Horticulture, Bellevue, WA) and the medium was wet with warm water. The use of a small, rounded dibble allowed the rhizomes to consistently be placed 2 cm down into the medium. A small amount of medium was used to cover the rhizome and the flat was watered a second time. Each flat was drenched with a fungicide solution of 75 µL/L Subdue and 150 mg/L Medallion (Syngenta, Greensboro, NC) to discourage fungal contamination. The flat was then placed in an environment specified by the experiment.

2.2.4 Rhizome harvest protocol

Rhizomes, removed from an experiment at a given time-point, were rinsed with water and placed into a small storage container (6-well-plate or scintillation vial, Fisher Scientific, Pittsburgh, PA). A label was placed on each vial/well indicating

date, experiment and time-point for the rhizome, and the container was stored at -20°C until processing. Rhizomes from any given experiment were stored until the experiment was completed so that they could be treated as a whole during processing in order to minimize variability in handling.

2.2.5 Processing rhizomes for quantitative carbohydrate assays

The rhizome was thawed and blotted on a paper towel to remove excess water, and weighed to the nearest milligram. A clean blade was used to cut the rhizome into small cubes (approx. 5 mm square) and the pieces placed in a labeled 15 mL (Fisher Scientific, Pittsburgh, PA) glass centrifuge tube. Five milliliters of water was added to the tube and the rhizome pieces were ground using a Polytron tissue grinder (Brinkmann, Westbury, NY) until a homogenous mixture was obtained. Examination, under a light microscope, of 5 varied samples of cell homogenate showed cell breakage of approximately 75 % of the cells. A plastic transfer pipette was used to transfer the resulting mixture to a labeled 12 mL disposable, polycarbonate, centrifuge tube (Fisher Scientific).

The rhizome homogenate was centrifuged at 12000 x g in a Sorvall (Ashville, NC) floor model RC5C centrifuge to pellet the solid plant material. The supernatant containing soluble sugars was transferred to tubes and a 50 µL aliquot was diluted 1:20 for HPLC analysis.

2.2.5.1 Colorimetric assay for starch

The remaining solid material was resuspended in 5 mL McIlvaine's buffer (0.1M citric acid & 0.1M sodium phosphate, pH 5) and centrifuged. All residual supernatant was removed. The pellet was resuspended in 7mL of McIlvaine's buffer

and the tube was vortexed to ensure the plant material was consistently suspended in the buffer. A 500μL aliquot of the suspension was pipette into a 2mL, Eppendorf (Westbury, NY) microcentrifuge tube and the solid material pelleted at full speed for 5min. in an Eppendorf, single speed microcentifuge (Microfuge E). The supernatant was removed and the pellet resuspended in 500μL of 0.25N potassium hydroxide (KOH). The sample was vortexed and placed in an 85°C water bath (Versa-Bath, Fisher, Pittsburgh, PA) for 30min. Following the incubation, the now basic suspension was neutralized with 50 μL of a 1.23 N solution of hydrochloric acid (HCl) and 500 μL of McIlvaine's buffer was added. Ten microliters of both α-amylase (50 mg/mL) and amyloglucosidase (25 mg/mL) were added to the tube. Because the enzymes were engineered to be heat tolerant, the tube was placed in a 55°C water bath for 20 hrs to facilitate starch digestion.

The following day, the tube was centrifuged at full speed and 20 μL of the resulting supernatant was pipetted into 12 mL glass test tubes. The tube volume was brought to 100 μL by an addition of 80 μL of water. Glucose standards were prepared at 25, 50, 100 and 200 μg. Three milliliters of a 1% solution of hydroxy-benzoic acid hydrazide (Sigma, St. Louis, MO) was added to each tube and the tubes were heated in a boiling water bath for 3 min to induce color development. The reaction was stopped by placing the tubes on ice for 5 minutes. Absorbance was read on an Agilent (Palo Alto, CA) UV-Visible Spectrophotometer 8453 at 410 ηm.

2.2.6 Methods Development

2.2.6.1 Starch stain

After any residual medium was washed off, the rhizome was cut in half through the apical shoot, and the freshly cut face dipped into Lugol's (I2/KI) solution for 20 seconds. After removal from the iodine solution the rhizome was rinsed for 20 seconds. Each rhizome was allowed to dry for approximately 5 minutes. Rhizomes were examined under a dissecting microscope and measured using a millimeter scale ruler. The enlarged image of the rhizome was then digitally photographed under the microscope, using a millimeter scale for reference

2.2.6.1.1 Titration of iodine stain to determine affects of starch density

To determine a concentration of iodine stain that would be dark enough to visibly stain starch, but dilute enough to contrast between varying amounts of starch in the rhizome, sections of rhizome were dipped into decreasing concentrations of iodine solution. The experiment was also used to determine whether a water rinse prior to staining would affect the ability of the stain to adhere to the section.

A single rhizome, approximately 2cm in diameter, was chosen directly from tissue culture. The rhizome was sectioned into 10 pieces of approximately equal size using a standard razor blade.

A 10 mL aliquot of 5% Lugol's solution (I2/KI) was titrated to obtain four, 2-fold serial dilutions resulting in five different concentrations of Lugol's solution (5%, 2.5%, 1.25%, 0.63%, and 0.31%). Five of the rhizome sections were rinsed in water before staining by holding the section with forceps and running under RO water for 10 seconds. Each of the sections was then placed in Lugol's solution for

20 seconds followed by a 20 second water rinse. Digital photographs of the sections were taken under a dissecting microscope.

2.2.6.1.2 Qualitative starch determination for rhizomes under varied conditions

To test for starch by iodine stain intensity, ten rhizomes of *T.maculatum* were removed from experiments at a variety of time points and conditions, for staining with iodine. Conditions included rhizomes direct from tissue culture on maintenance medium; rhizomes directly from maintenance medium that contained 5μM GA; rhizomes removed from tissue culture and chilled (4°C) in the dark for 8 months; rhizomes that were stratified under warm and cold for a total of 28 weeks (12wks. cold – 8 wks. warm – 8 wks. cold); and rhizomes that had been growing in the field for 3 years. Each rhizome was rinsed with water to remove residual medium and longitudinally cut in half through the apex. The cut face was placed in the iodine stain for 20 seconds and rinsed with water for an additional 20 seconds. The sections were digitally photographed under a dissecting microscope.

2.2.6.2 Quantitative colorimetric assay for starch

2.2.6.2.1 Comparison of starch content in rhizomes

For the quantitative colorimetric starch and HPLC soluble sugar assays, data are reported in micrograms of carbohydrate per milligram of rhizome fresh weight (μg/mg). For reference, the dry weight of tissue-cultured rhizomes of *T. maculatum* and *T. decumbens* was determined to be 9.9 % (standard deviation of +/- 0.9 %) of the fresh weight, on average.

In order to gain an initial quantitative understanding of the amount of starch in rhizomes known by qualitative analysis to contain very different

concentrations of starch, 8 rhizomes of *T. maculatum* were examined in a colormetric assay measuring units of glucose from digested starch. Four of the rhizomes were taken directly from tissue culture and the other four had been stored in StarPacs (AgriStar, Conroe, TX) at 4°C in the dark for 1 year. The rhizomes were chosen so that those that should have a relatively high concentration of starch (direct from tissue culture) could be compared to those that had little or no starch (those stored at 4°C for 1 year) as defined by the staining assay. Rhizomes were tested in the colorimetric assay as previously described above.

2.2.6.3 HPLC assay for sugars

The HPLC used was a Dionex (Sunnyvale, CA) DX600 system with an EG50 eluent generator. The column used was a CarboPac PA-10 (Dionex Corp., Sunnyvale, CA) 4 x 250 mm column, containing an ethylvinylbenzene - divinylbenzene (55% crosslinked) 10 μm resin. The resin was agglomerated with 460 ηm MicroBead difunctional quaternary ammonium ions (5% crosslinked). Samples were run at 30°C for 15 min and the injection volume set to 20 microliters.

2.2.6.3.1 *In vitro* assessment of carbohydrate at 4-day intervals for 1 month

A single *T. maculatum* rhizome was taken directly from tissue culture every 4 days, starting with day 0, for one month. The rhizomes were frozen at -20°C until the last time point was harvested. The eight rhizomes were thawed and ground to a gel-like consistency using a mortar and pestle. Three milliliters of water was added to each ground rhizome, and the mixture injected through a 0.2 μm filter into 2 mL HPLC vials (National Scientific Company, Rockwood, TN) and capped with

septum caps (National Scientific Company, Rockwood, TN). Samples were loaded into an auto-sampler and set to run continuously.

The method for preparing rhizomes for the HPLC was subsequently changed to that of using a tissue homogenizer as described above, simply for ease of high throughput processing.

2.2.7 Proliferation

2.2.7.1 Bead sterilizer vs. flame sterilization

Forty jars of 5 tissue cultured *T. maculatum* rhizomes each (n=100 per treatment) were included in the experiment. Rhizomes were transferred from 20 jars using forceps that had been sterilized by holding them over an open flame (Bunsen burner) for ten seconds until they were glowing hot, and allowing them to cool before insertion into another jar. For the remaining 20 jars, forceps were used that had been sterilized in a bead sterilizer (Steri 250 from Simon Keller Ltd., Burgdorf, Switzerland). The bead sterilizer had been allowed to heat to 250°C (482°F) prior to insertion of forceps. For both sterilization techniques, forceps were used for a single jar (5 rhizomes) and sterilized prior to re-use.

Jars were analyzed after 4 weeks in the lab, under normal tissue culturing conditions. Analysis consisted of visual observation of the agar-gelled medium for signs of fungal or bacterial contamination.

2.2.7.2 Rhizome shoot excision experiment

Rhizomes of *T.maculatum* and *T. sulcatum* proliferate slowly in culture. To determine if apical dominance has a role in suppressing the growth of mini-

rhizomes (MR), apical meristems were excised from 20 rhizomes of *T. maculatum* and *T. sulcatum*. Rhizomes having a single apical shoot meristem were chosen for the experiment. Twenty rhizomes of each were cut at the base of the apical shoot, one-third down between the base and the crown (Fig 2). Both the top and bottom portions of the rhizome were placed into jars. Twenty rhizomes of each species were also placed individually and uncut into jars, to be analyzed as controls. The experiment was conducted for 12 weeks and the rhizomes were transferred to new MM as usual, every 4 wks. At each transfer period, rhizomes were visually analyzed for number of axillary breaks (MR production) and morphological changes.

2.2.8 Influence of growth regulators on rhizome carbohydrates

2.2.8.1 *In vitro* experiment with auxin (IBA & NAA)

Twenty-eight *T. decumbens* rhizomes were placed in individual jars containing 5μM IBA and 2.7μM NAA (formulation described above) added to MM. An equal number of rhizomes were placed individually in jars containing MM only. Rhizomes were grown *in vitro* for 5 weeks and were then removed and planted into soilless medium as described by the above re-establishment protocol. The rhizomes were placed in a 4°C coldroom at Mt. Cuba, Inc. for an additional 8 weeks. Two rhizomes were harvested once per week for a total of 13 weeks.

2.2.8.2 *In vitro* experiment with cytokinin (BA)

T. decumbens rhizomes were placed in individual jars containing 0.0, 4.4(control, MM concentration) or 8.8μM BA, with 28 rhizomes per treatment. Rhizomes were grown *in vitro* for 5 weeks and were then removed and planted into soilless medium as described by the above re-establishment protocol. The rhizomes

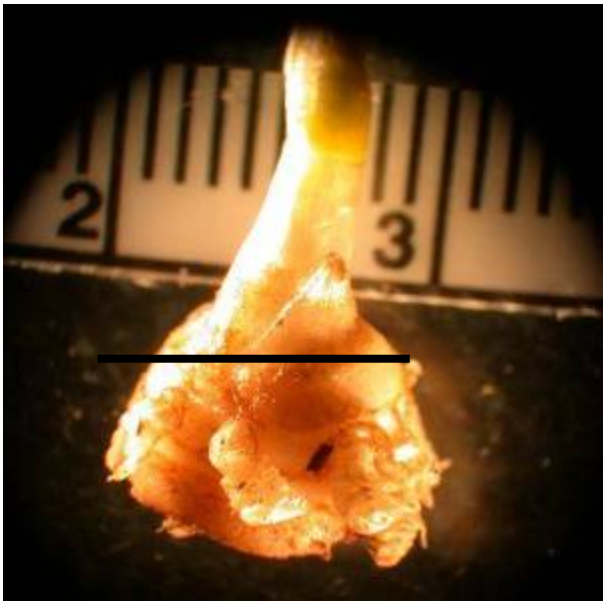


Figure 2. **Rhizome with black bar indicating area of excision. Scale: distance between dashes = 1mm**

were placed in a 4°C coldroom at Mt. Cuba, Inc., for an additional 8 weeks. Two rhizomes were harvested once per week for a total of 13 weeks.

2.2.8.3 Gibberellic acid

2.2.8.3.1 Preliminary GA experiment

Ten *T. maculatum* rhizomes were placed individually into jars containing MM to which 5 μM of GA had been added. As rhizomes from tissue culture have been shown to root when the apex is oriented parallel to the ground (Foster, 2002), rhizome orientation was also added as a variable. Following four weeks *in vitro*, the rhizomes were planted either upright or perpendicular to the ground, in soilless medium and stored in the greenhouse. Rhizomes treated using combinations of the variables as described above were harvested at either 3, 4, or 5 weeks. A single rhizome was used for each variation.

2.2.8.3.2 *In vitro* experiment with GA

Twenty-eight *T. decumbens* rhizomes were placed in individual jars containing 5μM gibberellic acid added to MM. An equal number of rhizomes were placed individually in jars containing MM only. Rhizomes were grown *in vitro* for 5 weeks and were then removed and planted into soilless medium as described by the above protocol. The rhizomes were placed in a 4°C coldroom at Mt. Cuba, Inc. for an additional 8 weeks. Two rhizomes were harvested once per week for a total of 13 weeks.

2.3 Results

2.3.1 Methods development

2.3.1.1 Starch stain

Staining various fresh rhizome cross sections with a potassium iodine dye (Lugol's) gave an indication of what sections of the rhizome were storing starch. Digital photographs of the rhizome cross sections that had undergone various treatments allowed for a general comparison between the carbohydrate storage patterns for each treatment. This qualitative evaluation gave an early indication of which rhizome treatments warranted further examination of carbohydrate composition by starch or soluble sugar quantification assays.

2.3.1.1.1 Titration of iodine stain to determine affects of starch density

Rhizome sections did not show differences in how well the sample stained for rinsed and un-rinsed samples. A titration effect was noted as the darkness of the stain decreased with decreasing concentration of stain (Fig 3). The stock solution at

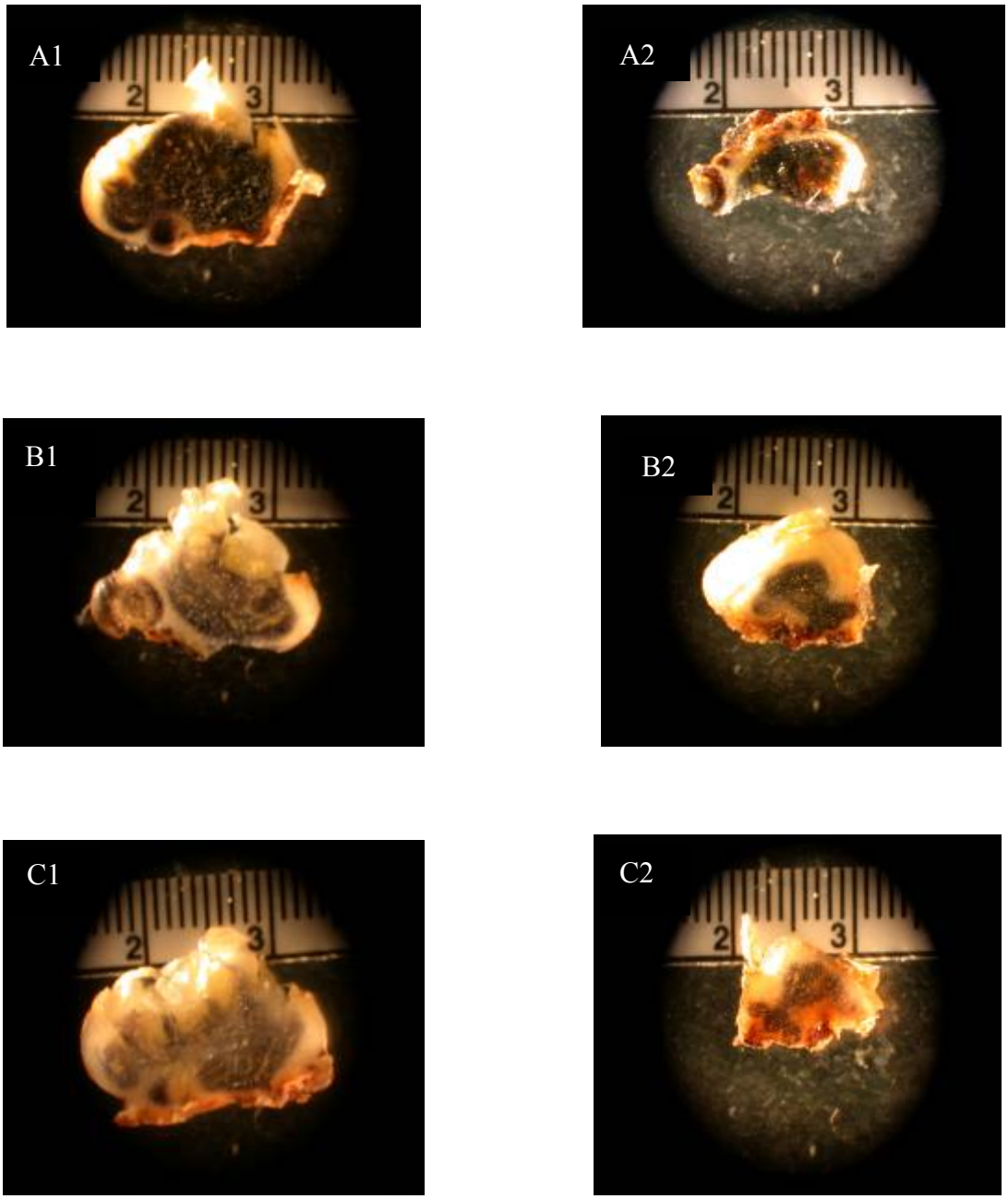
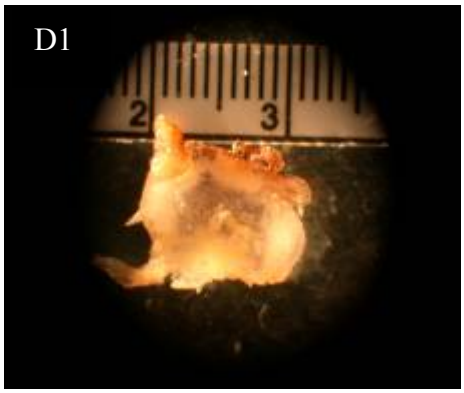
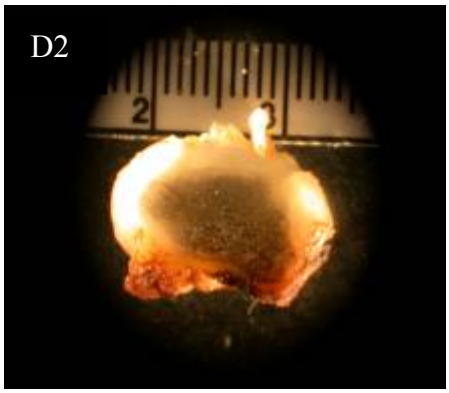
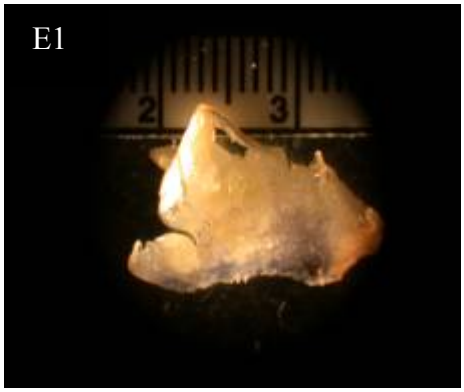


Figure 3. **Rinsed (left column) and un-rinsed (right column) rhizomes stained with decreasing concentrations of iodine solution (A1&2 5%, B1&2 2.5%, C1&2 1.25% D1&2 0.63%, E1&2 0.31%) Scale: distance between dashes = 1mm**







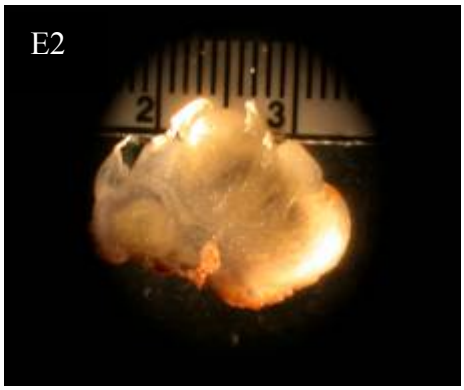


Figure 3. (continued)

5% iodine showed adequate delineation between samples of varied starch concentration, and, therefore, 5% was used for subsequent qualitative starch staining.

2.3.1.1.2 Qualitative starch determination for rhizomes under varied conditions

Digital images of rhizomes that had undergone various treatments (Fig.4) were shown to have contrasting qualitative staining differences. When taken directly from culture, whether on maintenance medium only or maintenance medium with GA, the rhizomes stained darkly for starch. Following dark, cold (4°C) storage for 8 months, starch was greatly diminished and therefore cells showed little to no staining. Similarly, a temperature stratification of 12 weeks in the cold followed by 8 weeks each in the greenhouse (22°C day and 15°C night) and in cold respectively, caused diminishing starch concentrations. Rhizomes grown *ex vitro* in the field for 3 years stained darkly for starch.

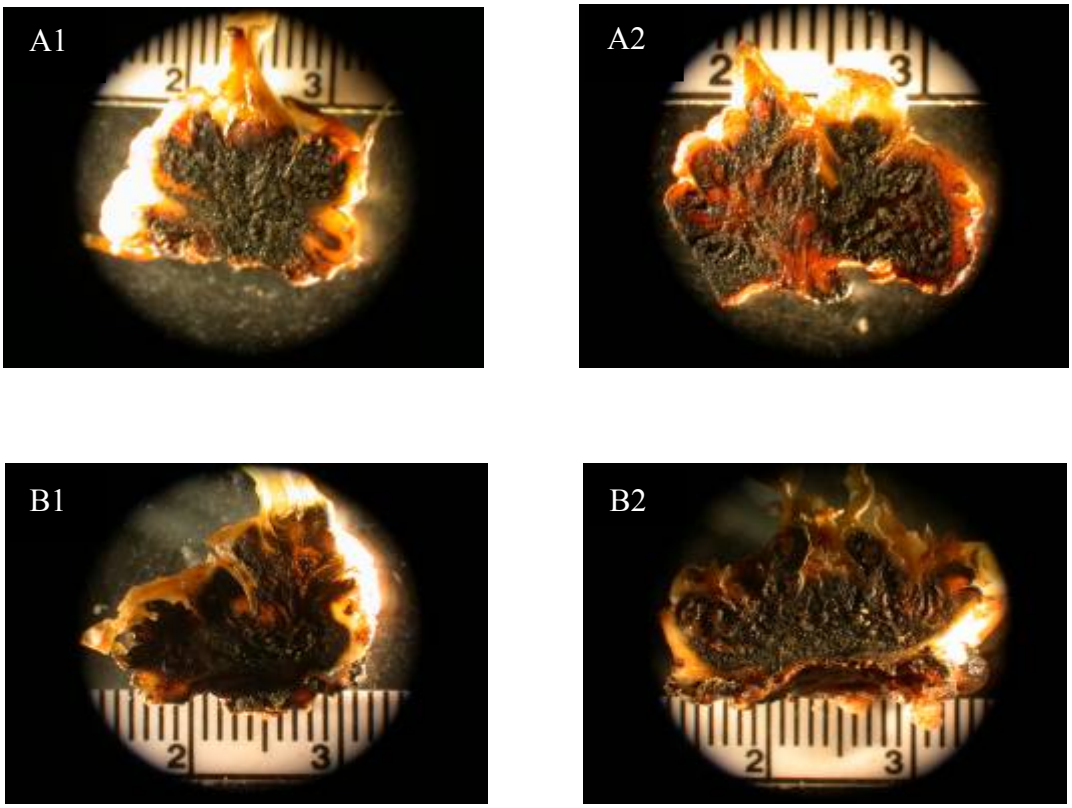


Figure 4. **Iodine stain of rhizomes from varied treatments (A1&2 – direct from tissue culture on maintenance medium, B1 – direct from tissue culture on maintenance medium containing GA, C1&2 – rhizomes from *ex vitro* dark storage at 4°C for 8 months, D1&2 – *ex vitro* temperature stratification (12wks cold – 8wks warm – 8 wks cold), E1 – field rhizome harvested in Feb2005, E2 – field rhizome harvested in Mar2005) Scale: distance between dashes = 1mm**

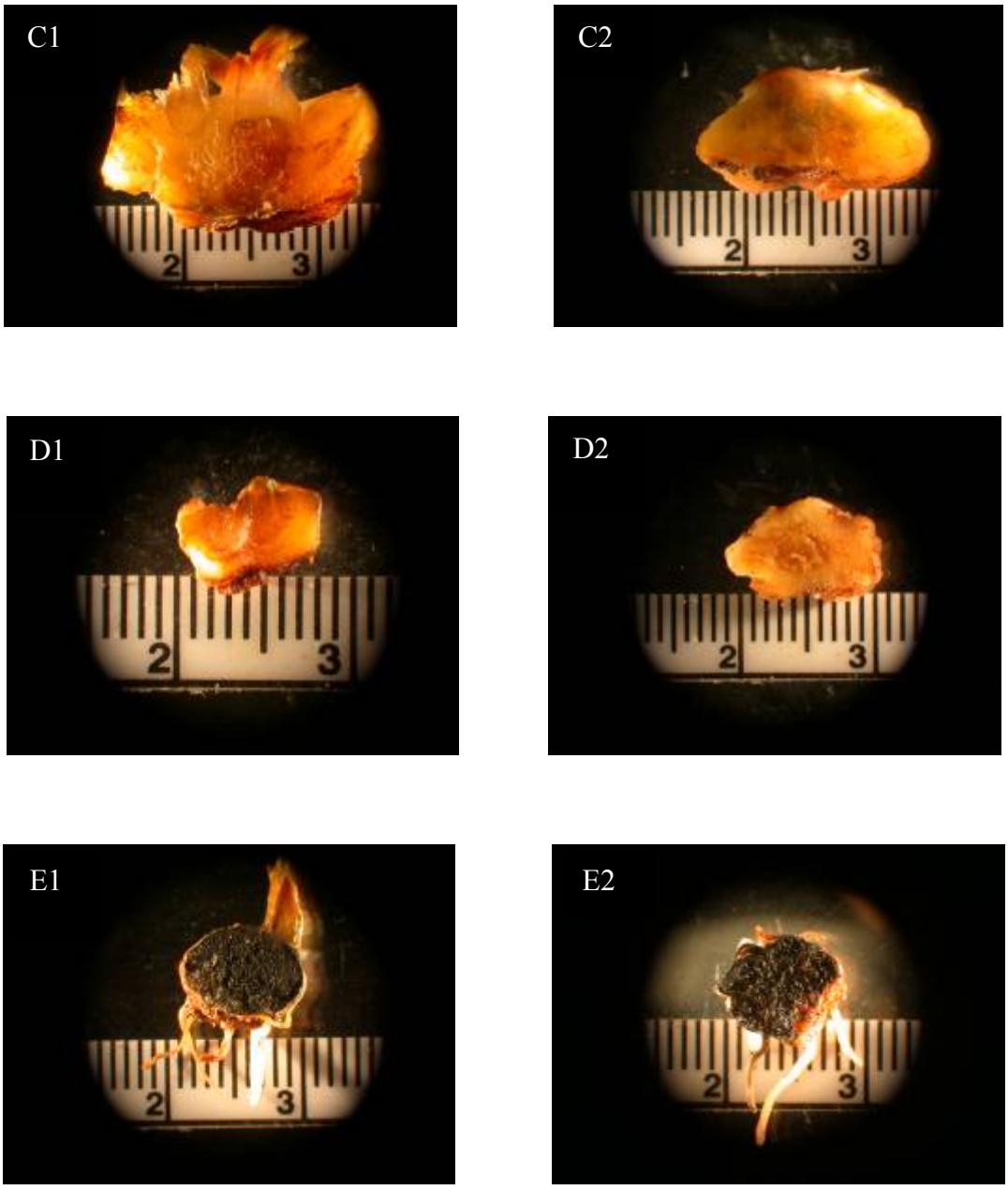


Figure 4. (continued)

2.3.1.2 Quantitative colorimetric assay for starch

2.3.1.2.1 Comparison of starch content in rhizomes

In the quantitative colorimetric starch assay, rhizomes directly from tissue culture had concentrations of starch with a range of 22 to 45 micrograms of starch per milligram of rhizome fresh weight (μg/mg). The average concentration of four rhizomes directly from culture was 32.3 μg/mg (standard deviation (SD) +/- 11.6) as compared to 4.2 μg/mg (SD +/- 1.9) of those stored at 4°C for 1 year (Fig. 5). Subsequent experiments used this colorimetric assay to measure starch concentration.

2.3.1.3 HPLC assay for sugars

2.3.1.3.1 *In vitro* assessment of carbohydrate at 4-day intervals for 1 month

Seven, four-day interval samples were run to obtain a preliminary status of the range of soluble sugar concentrations in tissue cultured trillium rhizomes. Rhizome weights were not initially recorded, so the samples could not be correlated back to fresh weight in proportion. Samples tested undiluted against known standard concentrations of glucose, fructose, ribose, and sucrose gave an indication that the peak range of the concentrations of sugar in the rhizomes were approximately 16 fold (glucose), 18 fold (fructose), and 11 fold (sucrose) higher than the highest concentrations of each sugar in the known standard. Ribose was below detectable levels for all of the samples in this set. From the data, a decision was made to analyze all of the samples on the HPLC at a 1:20 dilution for subsequent tests.

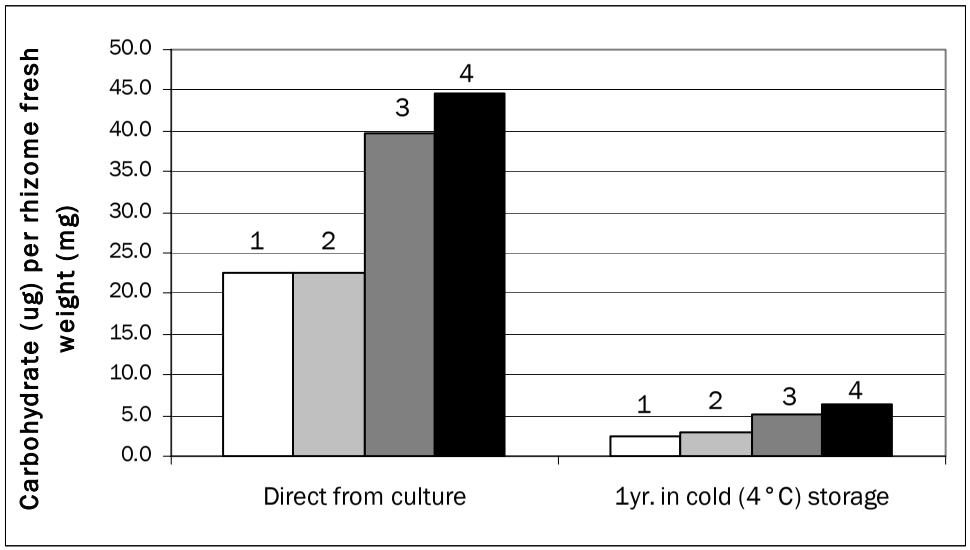


Figure 5. **Comparison of starch concentrations of four *T. maculatum* rhizomes direct from tissue culture and four stored at 4°C for 1 year (Each bar represents n=1)**

2.3.2 Proliferation

2.3.2.1 Bead sterilizer vs. flame sterilization

Four weeks after testing different sterilization techniques for the transfer instruments, none of the jars of rhizomes that had been transferred using the bead sterilized forceps had contamination. One of the jars that had been sterilized by flame became contaminated with a fungus. A standard operating procedure was written and bead sterilization was used for monthly transfer of tissue cultured rhizomes for the remainder of the experiments.

2.3.2.2 Rhizome shoot excision experiment

At the end of 12 weeks, 10 out of the 17 *T. sulcatum* rhizomes from which the apical shoot had been excised, formed breaks at the rhizome's basal portion, compared to 2 out of 17 of the uncut control rhizomes. Three rhizomes from each group became contaminated with a fungus over the 12-week period. For *T. decumbens*, only a single cut rhizome showed a clear indication of forming a break at its base. The remaining rhizomes often had an amorphous-type growth that did not indicate the emergence of defined breaks. The amorphous new growth on cut rhizomes had no clearly discernable apex or base at the end of 12 weeks. In the control, 11 out of 19 rhizomes (*T. decumbens*) had developed breaks at the basal portion, several of which had become almost as large as the original rhizome. These two species had very different growth habits in culture, defined in one way by the development of breaks on cut and uncut rhizomes.

T. sulcatum formed very upright rhizome, often with a single apex, with one or several leaves. *T. sulcatum* rhizomes may produce more MR when the apical

shoot is excised as was shown in these data, but further experimentation with this technique is necessary before being implemented as a standard procedure..

Rhizomes of *T. decumbens* were always less ideal (amorphous shape with a non-descript apex) in their shape and often seemed to grow as much or more sideways than upright. The formation of leaves was rare. From this experiment, it was not evident that rhizomes of *T. decumbens* would proliferate more quickly using this procedure.

2.3.3 Influence of growth regulators on rhizome carbohydrates

2.3.3.1 Auxin

Rhizomes cultured on MM with or without (control) supplemental auxin (IBA and NAA) displayed a trend in decreasing glucose and fructose concentrations over the 8-week cold period (Fig. 6A & B). Glucose was consistently higher than fructose, and fructose higher than ribose and sucrose. Ribose was consistently present, though no change was detected (Fig. 6C). Sucrose was undetectable except in weeks 0, 7, and 9 in the control sample (Fig. 6D).

At the end of the 8-week period of cold storage, the concentration of starch decreased approximately 10-fold but remained measurable. No difference was seen when comparing week vs. treatment at a p value of less than 0.05 (two-way ANOVA).

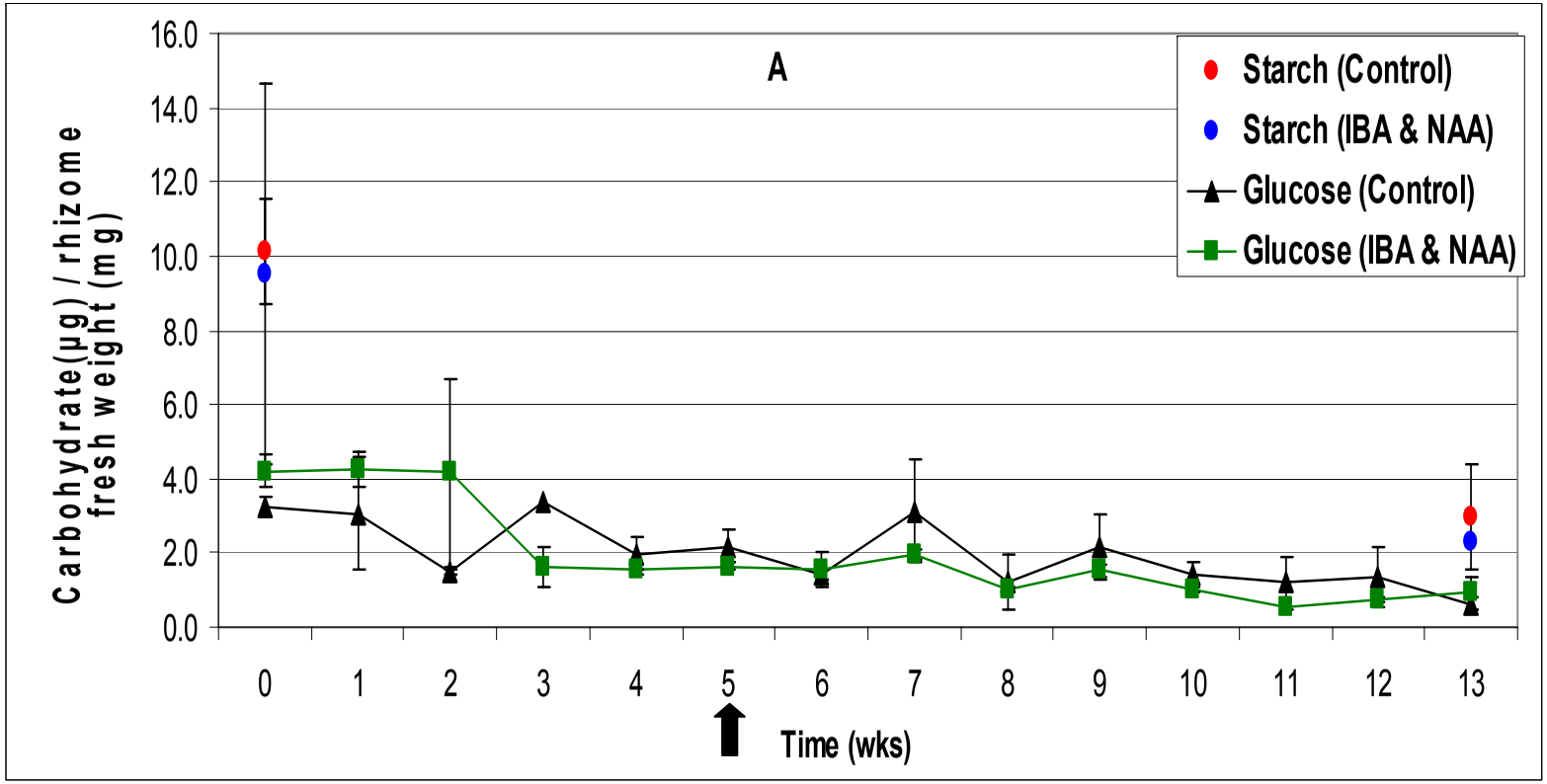


Figure 6A. **The effect of auxin (IBA and NAA) applied *in vitro* on glucose concentrations of *T. decumbens* rhizomes. After five weeks *in vitro,* the rhizomes were planted (see arrow) and placed in a 4°C coldroom. (n=2 for each point on the graph). Data were analyzed by two-way ANOVA. Error bars = +/- standard error.**

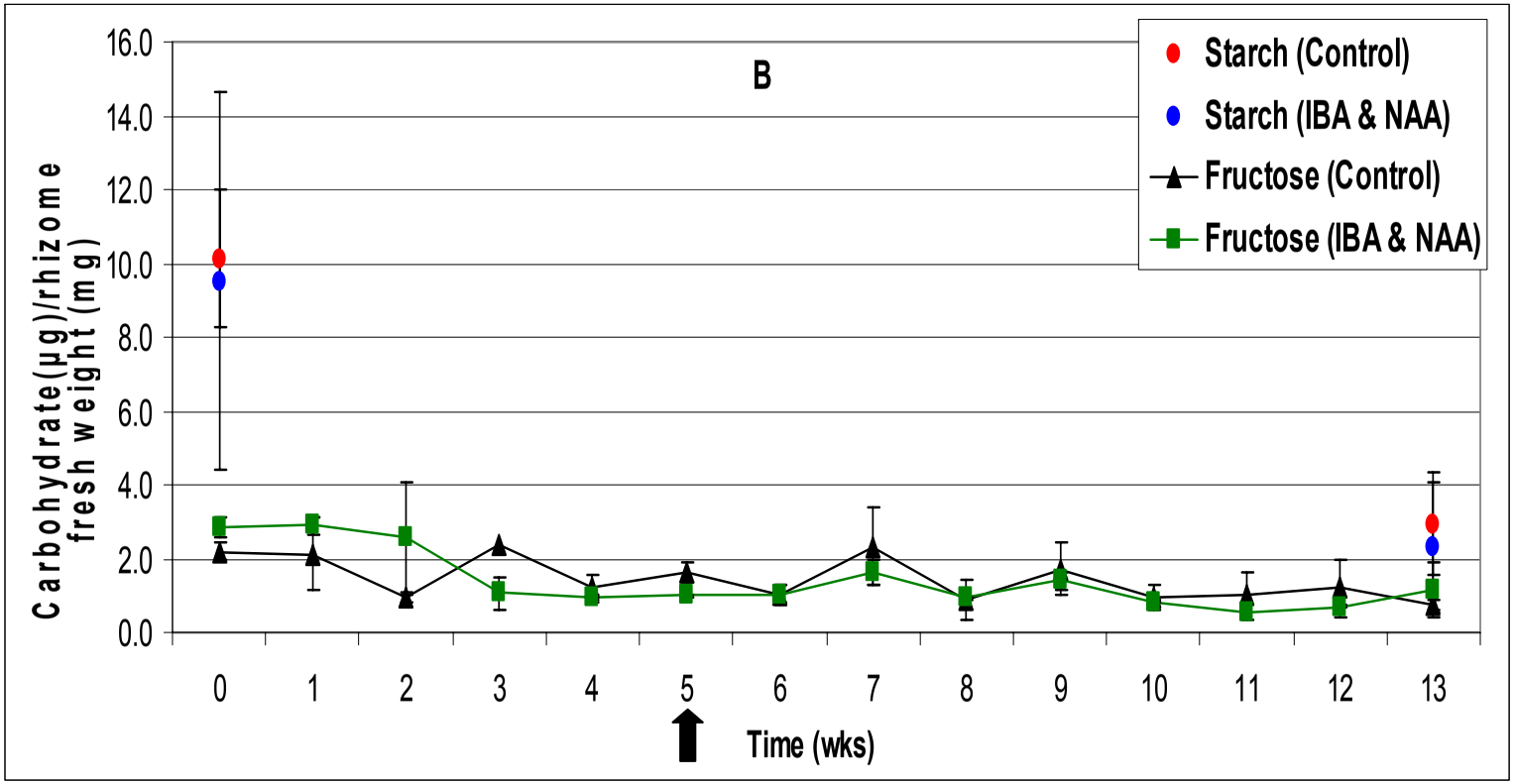


Figure 6B. **The effect of auxin (IBA and NAA) applied *in vitro* on fructose concentrations of *T. decumbens* rhizomes. After five weeks *in vitro*, the rhizomes were planted (see arrow) and placed in a 4°C coldroom. (n=2 for each point on the graph). Data were analyzed by two-way ANOVA. Error bars = +/- standard error.**

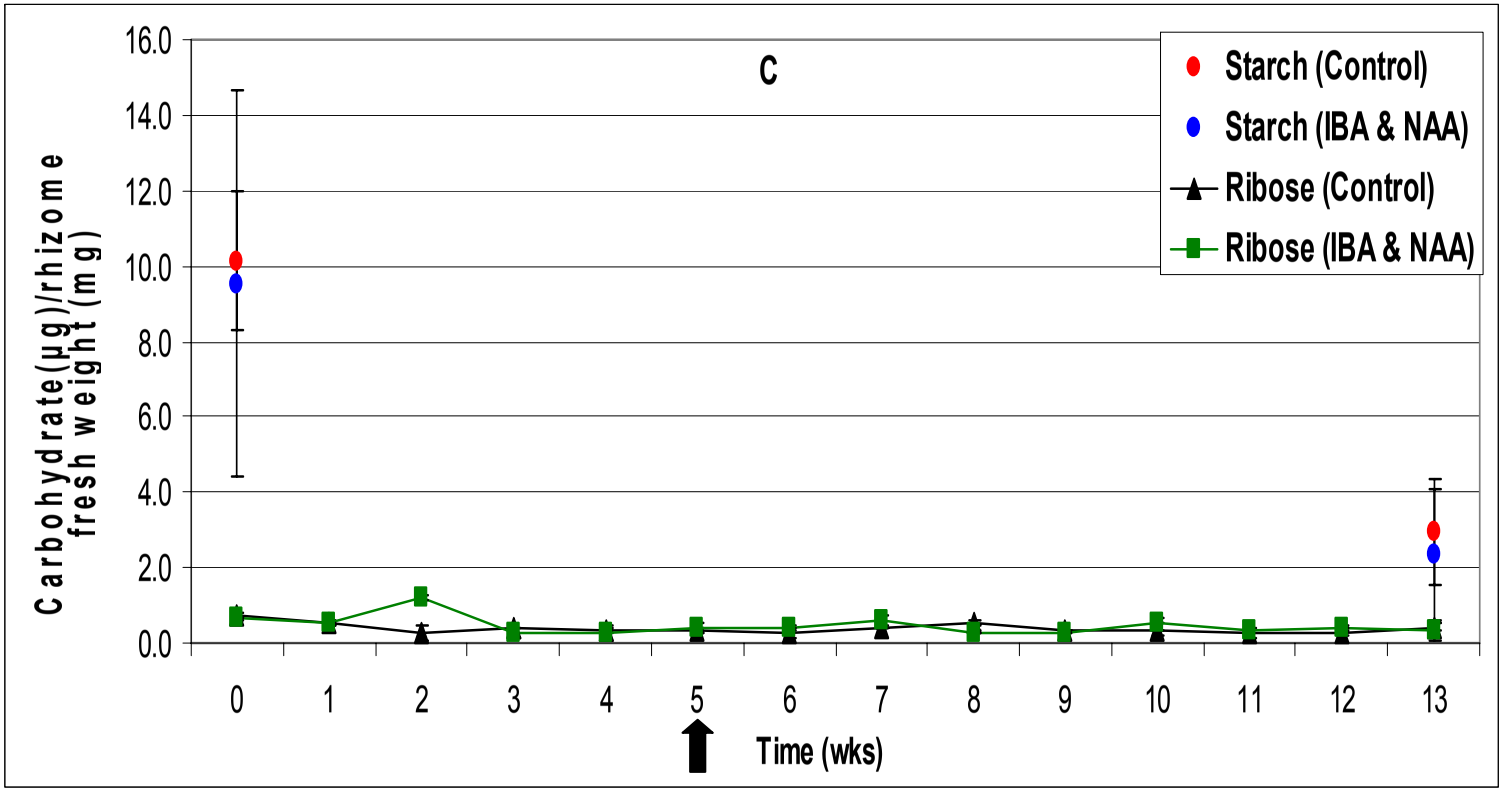


Figure 6C. **The effect of auxin (IBA and NAA) applied *in vitro* on ribose concentrations of *T. decumbens* rhizomes. After five weeks *in vitro,* the rhizomes were planted (see arrow) and placed in a 4°C coldroom. (n=2 for each point on the graph). Data were analyzed by two-way ANOVA. Error bars = +/- standard error.**

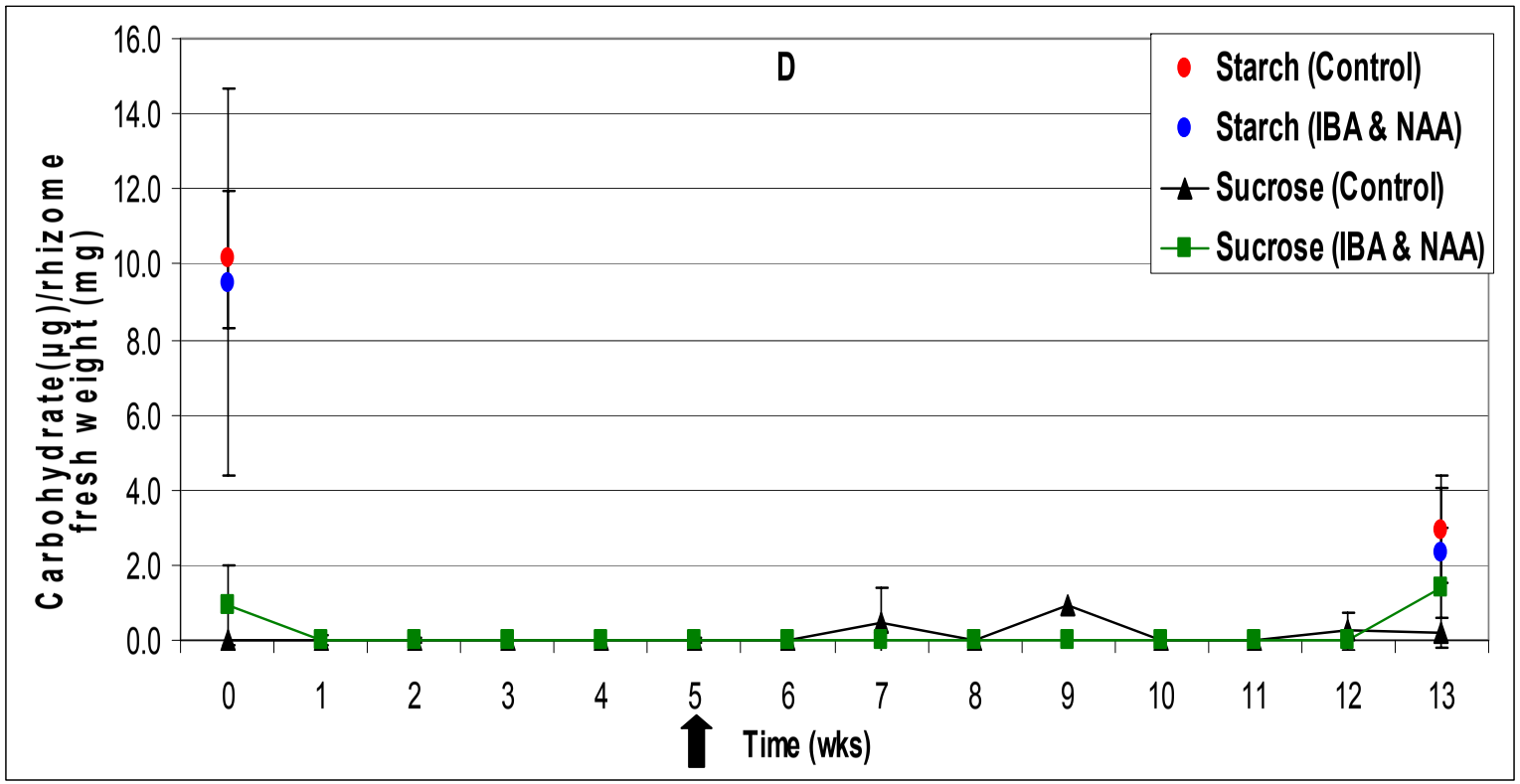


Figure 6D. **The effect of auxin (IBA and NAA) applied *in vitro* on sucrose concentrations of *T. decumbens* rhizomes. After five weeks *in vitro,* the rhizomes were planted (see arrow) and placed in a 4°C coldroom. (n=2 for each point on the graph). Data were analyzed by two-way ANOVA. Error bars = +/- standard error.**

2.3.3.2 Cytokinin

Rhizomes cultured on medium containing 0, 4.4, or 8.8μM BA showed a trend in decreased glucose, fructose and sucrose at the completion of the 8-week, greenhouse period (Fig. 7 A-C). Ribose was below detectable levels. Sucrose displayed a high degree of variability in concentration over the course of 13 weeks (Fig. 7C). A trend was observed in that glucose levels remained consistently higher than those of fructose. By 13 weeks, the concentration of all soluble sugars dropped to below detectable levels, though the level of starch was still detectable (Fig. 7D). There was a high degree of variability in starch concentration within the control samples in this experiment, which made it difficult to compare to experimental conditions.

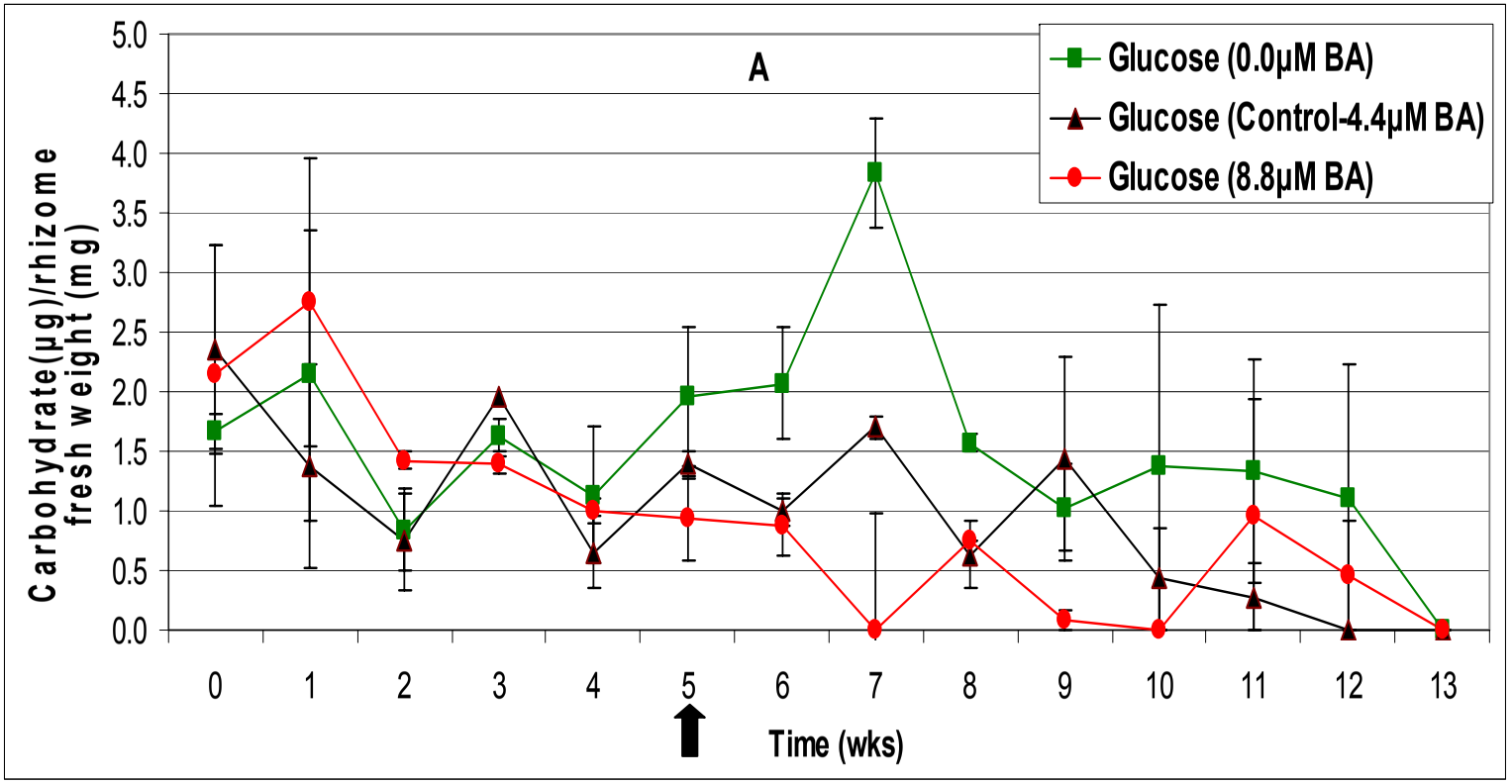


Figure 7A. **The affect of varied levels of cytokinin (BA) applied *in vitro* on glucose concentrations of *T. decumbens* rhizomes. After five weeks *in vitro*, the rhizomes were planted (see arrow) and placed in a 4°C coldroom. (n=2 for each point on the graph). Note different y-axis scales. Data were analyzed by two-way ANOVA. Error bars = +/- standard error.**

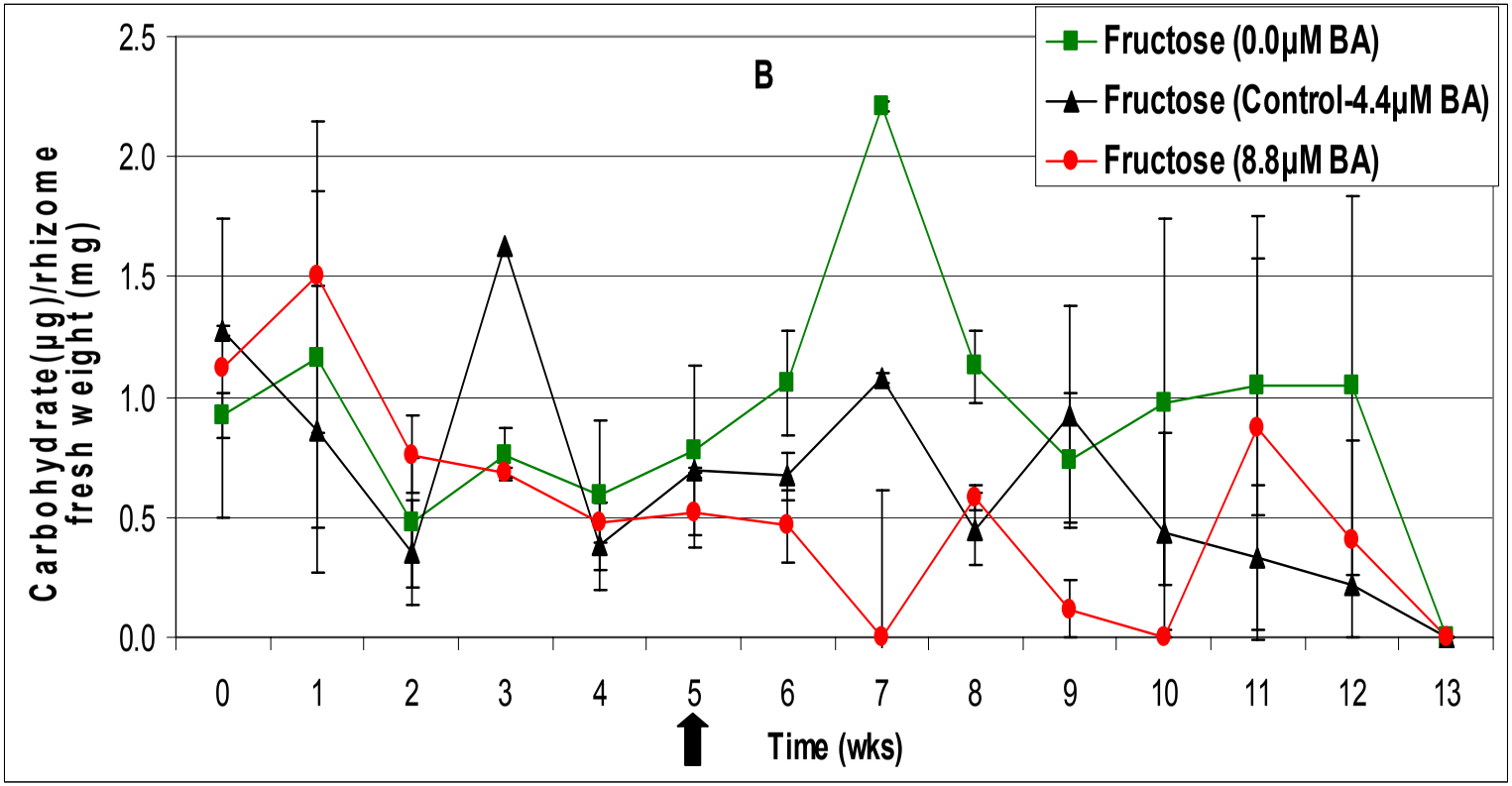


Figure 7B. **The affect of varied levels of cytokinin (BA) applied *in vitro* on fructose concentrations of *T. decumbens* rhizomes. After five weeks *in vitro*, the rhizomes were planted (see arrow) and placed in a 4°C coldroom. (n=2 for each point on the graph). Note different y-axis scales. Data were analyzed by two-way ANOVA. Error bars = +/- standard error.**

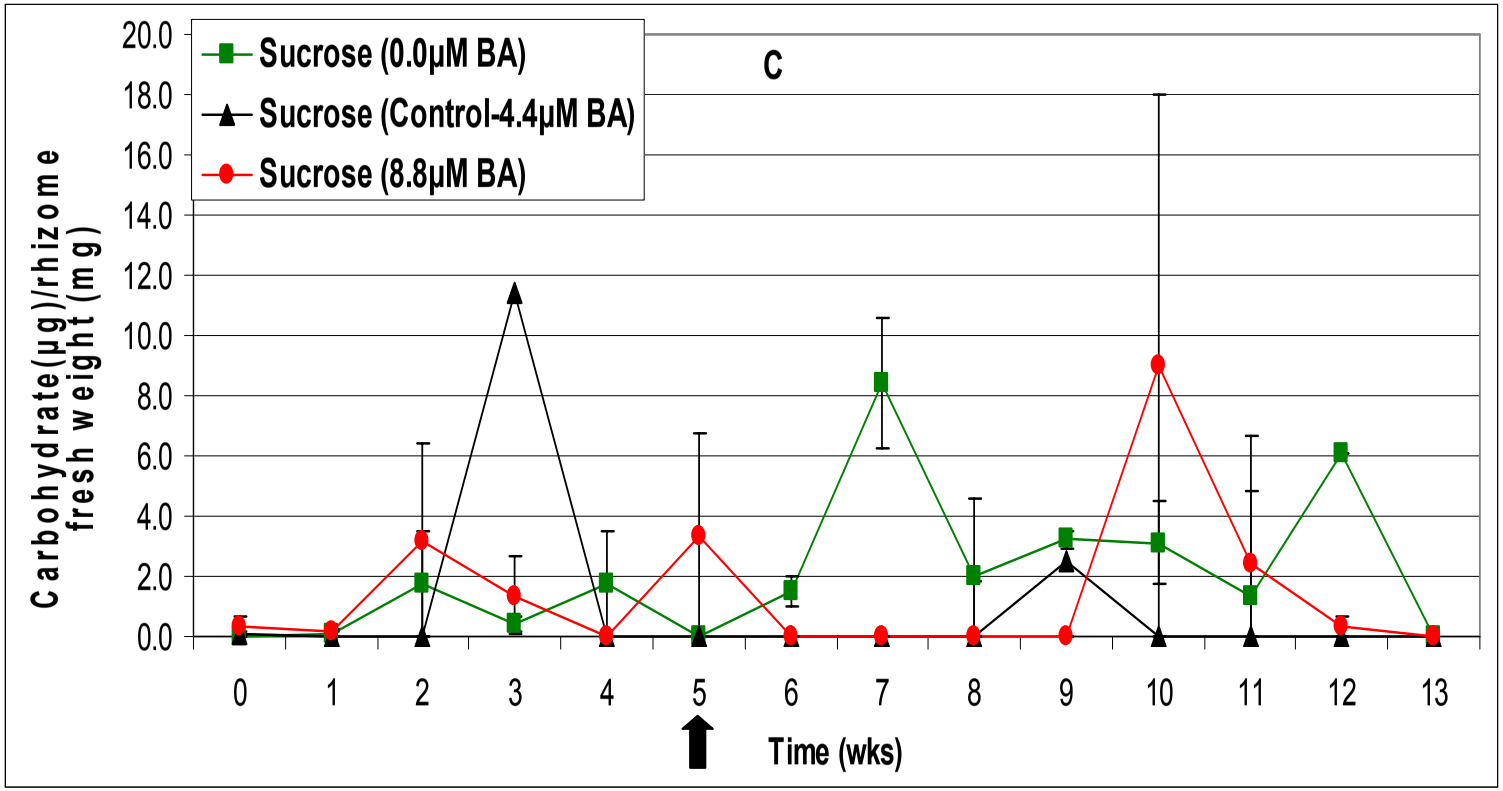


Figure 7C. **The affect of varied levels of cytokinin (BA) applied *in vitro* on sucrose concentrations of *T. decumbens* rhizomes. After five weeks *in vitro,* the rhizomes were planted (see arrow) and placed in a 4°C coldroom. (n=2 for each point on the graph). Note different y-axis scales. Data were analyzed by two-way ANOVA. Error bars = +/- standard error.**

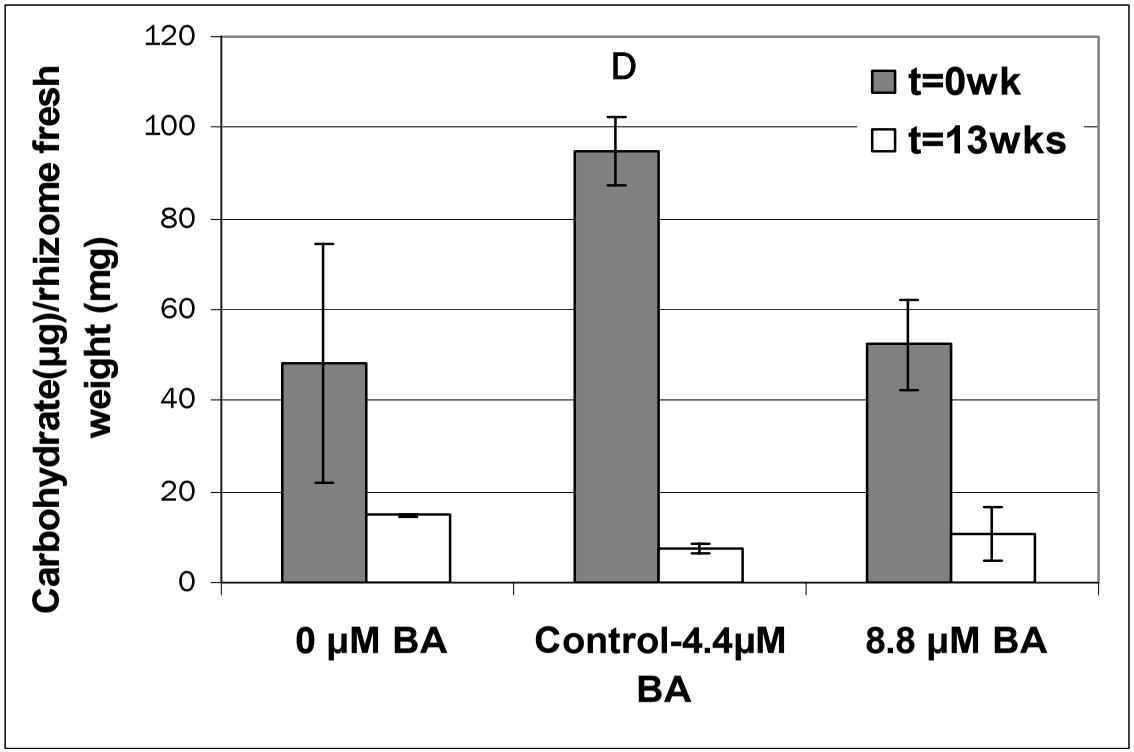


Figure 7D. **The affect of varied levels of cytokinin (BA) applied *in vitro* on starch concentrations of *T. decumbens* rhizomes. After five weeks *in vitro*, the rhizomes were planted and placed in a 4°C coldroom. (n=2 for each point on the graph). Note different y-axis scales. Data was analyzed by two-way ANOVA. Error bars = +/- standard error.**

2.3.3.3 Gibberellic acid

2.3.3.3.1 Preliminary GA experiment

There was no difference between MM with or without GA addition (data not shown). For all treatments n=1 and rhizomes were harvested at 3, 4, and 5 weeks.

2.3.3.3.2 *In vitro* experiment with GA

There was an overall trend toward decreasing carbohydrate concentrations in rhizomes treated and untreated (control) with GA (Fig. 8A-D). Once the rhizomes were removed from tissue culture and placed at 4°C in week 5, the levels of glucose, fructose, and ribose decreased and remained low through the following 8 weeks at 4°C. Another trend was seen in that glucose was consistently higher than fructose, and fructose higher than ribose. Ribose was consistently present, though no change was detected. Sucrose was only detectable before the rhizomes were transferred to the cold (Fig.8D). At the end of the 8-week period of cold storage, the concentration of starch remained measurable. No difference was seen when comparing week vs. treatment at a p value of less than 0.05 (two-way ANOVA).

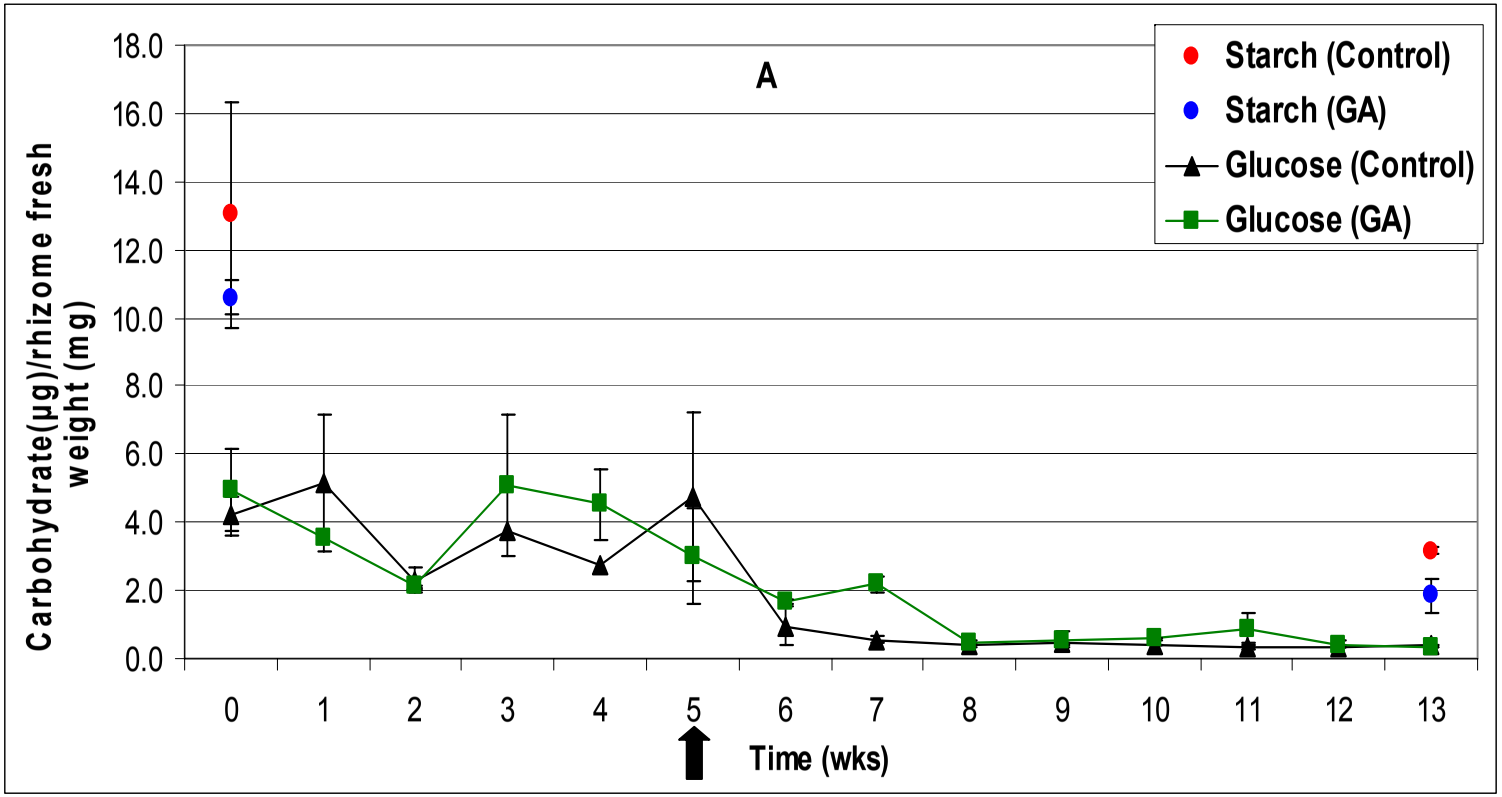


Figure 8A. **The effect of gibberellic acid (GA) applied *in vitro* on the glucose concentrations of *T. decumbens* rhizomes. After five weeks *in vitro,* the rhizomes were planted (see arrow) and placed in a 4°C coldroom. (n=2 for each point on the graph). Data was analyzed by two-way ANOVA. Error bars = +/- standard error.**

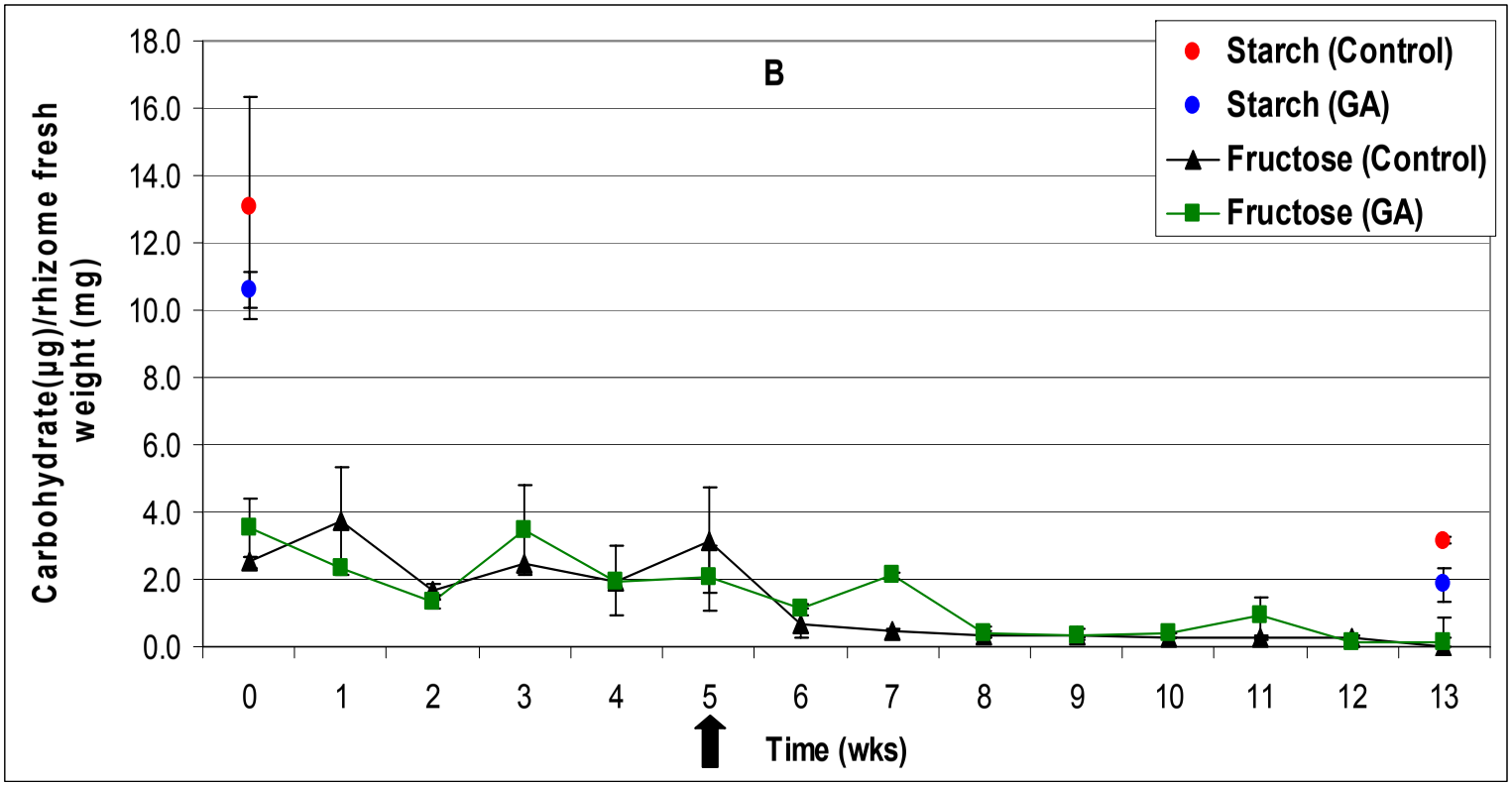


Figure 8B. **The effect of gibberellic acid (GA) applied *in vitro* on the fructose concentrations of *T. decumbens* rhizomes. After five weeks *in vitro,* the rhizomes were planted (see arrow) and placed in a 4°C coldroom. (n=2 for each point on the graph). Data was analyzed by two-way ANOVA. Error bars = +/- standard error.**

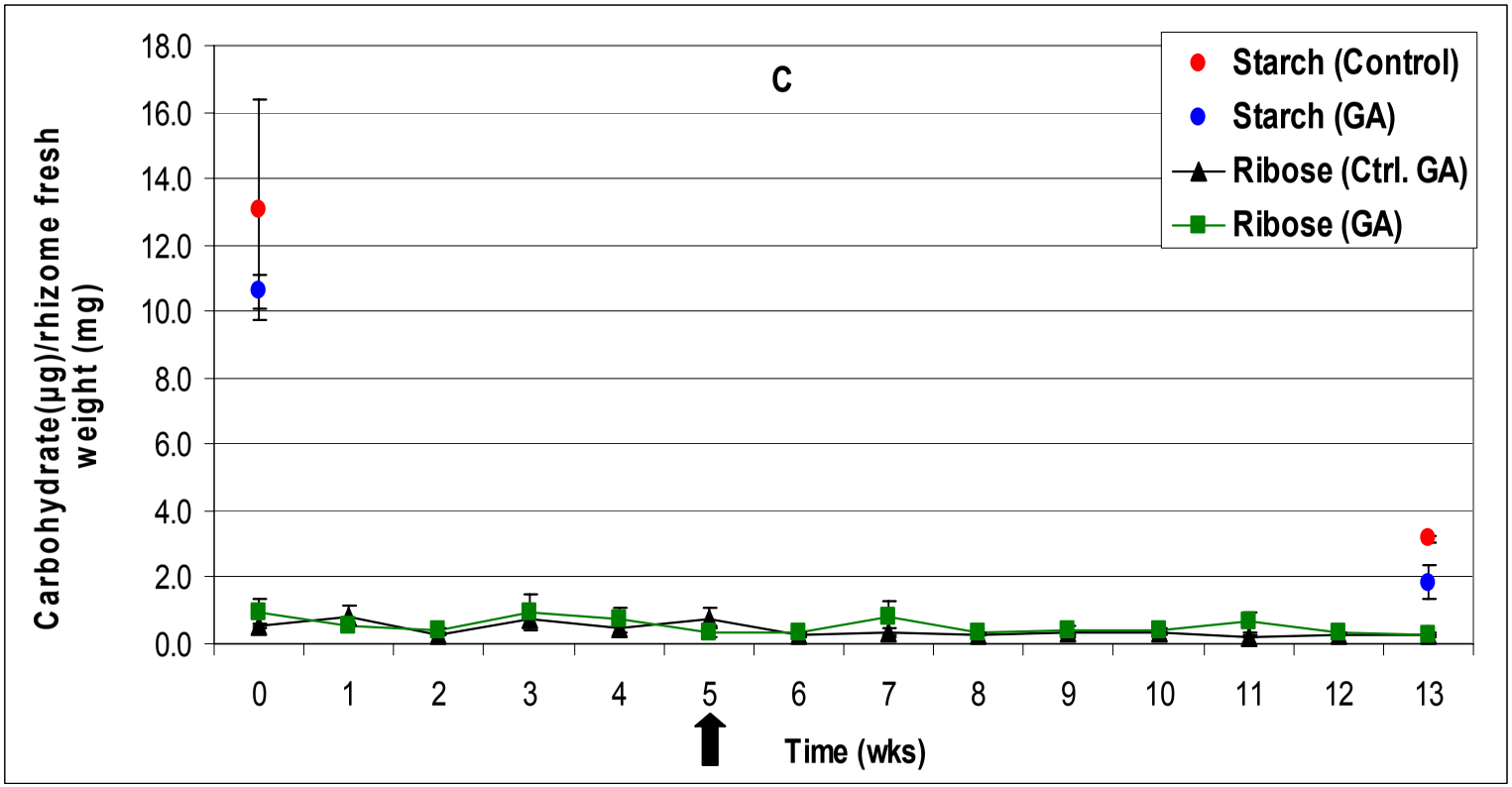


Figure 8C. **The effect of gibberellic acid (GA) applied *in vitro* on the ribose concentrations of *T. decumbens* rhizomes. After five weeks *in vitro*, the rhizomes were planted (see arrow) and placed in a 4°C coldroom. (n=2 for each point on the graph). Data was analyzed by two-way ANOVA. Error bars = +/- standard error.**

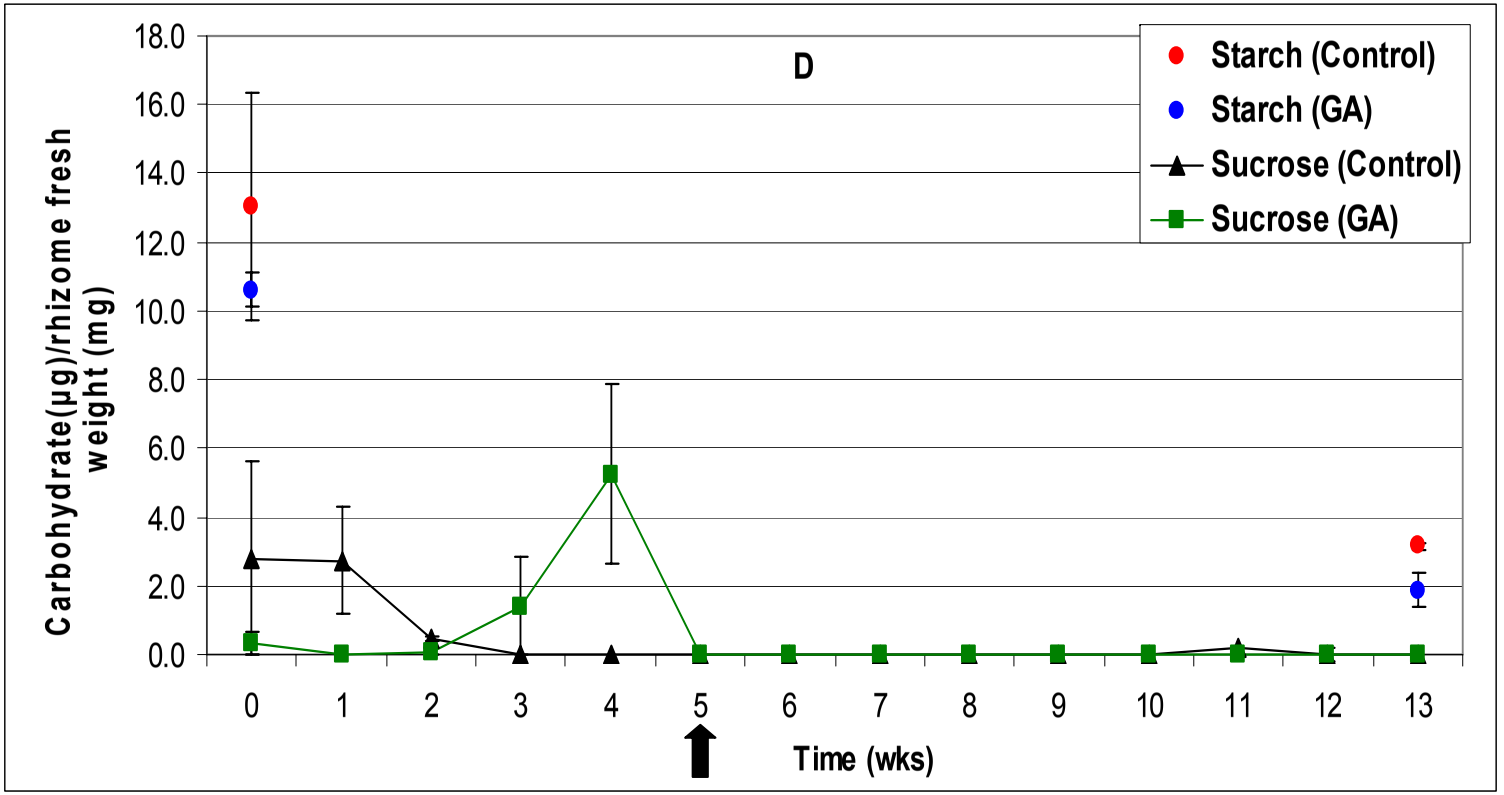


Figure 8D. **The effect of gibberellic acid (GA) applied *in vitro* on the sucrose concentrations of *T. decumbens* rhizomes. After five weeks *in vitro,* the rhizomes were planted (see arrow) and placed in a 4°C coldroom. (n=2 for each point on the graph). Data was analyzed by two-way ANOVA. Error bars = +/- standard error.**

2.4 Discussion

Trillium proliferates slowly in culture. Apical shoot excision was examined as a method to expedite the proliferation of MR. This method may further be helpful for producing rhizomes with an ideal shape – single base with apical shoot - to use in *ex vitro* experiments. Due to the fact that new growth in an apical meristem has been associated with a high relative concentration of soluble sugars (Ervin and Syperda, 1971), cultured rhizomes with single growing points should result in lower overall variability in soluble sugar concentrations. The benefits gained by shoot excision need to be further explored.

Staining with Lugol's iodine gave an adequate qualitative examination of starch accumulation in the rhizomes. It was difficult to say whether or not the staining was darker/lighter when comparing two rhizomes, but particular staining patterns on the cut face of the rhizome were evident.

The starch colorimetric assay and HPLC methods proved to be quick, reliable techniques for measuring starch and sugar, respectively. The starch assay required lengthy sample preparation due to enzymatic digestion of the sample prior to analysis, but sample duplicates were very consistent in measurement and the assay proved to be quite robust. Alternatively, rhizome preparation for the HPLC soluble sugar assay required a simple water extraction protocol, but, with *in vitro* trillium rhizomes, the results were more variable than with the starch assay. A higher degree of variation in the assay for sugars compared to that of the starch assay may be due to the high sensitivity of the HPLC assay as opposed to the colorimetric assay which requires the samples to be read on a spectrophotometer. Development of these assay methods helped to determine reliable assay methods for analyzing the carbohydrate profile of *in vitro* grown rhizomes.

High variability in glucose, fructose and sucrose concentrations may also be due to the fact that they are intermediate carbohydrates and not final storage products, as is starch.

Research has shown the influence of cytokinin on carbohydrate concentration in geophytes (Chu et al., 2002). In this study, concentrations of both starch and total soluble carbohydrates increased with increasing concentrations (0.44, 2.2, 4.4, 11 and 22μM) of BAP. The disagreement between the Chu et al. study and the trillium data may be due to the fact that the tubers of *Discorea* already had established leaves when acclimated outside of the lab. This would have allowed them to begin photosynthesis and accumulate carbohydrates much more readily than the leafless trillium rhizomes. By 13 weeks, the concentration of all soluble sugars dropped to below detectable levels in the cytokinin treatments but not with any of the other growth regulator treatments. Because it occurred for the duplicate samples and only at the 13 week time point, it may have been an anomaly affecting those samples only.

Auxin and gibberellic acid are known to affect storage organ size and number (Escalante and Langille, 1995; Xu et al., 1998; Sheelavantmath et al., 2000) in tissue culture, but little is known about their direct correlation to carbohydrate changes. A decrease in average starch concentration in trillium rhizomes was consistent throughout each of the experiments in which growth regulators were applied. The lack of a difference in soluble sugar concentrations amongst rhizome treatments for all growth regulators, was not necessarily evidence that IBA, NAA, BA, and GA did not influence the carbohydrate profile, but simply may be due to low sample numbers and highly variable rhizomes. The use of Dip ‘n Grow as a source of

auxin in tissue culture medium may have effected the results, as IBA and NAA were not pure, but were mixed with other inert ingredients.

Trillium rhizomes do not generate roots in tissue culture and, therefore, when planted in soilless medium, initially must absorb nutrients through cells in the body of the rhizome at the same time it is initiating roots and a shoot. An abundant carbon source is necessary to drive this *ex vitro* growth.

Several experiments might be designed to address these issues. First, an environment in the laboratory that would provide enough CO_2 and light to initiate photosynthesis in those rhizomes that grow leaves, may serve to accumulate carbohydrates before the rhizome is introduced to a stressful field condition.

Second, the type of soluble sugar that is added to the medium might be varied to study its effects on carbohydrate metabolism. Adding glucose, fructose, etc. might serve to shift the cells of the rhizome to different types of storage products or to increase the concentration of starch stored.

Finally, an increase in sucrose concentration may also drive the metabolism of the rhizome toward accumulation of higher concentrations of starch and make it more likely that the rhizome has an adequate carbon source to survive and even develop a new shoot during its acclimation *ex vitro*. In conjunction with varying sucrose concentrations, it would be important to test the amount of sucrose remaining in the medium following a transfer cycle (1 month) to determine how depleted the sucrose is after 1 month and whether the depletion is continuous throughout the medium. The amount of sucrose that is absorbed by the rhizome can then be compared with the increase in starch concentration over the same period of time. This

would indicate whether or not the rhizome is using another form of carbohydrate storage, such as fructan or glucosamine for example.

Fructans, as well as starch, are known to accumulate in rhizomes of asparagus (*Asparagus officinalis* L.) (Ernst and Krug, 1998). Some plants that store fructans do not store starch at all, as is the case with many Allium species (Darbyshire and Henry, 1981). A study of fructan concentration in trillium rhizomes would indicate whether fructan has any role in their carbohydrate fluctuation. There is a higher energy cost for polymerizing glucose molecules and storing starch, but the storage of starch does not influence the osmotic potential as does fructan.

Chapter 3

EX VITRO RE-ESTABLISHMENT

3.1 Introduction

Historically it has been reported that trillium seeds need at least a single period of cold prior to breaking their dormancy (Solt, 1996). With this in mind, it has been assumed that in transferring tissue culture grown trillium rhizomes to a true field condition that they must first be acclimated to conditions of an "external" environment to induce shoot growth.

Typically, *in vitro* grown plants, in which aerial plant parts arise from underground storage organs such as rhizomes, corms, bulbs, or tubers, have needed a period of cold storage – the temperature of the cold period being dependent on the native environment of the plant – to initiate active shoot growth (LeNard and DeHertogh, 1993; Yamagishi, 1993; Maqbool et al., 2004; Paz et al., 2005). With certain *in vitro* generated plant species, a cold period prior to field establishment hastened active shoot development, but was not imperative for growth (Gracie, 2000). Data from the aforementioned studies also demonstrated that the specific time period necessary to initiate shoot growth was very specific not only to a given genus, but even to species within a genus.

To look for a correlation between the condition of the *ex vitro* environment the rhizomes were exposed to (length of time and temperature they were stored at a given temperature) and the loss or accumulation of carbohydrates, a series

of experiments were designed to study carbohydrate concentration changes during varied time periods and temperatures for *ex vitro* growth.

3.2 Materials and Methods

3.2.1 Plant Material

Trillium maculatum and *T. decumbens* explants were received from Mt. Cuba Inc. on April 25th, 2001. The plants were initially cultured on regeneration medium (Foster, 2002), and subsequently subcultured on maintenance medium every four weeks. *T. dicolor* rhizomes were planted in soil on a field plot at the University of Delaware in 2002.

3.2.2 Tissue culture medium

All experiments in this chapter were carried out with rhizomes maintained on maintenance medium. See Chapter 2 for medium formulation.

3.2.3 Re-establishment protocol

All experiments in this chapter follow the re-establishment protocol as described in Chapter 2 to the point of coldroom and greenhouse storage. Several of the experiments in this chapter include the use of a coldroom and greenhouse at the facilities of the United States Department of Agriculture (USDA) in Beltsville, Maryland. The remainder of the experiments utilized the coldroom and greenhouse at the University of Delaware.

3.2.4 Processing rhizomes for analysis

3.2.5 Starch stain

3.2.6 Starch colorimetric assay

3.2.7 HPLC assay for sugars

Rhizomes were processed and assayed qualitatively and quantitatively as described in Chapter 2. This applies to topic headings 3.2.4, 3.2.5, 3.2.6, and 3.2.7.

3.2.8 Re-establishment

3.2.8.1 Acclimation

3.2.8.1.1 Rhizomes at 4°C for 12 wks/ greenhouse for 6wks

In March of 2004, three hundred rhizomes of *T. maculatum* were planted in MetroMix 510 (soilless medium) as described in Chapter 2, and placed in a 4° C coldroom at the University of Delaware for 12 weeks. At the end of 12 weeks, the flats of rhizomes were transferred to a non-temperature controlled greenhouse. Plants were visually monitored for shoot and leaf growth for 6 weeks following transfer to the greenhouse.

3.2.8.1.2 Comparison of different greenhouse temperatures

One-hundred and twenty rhizomes (two flats of sixty rhizomes each) of *T. maculatum* were planted in MetroMix 510 and placed in a 4°C coldroom at the University of Delaware for 12 weeks in July of 2004. Following 12 weeks in the cold, a single flat of rhizomes was transferred to a non-temperature controlled greenhouse at the University of Delaware and the other flat to a greenhouse set to maintain

approximately 26°C during the day, at the USDA in Beltsville, MD. Plants were visually monitored for shoot and leaf growth for 6 weeks following transfer to the greenhouse.

3.2.8.1.3 Comparison of 4°C coldrooms

A single flat (60 rhizomes) of *T. maculatum* was planted in MetroMix 510 and placed in a 4° C coldroom at the USDA, Beltsville site and another flat stored in the University of Delaware coldroom, in July of 2004. Following 12 weeks in the cold, the flat of rhizomes in the USDA coldroom was transferred to a USDA greenhouse. The flat stored at the University of Delaware coldroom was transferred to the greenhouse at the University. Plants were visually monitored for shoot and leaf growth for 6 weeks after transfer to the greenhouse.

3.2.8.1.4 Rhizomes at 4°C for 9wks

Tissue cultured rhizomes of *T. maculatum* were removed from culture and planted in soilless medium as described in Chapter 2. The rhizomes were placed in a 4°C coldroom for 9 weeks. Rhizomes were harvested once a week, beginning with t=0weeks, and processed for analysis upon completion. The entire experiment was run at three different times and every time point was analyzed for soluble sugars. The first (t=0wk) and final (t=9wk) time points were analyzed for starch content.

3.2.8.2 Temperature stratification

3.2.8.2.1 Rhizomes in preliminary experiment to examine an offset stratification scheme

Two sets of *T. maculatum* rhizomes were put into temperature stratification treatments so that one set was initially begun in a period (12 wks) of cold (4°C) and another set in an outdoor shade house (offset stratification scheme), in an attempt to initiate growth prior to planting in the field. The experiment was initiated by Mt. Cuba, Inc. researchers. The rhizomes were planted in MetroMix 560 and labeled with the accession numbers 20040156 A&B and 20040157 A&B. The set "A" rhizomes began with a period of 12 weeks in cold (4°C) followed by alternating 12-week periods of warm (outdoor shade house or greenhouse) and cold. The set "B" rhizomes were started with a 12-week warm period, followed by alternate cold and warm 12 week periods. Eight rhizomes were harvested at 24 weeks and stained with iodine for starch. At 36 weeks, four rhizomes were harvested and analyzed for starch by staining and assayed for soluble sugar concentrations by HPLC. A final harvest of four rhizomes was taken at 48 weeks and analyzed for sugar concentrations.

3.2.8.2.2 Rhizomes put through two offset stratification regimens for varied time intervals

To study the effect that time in and out of cold affects the carbohydrate concentrations in rhizomes, rhizomes were placed in an offset stratification scheme for varied lengths of time. In July of 2005, rhizomes from tissue culture were planted in MetroMix 510 and placed in either a coldroom (4°C) or an outdoor shade house (ambient temperature) at Mt. Cuba, Inc. for 4, 6, or 8 wk. periods. Two rhizomes were harvested at t=0 for each treatment and again at each warm or cold transfer point, through a total of 12 to16 weeks. Once the experiment was completed, each rhizome

was analyzed for soluble sugar concentrations and the start and end points were assayed for starch.

3.3 Results

3.3.1 Re-establishment

3.3.1.1 Acclimation

3.3.1.1.1 Rhizomes at 4°C for 12 wks/ greenhouse for 6wks

A total of 2 shoots with a single leaf each were observed out of 300 planted rhizomes, giving a 0.67% yield after a 12-week cold period and a period of 6 weeks in the greenhouse.

3.3.1.1.2 Comparison of different greenhouse temperatures

Whether rhizomes were placed in a temperature controlled USDA greenhouse or a non-temperature controlled greenhouse at the University of Delaware there was no indication of any stem or leaf growth from either flat of planted rhizomes. During the 6 weeks following a 12-week cold period, rhizomes at both greenhouses had a similar low yield of approximately 1%. This indicated that the warmer temperatures at the University of Delaware greenhouse were not responsible for the low stem/leaf growth rate seen in the first experiment.

3.3.1.1.3 Comparison of 4°C coldrooms

There was also no indication of a difference in number of rhizomes with stem or leaf growth after storage for 12 weeks in either the USDA or University of Delaware coldrooms, during the following 6 week period in respective greenhouses.

3.3.1.1.4 Rhizomes at 4°C for 9wks

Levels of glucose and fructose stayed relatively constant at low levels in rhizomes placed in the coldroom for 9 weeks, with a slight increasing concentration trend between weeks 6 through 9 (Fig. 9A & B). In rhizomes placed in the greenhouse, glucose and fructose rose from week 5 to week 7 when compared to those stored in the cold. This rise was not significantly correlated (p value was greater than 0.05) to the rise in sucrose levels over the same time period. Sucrose rose within the first week of subjection to cool (4°C) temperature (Fig. 9D) and, at weeks 6 and 9, levels displayed a trend toward increasing concentrations. For rhizomes in the greenhouse, sucrose concentration did not rise until the 5th week. Ribose was not present at significantly detectable levels throughout the 9-week time course (Fig. 9C). Starch was present in t=0 rhizomes at 4.48 μg and 4.13 μg starch/mg rhizome for cold and greenhouse stored rhizomes, respectively. By the 8th week, starch had been completely degraded under both experimental conditions to below detectable levels.

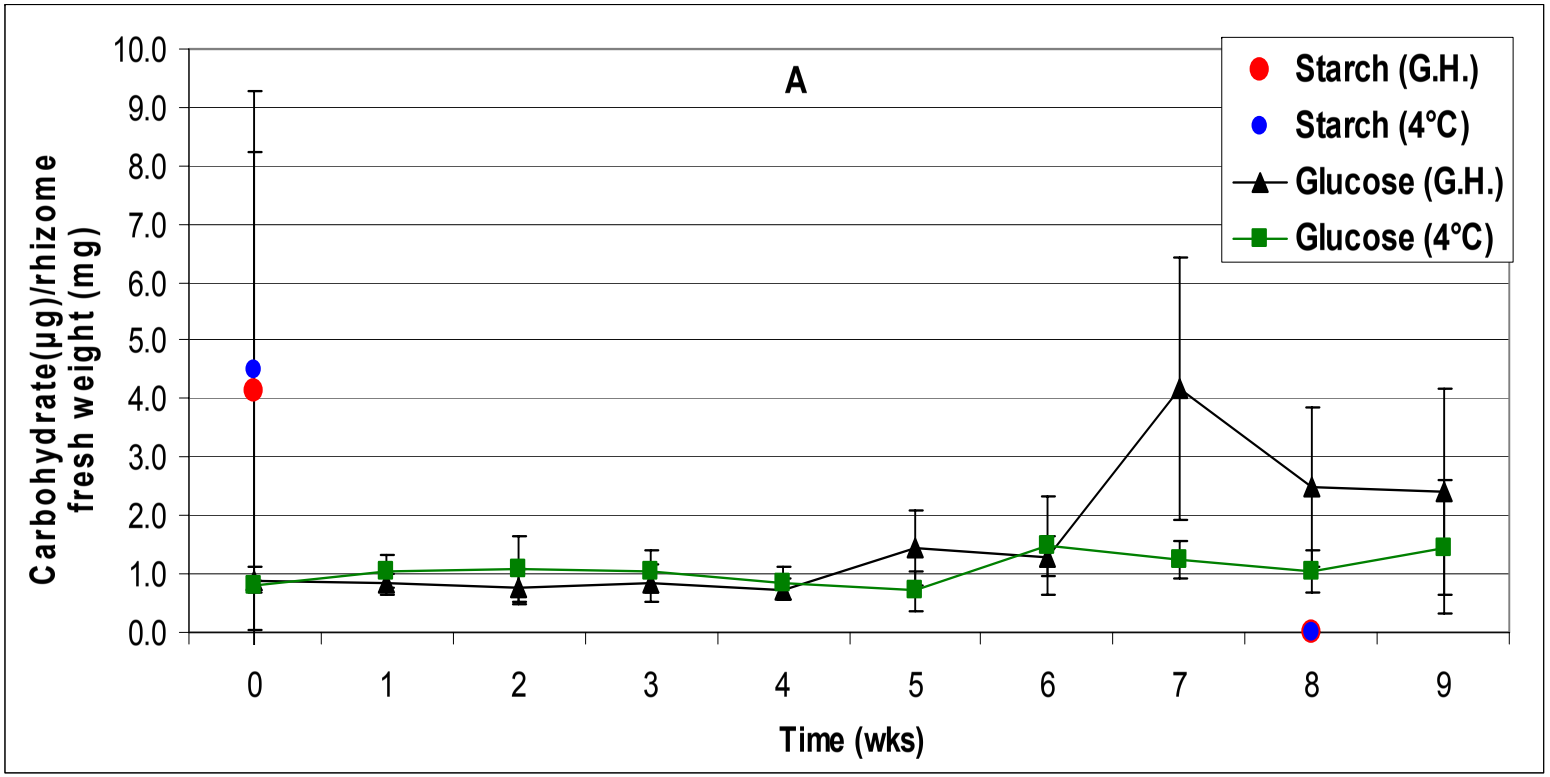


Figure 9A. **Changes in glucose concentrations in *T. maculatum* rhizomes planted and placed either at 4°C or in the greenhouse for 9 weeks. (n=3 for each point on the graph). Note different y-axis scales. Data was analyzed by two-way ANOVA. Error bars = +/- standard error. G.H. = greenhouse**

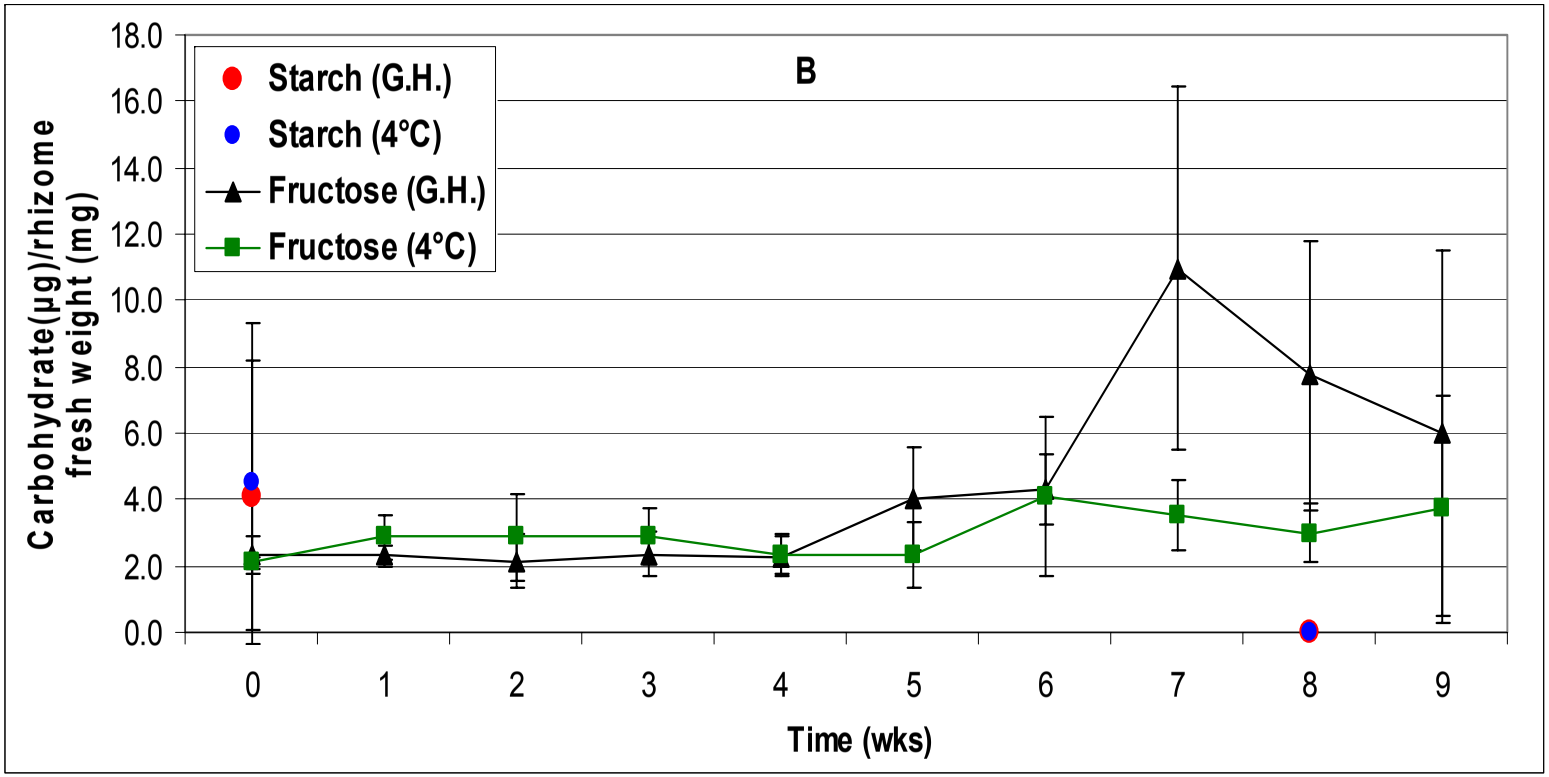


Figure 9B. **Changes in fructose concentrations in *T. maculatum* rhizomes planted and placed either at 4°C or in the greenhouse for 9 weeks. (n=3 for each point on the graph). Note different y-axis scales. Data was analyzed by two-way ANOVA. Error bars = +/- standard error. G.H. = greenhouse**

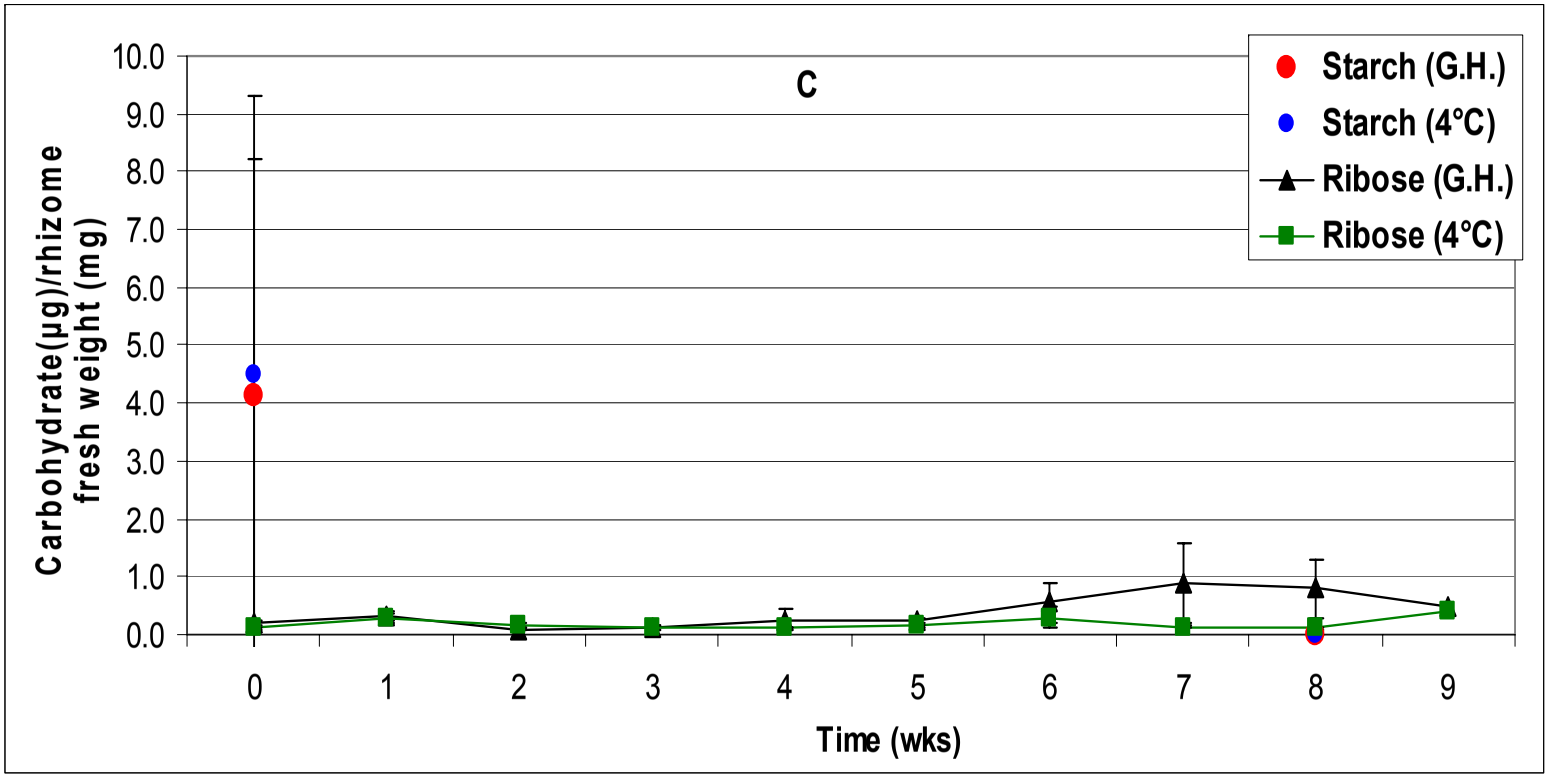


Figure 9C. **Changes in ribose concentrations in *T. maculatum* rhizomes planted and placed either at 4°C or in the greenhouse for 9 weeks. (n=3 for each point on the graph). Note different y-axis scales. Data was analyzed by two-way ANOVA. Error bars = +/- standard error. G.H. = greenhouse**

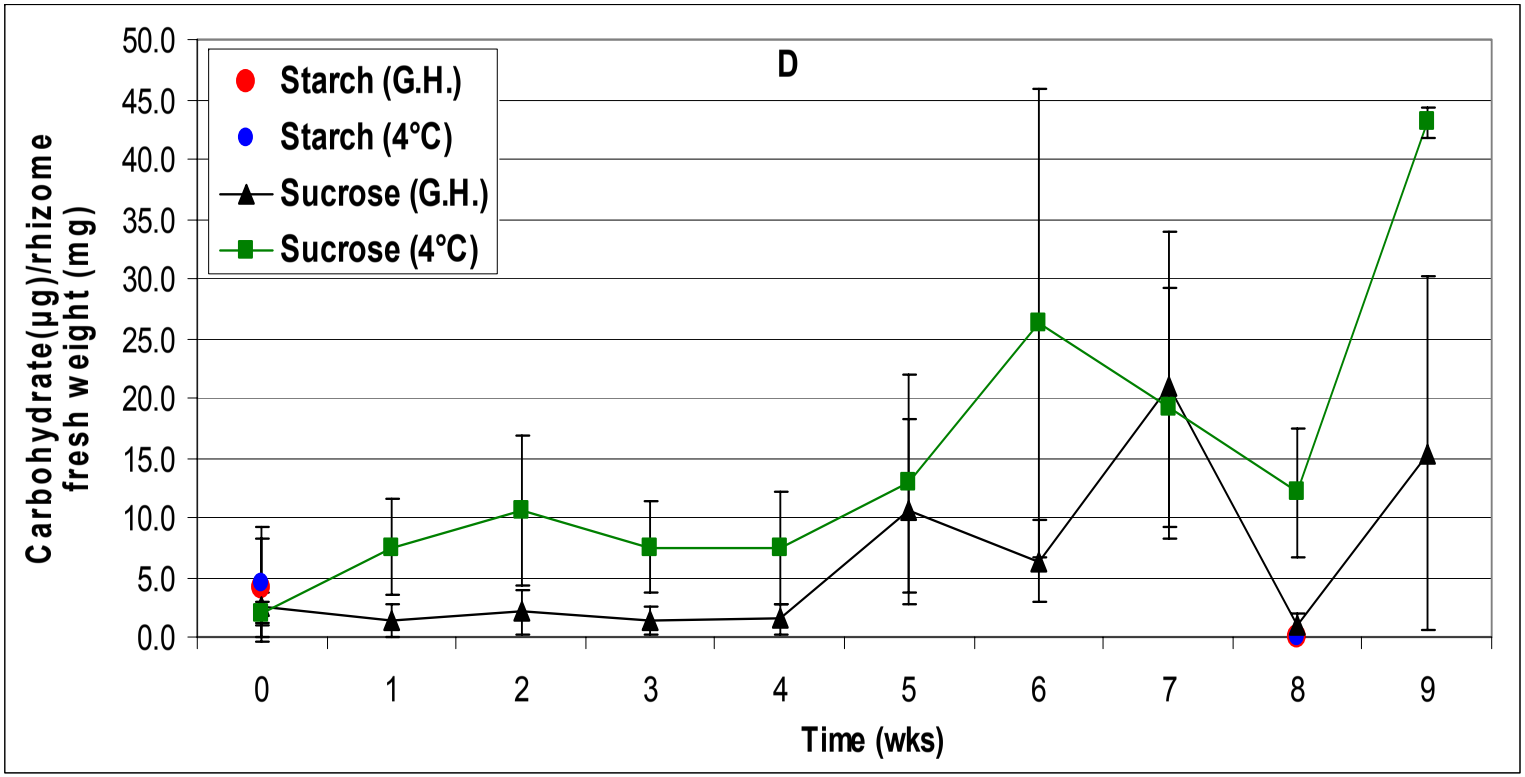


Figure 9D. **Changes in sucrose concentrations in *T. maculatum* rhizomes planted and placed either at 4°C or in the greenhouse for 9 weeks. (n=3 for each point on the graph). Note different y-axis scales. Data was analyzed by two-way ANOVA. Error bars = +/- standard error. G.H. = greenhouse**

3.3.1.2 Temperature stratification

3.3.1.2.1 Rhizomes in preliminary experiment to examine an offset stratification scheme

After 24 weeks, rhizomes that had started in 12 weeks of cold stained (Fig. 10A-Row A) darker and more consistently than those started in 12 weeks of warm storage (Fig. 10A-Row B). After undergoing another 12 weeks of either warm or cold storage (total of 36 weeks *ex vitro*), both those started in cold and in warm seemed to only retain starch around their apical meristems, evidenced by the dark staining at the base of what appears to be apical shoots (Fig. 10B). At 36 and 48 weeks of offset 12-week stratification the soluble sugars were quantified and were highly variable between stratification treatments (Fig. 11). This was an ongoing study from which rhizomes were harvested and, therefore, only provided a preliminary indication of carbohydrate concentration differences among treatments.

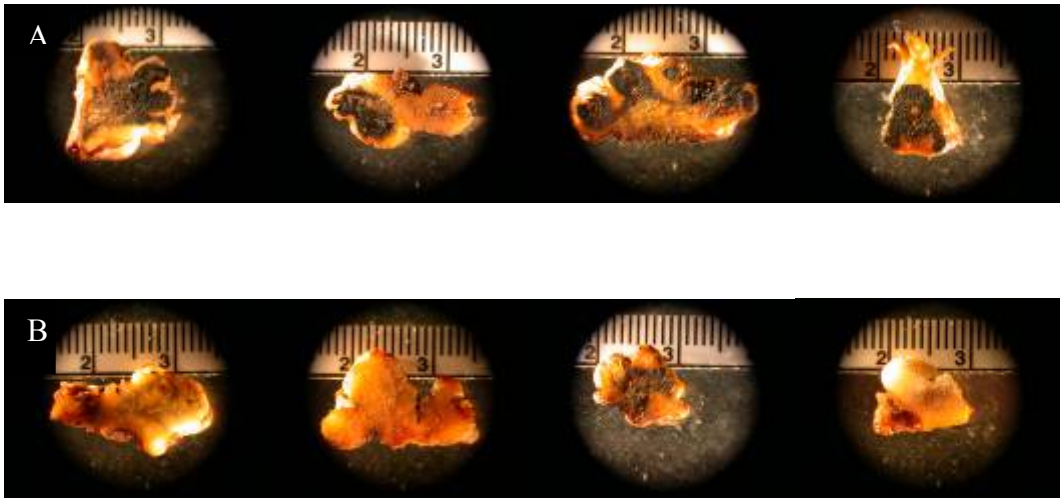


Figure 10A. Iodine stain of rhizomes harvested from Mt. Cuba Inc. at 24 weeks after planting. (A) Rhizomes were in the cold (4°C) for 12 weeks followed by 12 weeks of warm (outdoor shade house or greenhouse). (B) Rhizomes were in the warm for 12 weeks, followed by 12 weeks in cold. (Scale: distance between dashes = 1mm)

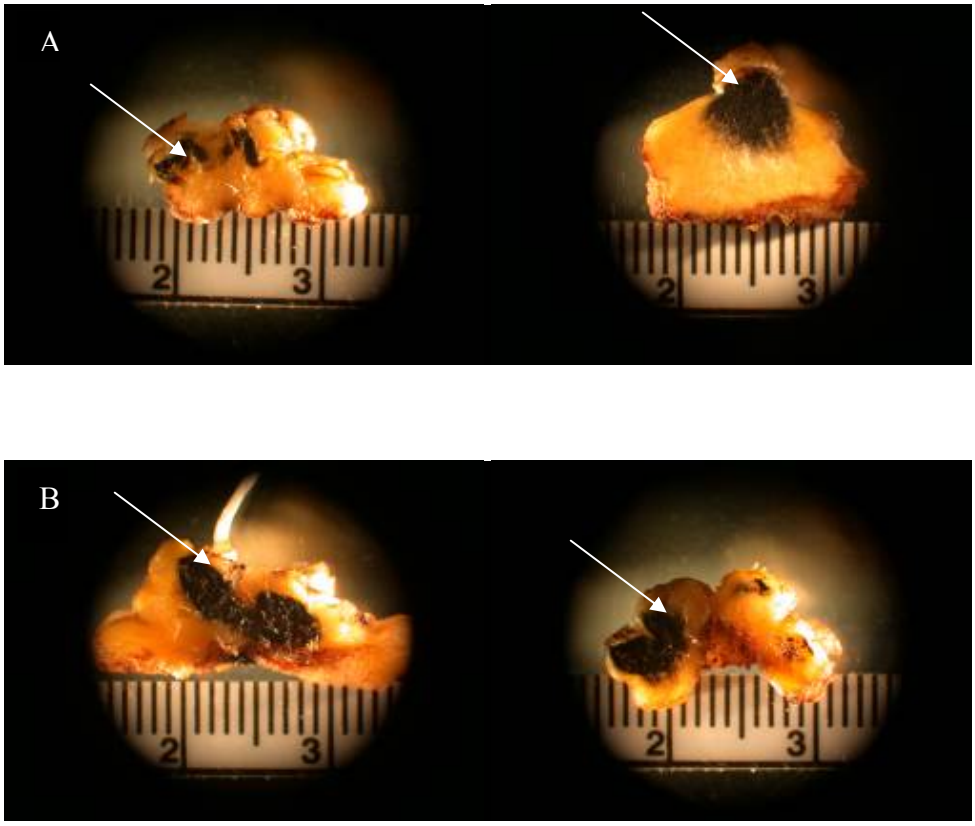


Figure 10B. Iodine stain of rhizomes harvested from Mt. Cuba Inc. at 36 weeks after planting. (A) Rhizomes were stratified by 12wks cold-12wks warm-12wks cold. (B) Rhizomes were stratified by 12 wks warm-12 wks cold-12 wks warm. (Scale: distance between dashes = 1mm). Arrow indicates base of apical shoot.

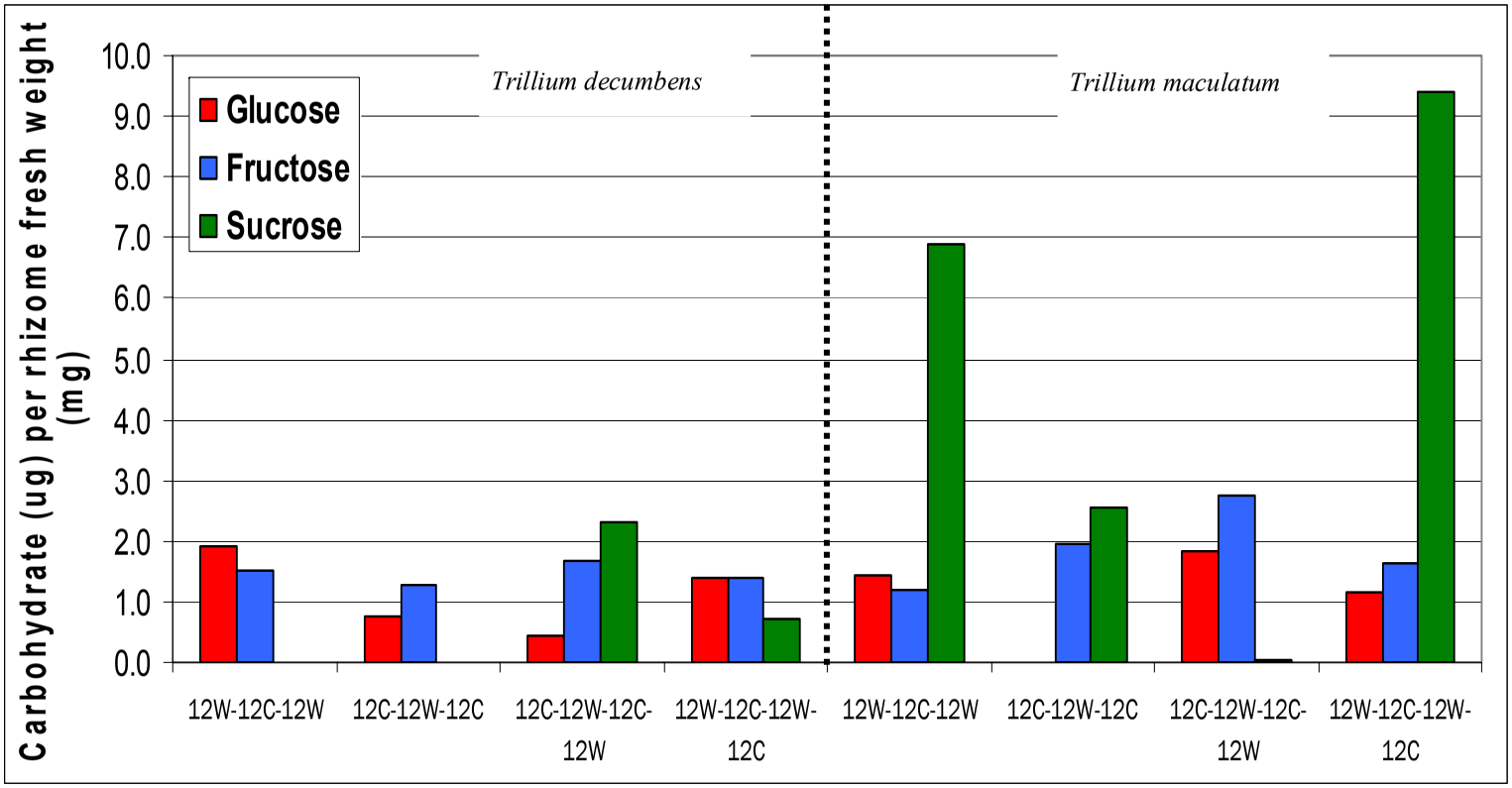


Figure 11. Comparison of stratification schemes and *ex vitro* duration of two trillium species (n=1 per bar).

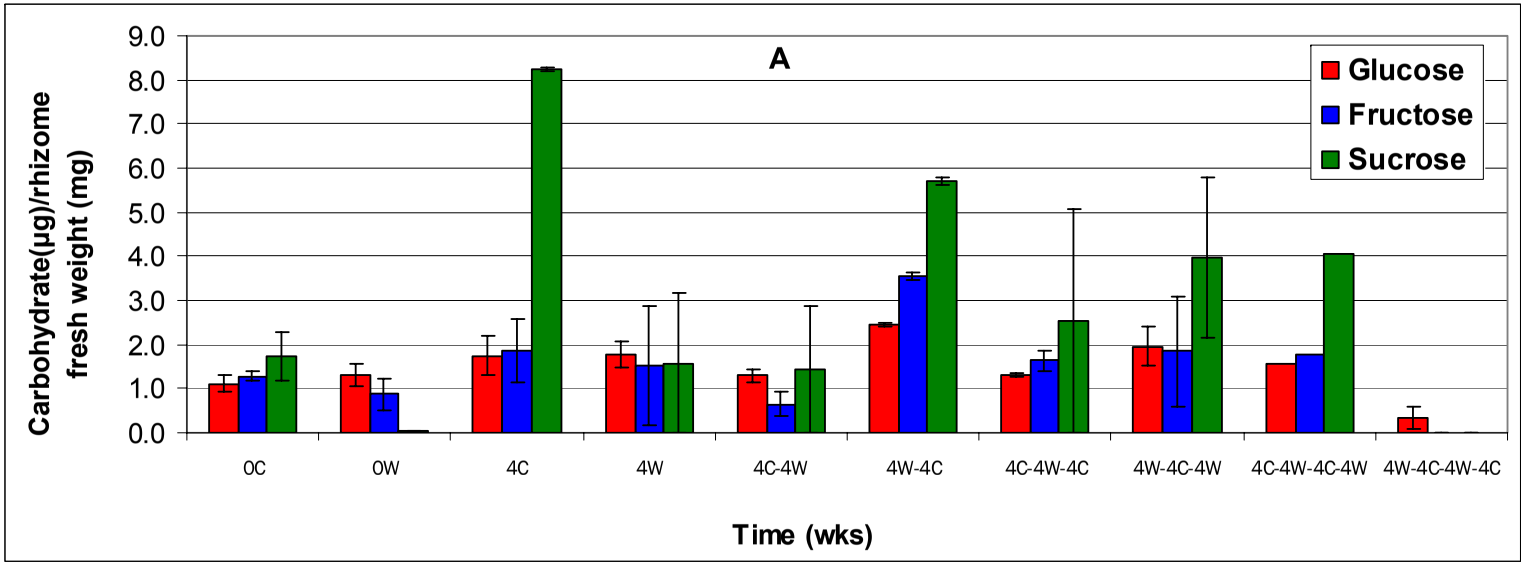
3.3.1.2.2 Rhizomes put through two offset stratification regimens for varied time intervals

For rhizomes stratified every 4 weeks and begun in cold, glucose, fructose, and sucrose concentrations fluctuated, but did not display a trend for any one treatment. For 4-week periods beginning in warm, concentrations of glucose, fructose and sucrose also fluctuated erratically, but by week 16, were below detectable levels. Starch was detectable after 16 weeks (Fig. 12).

For the six-week stratification at either cold or warm starting temperatures, levels of glucose and fructose concentration remained constant over 12 weeks, with levels of sucrose concentration varying erratically. Starch was consistently detectable at the conclusion of the time course, in rhizomes begun in either warm or cold (Fig. 13).

Those in an 8-week offset temperature stratification had a similar profile to those in 6-week periods. Levels of soluble sugars remained just above the detectable levels and varied erratically. Starch concentrations remained detectable at t=16 weeks (Fig. 14).

Ribose was below detectable levels for all stratification experiments.



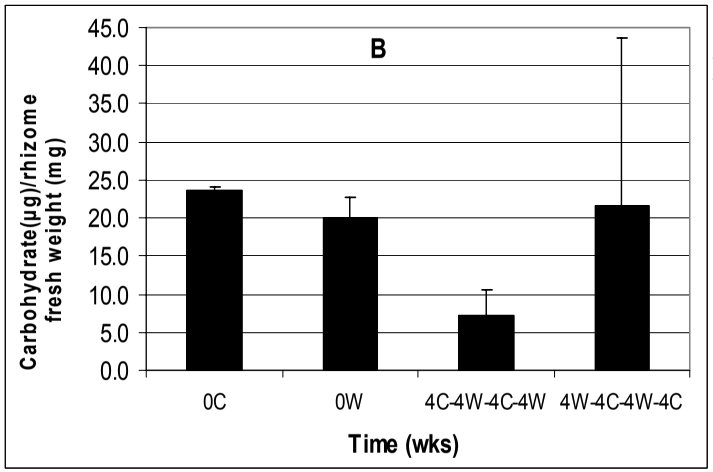
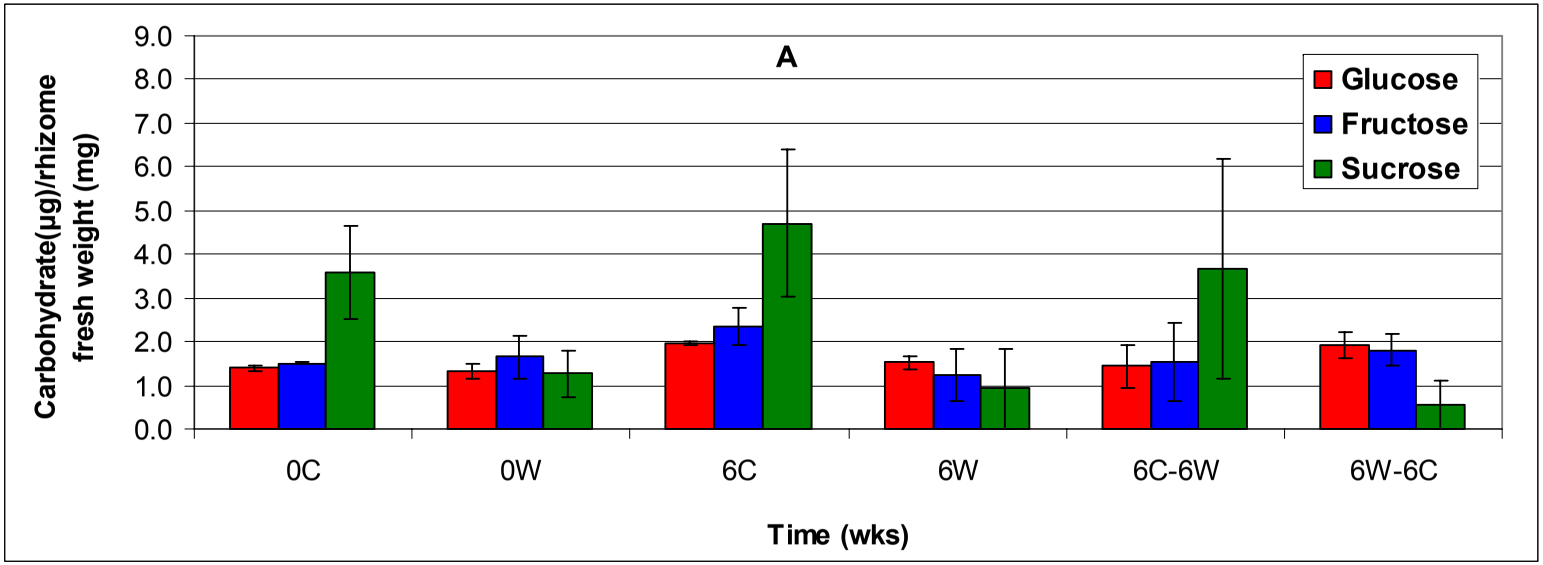


Figure 12A& B. **Soluble sugar (A) and starch (B) changes in rhizomes of *T. maculatum* subjected to two offset temperature stratification treatments at four - week intervals. Analyzed by one-way ANOVA. Error bars = +/- standard error.(n=2 per bar)**



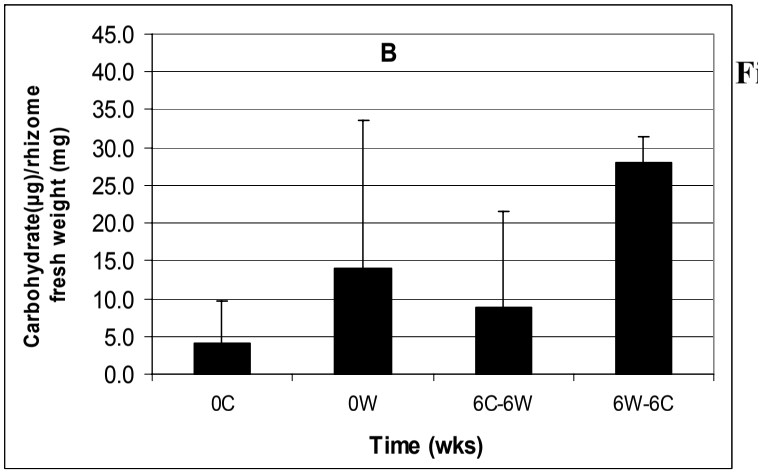
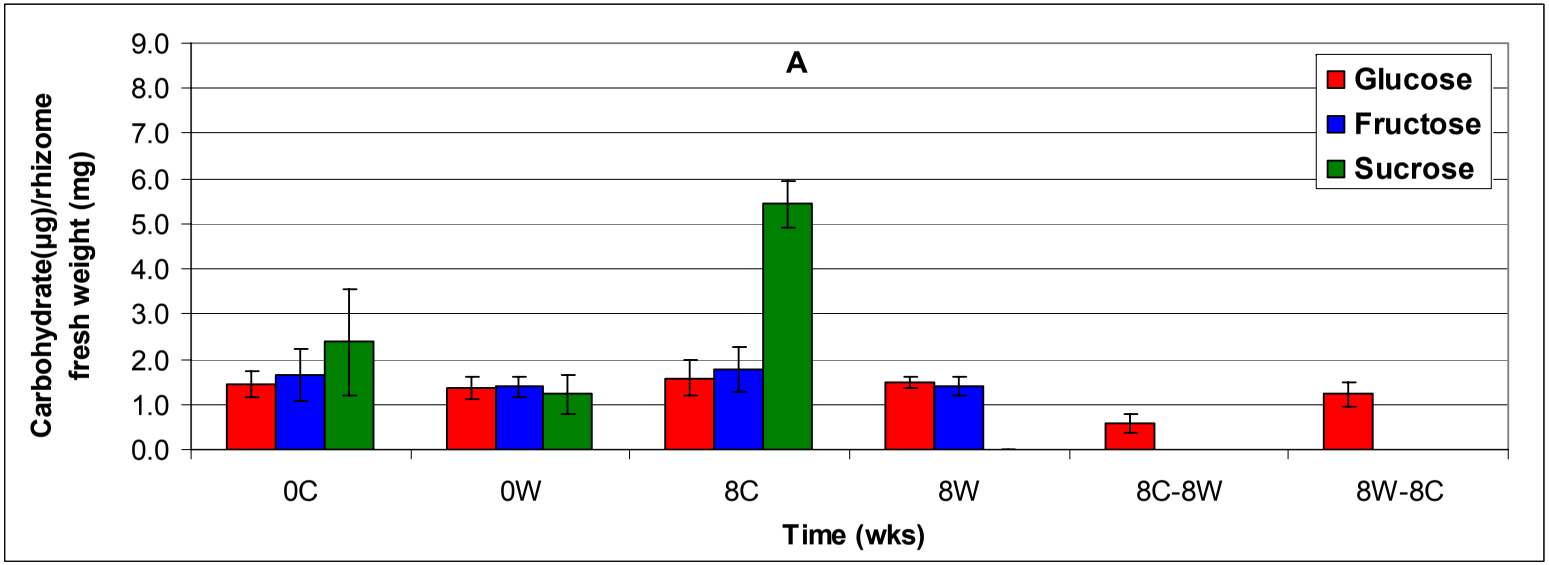


Figure 13A& B. **Soluble sugar (A) and starch (B) changes in rhizomes of *T. maculatum* subjected to two offset temperature stratification treatments at six - week intervals. Analyzed by one-way ANOVA. Error bars = +/- standard error.(n=2 per bar)**



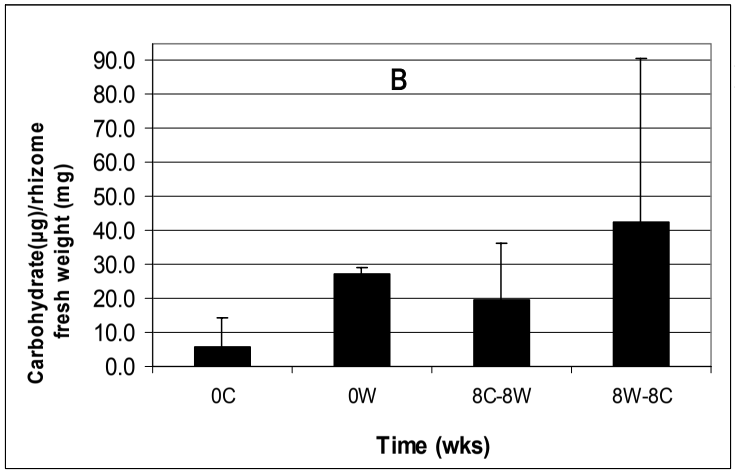


Figure 14A& B. **Soluble sugar (A) and starch (B) changes in rhizomes of *T. maculatum* subjected to two offset temperature stratification treatments at eight - week intervals. Analyzed by one-way ANOVA. Error bars = +/- standard error.(n=2 per bar)**

3.4 Discussion

Tissue cultured storage organs of many geophytes require a cold period (typically between 4 and 12 weeks depending on the species) prior to *ex vitro* initiation of new growth (LeNard and DeHertogh, 1993; Gracie et al., 2000; Foster, 2002; Maqbool, 2004). In a preliminary experiment, only 2 out of 300 rhizomes of *T. maculatum* became established and produced shoots in the greenhouse, following a 12-week cold period in a 4°C coldroom at the University of Delaware. Due to the fact that the greenhouse was warm and the coldroom had high levels of nitrogen, experiments were designed to test these factors as a cause for the low re-establishment rate. The follow-up experiments tested rhiozomes placed in 1 of 2 greenhouses or 1 of 2 coldrooms (University of Delaware or the USDA, Beltsville, MD). Rhizomes from each coldroom-greenhouse regime failed to re-establish and produce shoots. This suggested that though the coldroom used at the University of Delaware contained a high level of nitrogen, the nitrogen concentration was not solely responsible for the poor growth of rhizomes following the cold period.

At this time, a simple Lugol's iodine staining test disclosed that starch was depleted in rhizomes that had undergone various durations of cold and warm storage (Fig. 4), suggesting that conditions previously thought to be required for re-establishment of trillium were incorrect.

Though results from the offset stratification of *T. maculatum* and *T. decumbens* rhizomes at Mt. Cuba, Inc. had little significant quantitative data due to low sample number, it was instrumental in confirming the idea that 12-week stratification periods had a detrimental effect on carbohydrate concentrations and that stratification periods needed to be examined more closely.

Subsequent greenhouse and coldroom experiments to quantify starch content confirmed the depletion of starch after 9 weeks. One of the factors affecting this depletion could be that *T. maculatum,* being native to the southern United States, needs shorter periods of cold and warm to trigger physiological changes necessary for active growth under field conditions. The increase of sucrose in trillium rhizomes held in the cold may have indicated a physiological stress response to cold treatment in the rhizomes, with a possible further consequence on carbohydrate concentrations (Cai et al., 2004).

Therefore, three experiments to analyze cold/warm offset stratification at intervals of 4,6, and 8 weeks were set up. The lowest stratification interval, 4 weeks, showed more hopeful results, though stratification experiments still gave the indication that high variability in carbohydrate concentrations is a characteristic of tissues from *in vitro* rhizomes. Even though variability was still present, stratification treatments indicated an average higher starch content at the culmination of the treatment period (t=12 weeks for 6 week stratification and t=16 weeks for 4 and 8 week stratification) when compared to t=0 week analysis. Furthermore, stratification treatments beginning with a warm period consistently had an average starch concentration higher than those begun in cold, at the culmination of each experiment (Fig. 12 thru 14). This initial warm period may indicate an advantage in allowing the rhizome to slowly adjust to *ex vitro* conditions prior to imposing the physiological stresses associated with introduction to cold.

The reserves of starch in the rhizome provide carbon for growth. Without these reserves, the root-less and leaf-less rhizome will lose its capacity for growth. A decrease in duration of the *ex vitro* stratification period may prove to maintain

carbohydrate concentrations so that the rhizome produces a new shoot, in turn producing leaves and initiating photosynthesis.

Chapter 4

FIELD GROWTH

4.1 Introduction

The absence of seasonality factors such as daylength, temperature fluctuations, and drought, will most likely cause rhizomes grown *in vitro* to have a very different carbohydrate profile than field grown rhizomes that have been are subjected to such stresses and environmental cues. Seasonal carbohydrate content, most notably starch content, has been shown to fluctuate drastically over the course of May to November in wild grown *Trillium erectum*, according to a study conducted at the Universite Laval in Quebec, Canada (LaPointe, 1998). These changes are attributed to the different physiological states of dormancy, active growth, and senescence that the rhizome progressed through, throughout the year.

Knowing the seasonal carbohydrate changes in field grown (*in vivo*) trillium rhizomes during the period of active growth provided a benchmark to compare the effects of *in vitro* treatments to the profile of carbohydrate fluctuations *in vivo*.

4.2 Materials and Methods

4.2.1 Rhizome harvest

To determine the *in vivo* carbohydrate concentrations of field growth trillium rhizomes, two rhizomes of *Trillium discolor* were collected in the third week of every month, from January to October of 2005. Prior to processing the rhizomes as

described above (Chap. II), each one was rinsed of residual soil and all roots and above ground portions were removed from the plant so that the only contribution to carbohydrate measurements was the rhizome.

4.2.2 Starch stain

4.2.3 Starch colormetric assay

4.2.4 HPLC assay for sugars

Rhizomes were processed and assayed qualitatively and quantitatively as described in Chapter 2. This applies to topic headings 4.2.2, 4.2.3, and 4.2.4.

4.2.5 Field growth

4.2.5.1 Series of field grown rhizomes (*Trillium discolor*)

Rhizomes were cut and stained for starch from January to May of 2005. To conserve rhizomes, the staining procedure was discontinued after May because a qualitative difference in staining intensity was not evident. Starch and soluble sugar concentrations were determined quantitatively on replicate samples from January to October.

4.3 Results

4.3.1 Field growth

Cross-sections of rhizomes from January through May stained very dark with iodine; any contrast between months was indistinguishable (Fig. 4, E1 & E2). Colorimetrically, starch concentration decreased starting in September and rose

sharply upon the initiation of new growth and eventual flowering from March to May (Fig. 15 and 16). Sucrose concentration began at the same level as starch in January and then from this point, decreased 7-fold by April where it remained through October. The levels of glucose and fructose were markedly lower in concentration than either starch or sucrose. Except for a spike in glucose and fructose concentration in April, concentrations of both sugars decreased steadily from January to October. Ribose was below detectable levels. When analyzed by one-way ANOVA there was a significant (p-value < 0.05) change in the levels of all carbohydrates, over the course of the 10 month period. Furthermore, levels of glucose in the months of January and February were different from measurements taken from the period of August through October. In January and February, levels of fructose and sucrose were different from those detected June through October. Starch concentrations in February and March were lower when compared to concentrations in August and September.

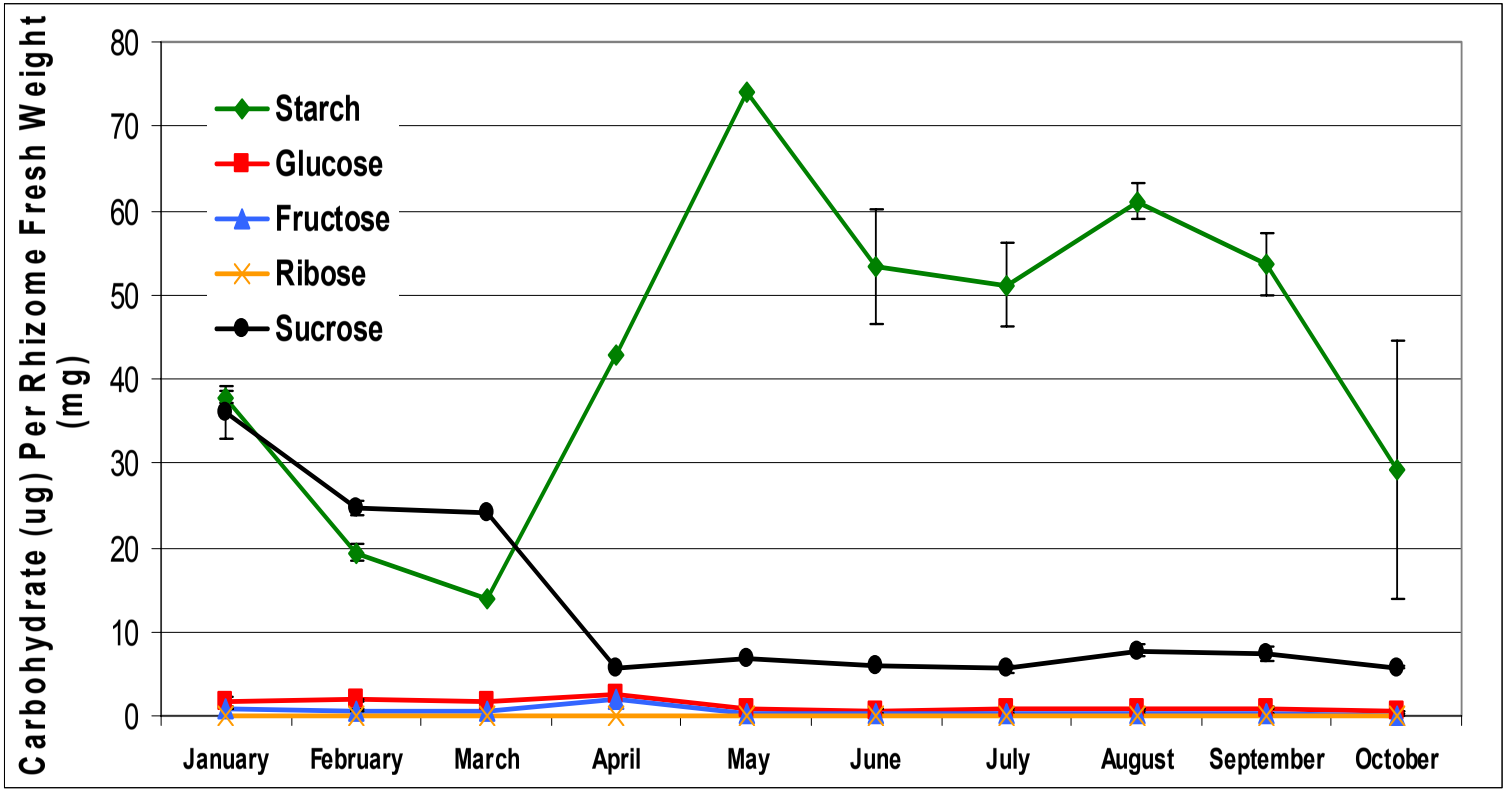


Figure 15. **Carbohydrate concentrations in field growth rhizomes of *T. discolor*, from January to October. (n=2 except for March, April and May, n=1) Analyzed by one-way ANOVA. Error bars = +/- standard error.**

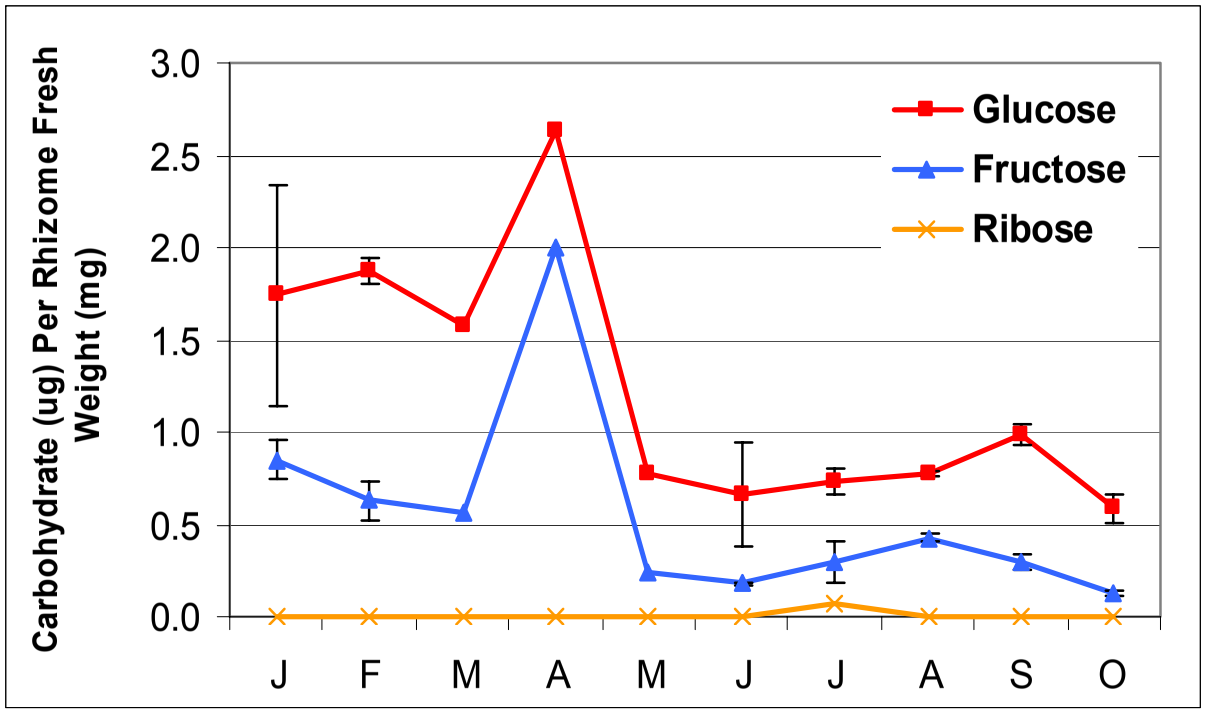


Figure 16. **Soluble sugar concentrations in field growth rhizomes of *T. discolor*, from January to October. Data is that shown in Figure 15, but the Y-axis scale has been adjusted to better resolve the differences in concentration among just the sugars. (n=2 except for March, April and May, n=1) Analyzed by one-way ANOVA. Error bars = +/- standard error.**

4.4 Discussion

Decrease in starch content of *T. discolor* after senescence (beginning in late summer) and into dormancy (late fall through winter) (Fig. 15), correlated well with data from LaPointe's study on *T. erectum* (1998). In general, dormant rhizomes (Jan. and Feb.) contained lower concentrations of carbohydrates compared to rhizomes that had ceased active growth and were progressing into dormancy (Aug. through Oct.), underscoring that carbohydrate changes exist amongst different physiological states.

Though starch is consistently present in tissue cultured rhizomes, starch is present in much higher concentrations in field grown rhizomes confirming a prior histological study on starch in the tissues of tissue cultured trillium rhizomes (Foster, 2002).

Studies have demonstrated that carbohydrate fluctuations correspond to the physiological states of dormancy, active growth, and senescence in many geophytic species that form underground storage organs. Risser and Cottam (1968) showed that carbohydrate differences in two species of *Erythronium* and two of *Dicentra*, peaked in starch concentration in the summer months of June and July. Starch decreased steadily into the winter until its lowest point in March when it was shown to be two-fold lower in concentration than in the summer months, and soluble sugars were present in concentrations inversely proportional to seasonal starch concentration. When the soluble sugars were individually quantified, it was found that sucrose and raffinose made up a significant percentage of the soluble sugars present in the bulbs. This seasonal pattern of high starch during active growth and relatively low concentrations in dormancy is common (Meyer and Hellwig, 1997).

The differences in concentrations of starch and soluble sugars, as well as the ratio between them, can be quite variable depending on the ratio of new growth to old tissue in rhizomes of various species. A study on the seasonal flux of carbohydrates in sectioned rhizomes of *Polygonatum canaliculatum*, showed that the younger portions of the rhizome contained higher concentrations of soluble sugars, while the older portions seemed to store mainly starch (Ervin and Syperda, 1971). Based on the fact that carbohydrate status within field grown rhizomes has been shown to be dependant on tissue age and location, it may be expected that *in vitro* grown trillium rhizomes, being less anatomically defined and organized, would be even more variable in their carbohydrate profile.

In vitro grown rhizomes are assumed to be at a certain level of maturity and competency for flowering. If the distinct *in situ* physiological states could be duplicated in a laboratory environment, a cycle might be set in motion for accumulating starch with subsequent shoot and possibly floral meristem development. As starch concentration was persistent after completion (t=12 or 16 weeks) of warm start offset stratifications at 4, 6, and 8 weeks, it may be beneficial to introduce *in vitro* grown rhizomes to these same offset stratification periods while they are still in culture and being transferred monthly to fresh medium. This may help to shift the metabolism to that of biochemical preparation for active growth rather than carbohydrate storage simply for the maintenance of tissues.

It may prove useful to harvest and test rhizomes at points between months, especially during the dynamic carbohydrate changes of active growth and senescence. Data from more frequent timepoints will establish an accurate pattern of carbohydrate

fluctuations, and establish at what point in the season photosynthesis is vital for replenishing carbohydrate reserves.

Field rhizomes have a different orientation/morphology than those grown in the lab. The apical shoot grows from a horizontally oriented end of a field rhizome, as opposed to a vertical shoot orientation arising from the center of the rhizome's top portion in tissue-cultured samples. This phenomenon could be further studied to determine if this orientation is crucial to rhizome shoot growth, and if so, how to orient the rhizome in such a way while it is in tissue culture.

Conclusion

Apical shoot decapitation produced more mini-rhizomes (MR) in *T. sulcatum* rhizomes when compared to the control, but research is required to further define this technique.

Carbohydrates are the main metabolic components in the tissues of most underground storage organs. The use of a colorimetric assay for starch combined with an HPLC assay for soluble sugars were adequate techniques for profiling rhizome total carbohydrate content, both in and out of an *in vitro* environment. After initially failing to establish the cultured rhizomes in an *ex vitro* environment, treatment variations on rhizomes were measured by these assay methods. Growth regulator treatments failed to induce a carbohydrate concentration increase in trillium rhizomes. A warm-cold period of 4 weeks was adequate to sustain rhizome starch in experiments using offset stratification at varied intervals. Field grown trillium rhizomes had distinct changes in carbohydrate concentrations during seasonally induced physiological states; confirming other studies of the seasonal carbohydrate content of underground storage organs.

Subsequent research on tissue cultured trillium rhizomes would benefit from analysis of starch alone. Starch seems to be the main indicator of the potential for successful field growth, and while the fluctuations in soluble sugar content was scientifically interesting, the best prospective rhizome treatments could be adequately determined solely by starch analysis. Furthermore, statistical power would be gained

by simply splitting the samples into pre- and post-treatment groups, as opposed to lower sample sizes at weekly timepoints.

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