INFLUENCE OF EXOGENOUS EFFECTORS OF INVERTASE ACTIVITY ON RICE (*Oryza sativa* L.) PHYSIOLOGY AND GROWTH

A Thesis

by

ELLIOTT WILSON ROUNDS

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

May 2008

Major Subject: Molecular and Environmental Plant Sciences

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Approved by:

Chair of Committee,	Lee Tarpley
Committee Members,	J. Tom Cothren
	William D. Park
Chair of MEPS Faculty,	Jean H. Gould

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ABSTRACT

Influence of Exogenous Effectors of Invertase Activity on Rice (*Oryza sativa* L.)

Physiology and Growth. (May 2008)

Elliott Wilson Rounds, B.S., Oklahoma State University

Chair of Advisory Committee: Dr. Lee Tarpley

Carbon flow into developing ovaries has been reported to be important in seed retention and seed size. Invertase, which cleaves sucrose into glucose and fructose has been shown to be important in rapidly expanding tissue, such as early root growth or during tiller expansion. The manipulation of invertase activity with over-the-top applications of agrochemicals may prevent the detrimental effects of abiotic stress by altering the source/sink relationship.

These experiments examined economically important tissues in rice production during critical developmental stages under abiotic stress. Field and greenhouse studies were conducted under normal growing conditions using local management practices. Plants were treated with exogenous chemicals that affect the activity of invertase during the early-grain fill stage on field grown plants. Other plants were exposed to elevated nighttime temperature of 30°C for 4 d using a free-air, infrared heating device in the greenhouse. Rice was also treated at mid- to late-grain fill stage of the main crop to identify the impact of the exogenous chemicals on developing ratoon tiller buds. The

activity of soluble acid invertase (SAI), concentrations of glucose, fructose, sucrose, and starch were determined in penultimate leaves, panicles, and main-crop stem segments during ratoon tiller bud expansion, using the enzyme-coupled stoichiometric production of NADH measured spectrophotometrically at 340nm.

The results suggest SAI, carbohydrates, and agronomic characters are influenced by exogenous chemicals at the applied rates. The thidiazuron treatment caused an unidentified stress event. The stress was confirmed by increased hexose concentration and the proportion of hexose concentration to sucrose concentration. This stress reduced the main-crop grain yield, but not the ratoon yield or total grain yield.

An interaction between the ammonium molybdate treatment and high nighttime temperature was seen in the panicle. The ranked difference was reduced by the high nighttime temperature from the ambient nighttime temperature for the SAI activity, TSC content, starch content, and TNC content.

The tested chemicals and rates are not appropriate for commercial rice production because the effect of the exogenous chemicals do not appear to consistently aid rice plants to counteract the detrimental effects of abiotic stress.

DEDICATION

For my family

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A special thank you goes to my family for their support during my degree. They all have put up with the late nights.

NOMENCLATURE

Ψ	Water Potential
ANOVA	Analysis of Variance
ANT	Ambient Nighttime Temperature
CWI	Cell Wall Invertase
d	days
da	Daltons
dat	Days After Treatment
diH_2O	Deionized Water
DW	Dry Weight
DM	Dry Matter
FW	Fresh Weight
HNT	High Nighttime Temperature
NI	Neutral Invertase
SAI	Soluble Acid Invertase
TMT	Thousand Metric Tonnes
TNC	Total Nonstructural Carbohydrates
TSC	Total Soluble Carbohydrates

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CHAPTER I

INTRODUCTION

Worldwide, cereal crops provided the greatest share of the dietary component between 2001 and 2003 with nearly 50% provided by the cereal category. Rice (*Oryza sativa* L.) provides the largest amount of energy on a per person basis with an average of 557 kcal person^{-1} day^{-1} with wheat (*Triticum aestivum* L.), sugar products, and maize (*Zea mays* L.) providing 521 kcal person^{-1} day^{-1}, 202 kcal person^{-1} day^{-1}, and 147 kcal person^{-1} day^{-1}, respectively. In 2004, the production of rice ranked third worldwide with 608,368 thousand metric tonnes (TMT) behind maize 724,515 TMT and wheat 629,873 TMT. The US produced 10,470 TMT in 2004 (FAO, 2007). The U.S. exports the majority of the rice produced and is one of the top three rice exporting countries in the world behind Thailand and Vietnam (Childs and Livezey, 2006). The value of the exported rice in 2004 was estimated to be $1.1 billion (FAO, 2007).

The U.S. Gulf Coast provides a favorable environment for rice production. In Texas, the production of rice in 2004 was 676.1 TMT (USDA, 2007) with the highest yields per acre in the mid-south rice-growing regions.

This thesis follows the style of Crop Science.

Sucrose, the primary sugar transported among different tissues of the plant; is cleaved by two enzymes, invertase (β-fructofuranoside fructohydrolase, E.C. 3.2.1.26) and sucrose synthase (D-fructose 2-glucosyltransferase, E.C. 3.2.1.13). Invertase hydrolyzes sucrose into glucose and fructose. Although this reaction is essentially irreversible, sucrose hydrolysis by sucrose synthase produces UDP-glucose and fructose, a reversible reaction.

The activity of invertase appears to be very important during seed set of maize. When sucrose supply to the developing ovary was decreased by reduced photosynthesis, increases in kernel abortion and a reduction in kernel starch were observed. The invertase activity was hypothesized to be important in the establishment and maintenance of sink strength of the developing maize seed. Sucrose synthase activity was not observable until later in development (about 13 days after pollination) (Zinselmeier et al., 1995b; Zinselmeier et al., 1999).

In this study, I assume that a physiological requirement exists to maintain invertase activity within a certain range during seed set for proper grain development and growth, which is needed for desirable yields. The possibility of using exogenous (over-the-top) chemicals to influence invertase activity and artificially maintain its activity within a desirable range in developmental periods, such as early grain filling, that might possess an innate susceptibility to stresses impacting yield, is tested. Further, the question was addressed as to whether the activity of invertase was influenced during grain filling by

seasonally high nighttime temperatures and during the development of ratoon tillers in the main crop.

A concern for rice producers in the U.S. Gulf Coast rice growing region is the effect of seasonally high nighttime temperatures on grain yield (Fig. 1). High temperatures have many effects on the growth and development of many crops. High daytime temperature reduces the grain development, yield, and yield quality of maize (Wilhelm et al., 1999). High nighttime temperature decreases the seed size and yield of soybeans (*Glycine max* L.) (Thomas and Raper, 1978; Gibson and Mullen, 1996). Not only does elevated temperature influence the economic yield of crops, but enzymes have also been shown to be influenced. An example is activity of invertase, which was reduced in maize kernels during early seed set under heat stress (Cheikh and Jones, 1995).

The environment along the U.S. Gulf Coast is favorable for ratoon cropping of rice, the second cutting of rice grain. Many producers have expressed concern for the lack of consistency in the ratoon stand and yield, appearing even under identical main-crop production practices. Nitrogen fertilization, main-crop stubble cutting height, and permanent ratoon flood timing have provided the greatest improvement to ratoon yield, indicating the possibility that maintaining the crop plants within a range of favorable environmental and physiological conditions is important for obtaining good ratoon yield. Other research has addressed the application of plant growth hormones to improve the ratoon-crop yields by increasing ratoon stand and yield consistency.

The over-the-top applications of chemicals that influence the activity of invertase may further influence the ability to maintain the optimal delivery of photoassimilate to the developing tillers. If so, these chemicals may influence the economic yield of rice.

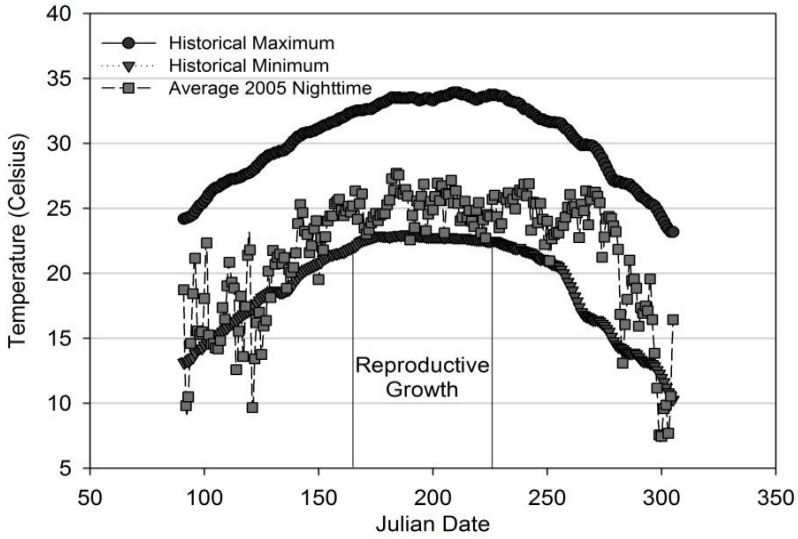

Figure 1. The historical minimum (bottom line) and maximum (top line) temperatures during the growing season for rice at Beaumont, Texas. The average nighttime temperature during the 2005 growing season between 2000h and 0600h (squares) (Wilson et al., 2007).

This research was divided into three major studies. The early grain fill study examined the influence of invertase effectors applied to the main-crop at early grain fill stage in a field environment to determine if the invertase activity could be manipulated at early grain fill and if the effector chemicals could positively impact yield or alter the physiology as determined by observed alteration of the carbohydrate pool. The heat

stress study focused on the effects of high nighttime temperature at the early grain fill developmental stage and how the above chemical applications influenced invertase activity and the physiology/carbohydrate profile of greenhouse-grown plants. In the ratoon study, the application of the invertase effector chemicals were applied at mid- to late-grain fill of the main crop 1) to determine if invertase activity during ratoon tiller bud initiation in the main crop stem segments could be manipulated and 2) to examine the effects of the chemicals on the physiology/carbohydrate profile in the stem segments.

The objectives of this study were to: 1) evaluate a hormone activator of invertase, thidiazuron; a chemical inhibitor, ammonium molybdate; and a chemical activator, concanavalin A, under field conditions in the early grain fill study, and for the ratoon study and the heat stress study in the greenhouse, 2) determine if invertase manipulation at the above stages influenced yield and yield characters of rice, and 3) determine how invertase manipulation affected the physiology of the rice plants as determined by a change in the glucose, fructose, sucrose, and starch concentration and their distribution in the rice plant.

CHAPTER II

LITERATURE REVIEW

Previous Work

Carbon Flow to the Developing Grain

The reproductive stage in most, if not all, commercially grown cereal crops is the most important stage for economic grain yield. For example, during pollination crops appear to be very sensitive to environmental stresses, such as drought, which can lead to reductions in grain yield through various means. The reproductive growth in maize was reduced by a low water potential (Ψ_w) treatment, probably due to a drop of carbon influx to the developing ovary. In order to meet the carbohydrate requirements of the young ovary, starch reserves were metabolized to meet the metabolic requirements (Zinselmeier et al., 1995b). The ovary appears to be an important sink for carbohydrates during growth and development of the kernel (Mäkelä et al., 2005).

Disruption in the carbon flow into developing tissues during key stages may be the cause of this and other detrimental effects. Studies that used stem infusion to supply additional sucrose during critical developmental stages were performed to determine the effect that changes in photoassimilate supply had during important growth stages (Zinselmeier et al., 1999). The respiration rate in roses (*Rosa hybrida* L.) was increased by three-fold with sucrose in the infusion medium compared to the infusion treatment with distilled water (diH_2O) in stems with one rose bud. The other flower buds were removed so that

only one flower bud in each plant was used. A positive correlation between flower respiration and starch content was further identified with the sucrose infusion (Monteiro et al., 2002).

Low Ψ_w inhibits photosynthesis and the sucrose supplied to sink tissue (Mäkelä et al., 2005). This method was used to determine the effects of reduced photoassimilate supply to developing tissues, such as the ovary. To ensure that results in low Ψ_w were attributed only to low Ψ_w, a shade study on maize found similar results for the shade treatments and low Ψ_w treatments (Mäkelä et al., 2005).

Reduced photoassimilate supply has many effects on the growth and development of plants. The dry matter (DM) content of maize ovaries was reduced in the low Ψ_w compared to the normal Ψ_w (Zinselmeier 1995a). When sucrose was supplied in the infusion medium in the low Ψ_w treatment, the ovary DM was maintained at levels comparable to the normal Ψ_w. The recovery of plants due to the sucrose infusion was due to the addition of sucrose and not by rehydration of the plant. These results suggest photoassimilate transport into developing tissues is an important physiological process for grain development and yield.

Ovary Abortion/Kernel Retention

A major target for enhancing economic grain yield is to reduce the number of aborted kernels. Zinselmeier et al. (1999) identified starch degradation in the ovary as the

primary factor in kernel abortion in maize. They found that water stress applied during silk emergence significantly lowered ovary starch without recovery. They suggested the ovary was utilizing the starch reserves for growth because carbohydrate availability was low. These results were challenged in maize; i.e., starch level was increased during a drought period (low Ψ_w) with an increase in starch seen as early as minus 6 days after pollination (dap) or 6 days prior to pollination (Andersen et al., 2002).

Sucrose was identified as the primary limiting factor among the transported nutrients for starch accumulation in maize (Zinselmeier et al., 1995a; and Zinselmeier et al., 1999). When sucrose was omitted from the infusion medium in the low Ψ_w, the starch content was low, but when sucrose was fed, the starch content partially recovered and prevented most, but not all, of the starch degradation. A correlation between total grain yield and ovary starch at plant maturity was identified.

Ovary abortion in maize has been linked to the carbohydrate concentration and possibly to the concentration of glucose. Glucose concentration of the ovary was low in response to the low Ψ_w, but when sucrose was supplied by stem infusion, the glucose levels were maintained at the normal Ψ_w levels (McLaughlin and Boyer, 2004a). It has been proposed that the abortion of maize kernels is due to the reduced photosynthesis that in turn lowered the photoassimilate supply (Mäkelä et al., 2005).

The inhibition of photosynthesis by low Ψ_w reduced the sucrose content, altered the sucrose metabolism, and diminished sucrose transport. When sucrose was fed by stem infusion to the low Ψ_w treated maize, the sucrose concentration was higher in ovaries of these plants than in the normal Ψ_w treated maize. Sucrose was identified as the active ingredient of the infusion medium. The other components in the infusion medium were found to be inactive in the plant. The inactivity could be due to low concentrations of these components as osmoticum compared to the sucrose concentration (Zinselmeier et al., 1995a). In another study, the sucrose concentration in normal plants decreased and little change was observed in moderately water-stressed plants, but the sucrose concentration in ovary tissue increased with severe water stress (Andersen et al., 2002). Of the sucrose that entered the normal-Ψ_w ovary tissue, 40% was proportioned to the glucose pool and 60% for starch production (McLaughlin and Boyer, 2004a). Clearly, interactions exist between some environmental stresses, sucrose supply to the ovary, and carbohydrate metabolism in the ovary.

Invertase Activity During Early Grain Development

The activity of soluble acid invertase (SAI) likely influences the survivability of the zygote during stresses at this stage (Andersen et al., 2002). The flow of carbon into the ovary was primarily used in the production of starch. A reduction in photoassimilate supply to the ovary was associated with decreased starch synthesis (Zinselmeier et al., 1999). This suggests that starch synthesis is important for grain size and number of retained seeds, both of which contribute to yield.

The activity of invertase is likely a key enzyme in the hydrolysis of sucrose for starch production in ovaries. Invertase is the enzyme that initiates this process by producing substrates upstream of starch synthesis (Zinselmeier et al., 1999). Low Ψ_w treatment decreased invertase activity (Zinselmeier et al., 1999; and Zinselmeier et al., 1995b).

Sucrose is the primary sugar transported in plants (Nelson and Cox, 2003), and invertase activity is suspected to have a large influence during early ovary development for kernel retention and starch concentration. The glucose concentration in the upper pedicel tissue of maize ovaries was high under normal Ψ_w but reduced in the low Ψ_w. The sucrose cleavage by invertase was suspected to be reduced because there was no observed accumulation of glucose (McLaughlin and Boyer 2004a). Concentration of glucose in the upper pedicel tissue of maize ovaries, was taken as an indication of the site of sucrose hydrolysis (Mäkelä et al., 2005).

The growth and development of the maize ovary may be dependent on the production and transport of glucose produced by invertase cleavage of sucrose. Metabolism utilizing glucose could be limited because of reductions in the activities of certain invertase isoforms (McLaughlin and Boyer, 2004b). Zinselmeier et al. (1995b) found that in plants with low SAI activity, sucrose accumulated in the symplasm and apoplasm of the pedicel after the sucrose was unloaded from the phloem. These authors suggested that invertase activity establishes and maintains sink strength of maize ovaries.

The primary enzyme responsible for sucrose hydrolysis during pollination and the early kernel growth of maize appears to be SAI. The activity of sucrose synthase was not seen until +13 dap in water stress maize plants (Zinselmeir et al, 1995b). The activity of SAI had peak activity at 8 dap, which corresponded with observable increases in the protein content and starch synthesis (Zinselmeier et al., 1995b). Andersen et al. (2002) found the activity of SAI under normal Ψ_w increased between -6 to +3 dap. However, under low Ψ_w the range of increasing SAI activity was reduced to -4 to +3 dap. A correlation between the activity of SAI and the proportion of hexose sugar to sucrose was observed in ovaries of maize. This correlation was not seen with the activity of cell wall bound invertase (CWI) (Andersen et al., 2002).

Genetic Expression of Invertase During Ovary Development

The activities of SAI and CWI were investigated to determine the genetic regulation of the enzymes. The genes that code for the messenger ribonucleic acid (mRNA) of SAI and CWI in maize are *Ivr2* and *Incw2*, respectively (McLaughlin and Boyer, 2004b; Andersen et al., 2002, respectively). The level of transcripts for *Ivr2* and *Incw2* regulated the activities of SAI and CWI and the *Ivr2* transcript levels were affected by the Ψ_w of the maize plants. The SAI activity and the relative amounts of *Ivr2* mRNA were also correlated (Andersen et al., 2002).

The reduction of photoassimilate supply due to the low Ψ_w further lowered the mRNA for *Ivr2* and the *Incw2*. This suggests the sucrose supply regulates genetic expression of invertase. The genes were found to be responsive to stem-infused sucrose. The *Incw2* mRNA levels were reduced in the low Ψ_w compared to the normal Ψ_w. When sucrose was fed by stem infusion the mRNA levels for *Incw2* were intermediate between the high mRNA amount (normal Ψ_w) and the low mRNA amount (low Ψ_w) (McLaughlin and Boyer, 2004b).

The genes that code for the sucrose synthase enzyme were also investigated to determine the effects of reduced photoassimilate supply on the expression of the mRNA. The genes *SS1* and *SS2* code for the sucrose synthase isoforms in maize. The transcript levels of both genes were decreased with the reduced Ψ_w compared to the normal Ψ_w and infused sucrose did not influence the amount of mRNA (McLaughlin and Boyer 2004b). The observation that the sucrose synthase genes are not influenced by sucrose suggests the pretranslational repression of sucrose synthase is influenced by developmental stage, not by an environmental change.

Since the activity of invertase during pollination was established in maize, I wanted to investigate its activity later in the development of the rice kernel, specifically during early grain fill. Further, photoassimilate supply has been shown to affect the activity of invertase, not only the enzyme activity but also the mRNA levels. I wanted to determine

if the activity could be influenced by over-the-top applications of chemicals that target invertase.

Environmental Influence on Invertase Activity

Invertase activity has been reported to be sensitive to environmental stress, such as water stress and temperature. Sheoran and Saini (1996) found that during water stress in rice, SAI activity was reduced 4-fold with no apparent recovery once the water stress was removed. They also found that when the stress was applied during meiosis the SAI activity was reduced during and after the stress period. They further found that water stress at meiosis of the pollen mother cells was associated with a reduction in grain set for rice due to pollen sterility.

An international and Texas concern in rice production is the impact of high nighttime temperatures during seed set and grain development on yield. The effects of elevated temperature on morphological characters associated with growth and development of many plant species have been documented. Studies have further identified the effects of temperature on the activities of enzymes and the carbohydrate profile. I wanted to determine if nighttime temperatures of 30°C during a 4-night period at early grain fill have a measurable effect on the activity of invertase and the carbohydrate pool. A further objective was to identify if the application of over-the-top applications of chemicals can manipulate the activity of invertase and the carbohydrate pool.

High nighttime temperature affects plant growth, development, and other physiological processes. Frantz et al. (2004) found high nighttime temperatures significantly increased the nighttime respiration in lettuce (*Lactuca sativa* L.), tomato (*Lycopersicum esculentum* Mill), and soybean. This study also analyzed the effects of nighttime temperature on daytime photosynthesis and found no association between high nighttime temperatures and daytime photosynthesis. However, cool days and warm nights in soybean caused a decrease in the photosynthetic capacity of soybean (Thomas and Raper, 1978)

Elevated nighttime temperature affects many morphological traits that contribute to the economical yield of soybeans. The leaf area and specific leaf weights were reduced by the high nighttime temperature, which also reduced the individual plant vegetative dry weight (DW) (Thomas and Raper, 1978). Gibson and Mullen (1996) found that soybeans exposed to high nighttime temperatures increased the number of pods plant^{-1} but reduced the seeds pod^{-1} and the individual seed weight.

High temperatures have been shown to have detrimental effects on early grain development. Wilhelm et al, (1999) found that the grain-fill rate of maize increased 19% with high daytime temperature, but the duration of the grain fill period was reduced by 22%. However, in soybeans, the nighttime temperature increased the seed fill duration by 4 d (Gibson and Mullen, 1996). The conclusion drawn from the Wilhelm et al. (1999) study was that the grain fill duration was reduced by heat stress and caused

reductions in kernel size. The same response to heat stress was found in rice with an Indica and a Japonica variety (Yoshida and Hara, 1977).

The effects of increased temperature on grain development are numerous. The reduction in kernel density from heat stress of maize seed was attributed to differences in starch, protein, and oil concentrations, but not in their proportions (Wilhelm et al., 1999). Viable seed was reduced by greater than 95% when day/night temperatures were 37/29°C in 17 tested rice cultivars (Ziska et al., 1996). High temperatures have also been reported to reduce the kernel DW and induce kernel abortion in maize (Cheikh and Jones, 1995). In wheat, the kernel weight was lowered with day/night temperatures of 35/25°C with no difference in total above ground biomass (Banowetz et al., 1999).

The rice plant is tolerant to moderately high temperature throughout most of the plant's development cycle; however, heat stress at flowering and early grain development could produce many problems associated with the economic grain yield in rice. During meiosis, high temperatures reduced the germination of mature pollen grains in bell pepper (*Capsicum annuum* L.) as well as the number of pollen grains available (Karni and Aloni, 2002). Cheikh and Jones (1995) found the fresh weights (FW) of maize endosperm and pericarp were reduced by high temperature stress, and that the DW of the pericarp was increased while the endosperm was not affected by the temperature treatment. With increasing nighttime temperatures, the starch concentration was decreased in leaves of *Populus deltoides* (Bartr. Ex Marsh). This trend was observed at

both sunrise and sunset (Turnbull et al., 2002). This suggests that sucrose hydrolysis is inhibited by temperature in the basal portion of the kernel, further indicating that invertase activity is correlated to temperature.

Other enzymes in plants were found to be sensitive to elevated temperatures. For example, in the endosperm of maize, heat stress reduced the activity of adenosine diphosphate-glucose pyrophosphorylase (E.C. 2.7.7.27), glucokinase (E.C. 2.7.1.1.), sucrose synthase and soluble starch synthase (E.C. 2.4.1.21). However, when the enzymes were adjusted for Q_{10}, adenosine diphosphate-glucose pyrophosphorylase was the enzyme with the largest proportional reduction due to heat stress (Wilhelm et al., 1999). The Q_{10} is the doubling of the enzyme reaction rate for each 10˚C increase in temperature (Hopkins and Hüner, 2004).

All enzymes have optimal temperature ranges for maximum catalytic activity. For many plant enzymes, this range is between 25 and 35ºC. Once temperatures approach 40ºC and higher, enzyme activity declines and further increases in temperature can cause the protein to become inactivated and the enzyme to denature. When temperatures exceed 55 to 60°C, most enzymes become inactivated (Lehninger, 1975).

Invertase activity has been shown to be affected by elevated temperature in a variety of plants. In maize, SAI activity was reduced at 35ºC and the CWI inhibited only by long-term temperature stress at the same temperature. The lack of short-term CWI inhibition was attributed to the thermostability of the enzyme (Cheikh and Jones 1995). Aloni et al. (1992) found that heat stress in transplanted bell peppers significantly decreased the activity of SAI in the roots but significantly increased the SAI activity in the leaves. However, SAI and NI activities were not significantly influenced by heat stress in young growing leaves of potato (*Solanum tuberosum* L.) (Lorenzen and Lafta, 1996). Maize SAI did not show significant reduction in activity until between 15 and 25 dap in a high temperature environment (Cheikh and Jones, 1995).

Importance of Ratoon Rice Production

The activity of invertase during pollination has been shown to be important in maize. I wanted to study invertase activity at ratoon tiller bud expansion of rice in main-crop stem segments. I hypothesize that the over-the-top application of invertase effectors may increase the number of tillers and other important yield characters in the ratoon crop by promoting more sucrose cleavage for development of vegetative nonstructural carbohydrate reserves and biosynthetic reactions.

Ratoon rice production is practiced along the U.S. Gulf Coast, including southeast Texas, southwest Louisiana, and parts of Florida, due to the long growing season favorable for this practice. Ratoon rice production is the second cutting of rice which

occurs between 60 and 90 d after main crop harvest. Ratoon-crop yields often can reach nearly 50% of main-crop yields (Street and Bollich, 2003). Ratoon yields in rice production fields have been previously reported to have large variability between adjacent fields, even with identical production practices. These observations were seen even when the main crop yield was consistent throughout the field (McCauley et al., 2002).

Scientists across the U.S. Gulf Coast have addressed the inconsistent ratoon yields. The primary factors identified for maximal ratoon yield are nitrogen fertilization, flood water management (Bollich et al., 2002b), and cutting height (Jund et al., 2002; and Bond and Dunand, 2007). It was found that 34 to 101 kg ha^{-1} of nitrogen applied prior to permanent flood after main-crop harvest increased ratoon-crop yield. The nitrogen rate of 34 kg ha^{-1} was the most advantageous for ratoon-crop yield in field studies from 1995 to 1997 in southwest Louisiana (Bollich et al., 2002b).

The timing of ratoon permanent flood was identified to be very important in the maximization of ratoon-crop yield. If the permanent flood was delayed, there was consistent reduction in ratoon tiller production and crop maturity for the tested varieties. When the permanent flood was applied immediately after main crop harvest, the ratoon yields were increased (Bollich et al., 1998).

The height of cutting at main crop harvest has been reported to be important for maximal ratoon crop yield. A lower cutting height increased the ratoon crop stand, but delayed the ratoon crop maturity (Jund et al., 2002; Bond and Dunand, 2007). The increase in ratoon stand when a low cutting height was achieved by flail mowing was possibly due to the reduction in biomass that could inhibit ratoon growth because of the straw matting, along with encouragement of basal tillering (Tarpley et al., 2007). A lower cutting height appeared to lower ratoon yields of cultivars with low ratoon yield potential. However, for cultivars with a high ratoon yield potential, the ratoon yield was increased when the cutting height was lowered from 50 cm to a height of 20 to 30 cm (Jund et al., 2002). In another study, most of the tested cultivars responded well to the main crop cutting height of 15 to 20 cm (Bollich et al., 2002b).

Various agrochemicals have been studied to address the ratoon yield inconsistencies and poor stand. The timely application of desiccants to the main crop minimized the negative effects of paraquat dichloride and sodium chlorate on grain yield and milling quality when applied 3 d before harvest compared to the application at 7 d before harvest. Further, the higher rate (0.14 kg ha^{-1}) of paraquat dichloride lowered ratoon crop yield and main crop milling quality. The low rate (0.067 kg ha^{-1}) of paraquate dichloride appears to be the best tested rate for consistent yield and milling quality of the ratoon crop (Bollich et al., 2002a). The application of the fungicides Quadris and Tilt during the boot stage of the main crop increased the ratoon yield. This ratoon-crop yield

increase was dependent on the ratoon yield potential of the cultivar, disease pressure, and the efficacy of the fungicides (Jund et al., 2002).

Application of plant hormones has been tested to increase the consistency of the ratoon crop stand and yield. The application of gibberellins (GA) to the main crop of rice was able to increase the ratoon crop yield (Rounds et al., 2007) and ratoon crop tiller numbers (Tarpley et al., 2002). Further, the application of cytokinin hormones, specifically benzyl adenine, to the main crop of rice improved the numbers of ratoon tillers with panicles (Tarpley et al., 2002).

I hypothesize that applications of exogenous chemical application, that influence the activity of invertase, to the main crop of rice during the mid- to late- grain fill developmental stage will influence the activity of invertase, the carbohydrate pool, yield, and yield characters of the ratoon crop in field grown rice.

Plant Hormones and their Effect on Invertase Activity

Plant hormones influence many physiological processes in plants. Further, they have been shown to alter emzyme activities, expression of genes, and other events in the growth and development of plants. Two classes of hormones that influence the activity of invertase are the GAs and the cytokinins.

Gibberellins are plant hormones that are derivatives of terpenoid compounds with isoprene units (Taiz and Zeiger, 2002). They are responsible for stem growth due to increased cell division and cell elongation (Davies, 2004) and internode elongation (Taiz and Zeiger, 2002). Gibberellins have been shown to increase seed germination in plants that required cold or light for germination and have been used to stimulate the germination of seeds (Davies, 2004). The enhanced seed germination has been linked to enzymes of the aleurone layer. In the aleurone layer of cells, GA has been shown to stimulate many hydrolytic enzymes. The primary enzyme is α-amylase (E.C. 3.2.1.1) (Woodger et al., 2004).

Gibberellin treatments have been shown to increase the level of invertase activity in oat (*Avena sativa* L.). The GA treatment led to a 9-fold increase of growth in the oat stem compared to the control. A synergistic or additive effect was observed for the activity of invertase treated with GA in the presence of sucrose compared to the invertase activity with GA when sucrose was not present in the system. There appears to be an association between invertase activity and GA-mediated growth (Kaufman et al., 1973).

Cytokinins are a category of plant hormone responsible for cell division, leaf expansion, enhancement of stomatal opening, promotion of shoot initiation and the growth of lateral buds (Mok and Mok, 2001; Taiz and Zeiger, 2002; Davies, 2004). Cytokinin hormones are derivatives of the deoxyribonucleic acid (DNA) base adenine (Mok and Mok, 2001). The cytokinin hormones have been identified to work synergistically or antagonistically

with the auxin class of hormones for the production of ethylene (Binns 1994; Deikman, 1997; Vogel et al., 1998). A hypothesis was presented that cytokinin levels were related to seed fill, seed size, and yield (Mok and Mok, 2001). However, the kernel mass was correlated with cytokinin content and when the cytokinin levels were low, the kernel mass was low. The exogenous application of cytokinin to soft white winter wheat increased the cytokinin content in the kernel, but did not change kernel mass (Banowetz et al., 1999).

Cytokinins can influence the activity of invertase. Cytokinins have also been shown to increase the translatable mRNA of invertase in tobacco (*Nicotiana tabacum* L.) (Abdelghani et al., 1991) and in *Chenopodium rubrum* (Ehneb and Roitsch, 1997). The cytokinins increase the mRNA above the control for the *CIN1* gene that encodes for extracellular invertase in *C. rubrum* while not affecting the gene that encodes for sucrose synthase. In the study by Abdelghani et al. (1991), thidiazuron, benzylaminopurine (6-BA), and kinetin were evaluated. All these three tested cytokinins increased the mRNA for the *CIN1* gene. This study also showed that some auxin hormones may increase invertase mRNA. The auxin herbicide, (2,4-dichlorophenoxy)acetic acid, increased the expression of the *CIN1* mRNA. The *CIN* family of genes appear to be upstream of the *AGS5* in the biosynthesis of ethylene induced by cytokinin (Estelle, 1998). The *AGS5* is the gene that encodes for the enzyme which is a part of the 2-step conversion of S-adenoxylmethionine (ACC) to ethylene; 1-aminocyclopropane-1-carboxylate synthase, ACC synthase (E.C. 4.4.1.14) (Berg et al., 2002).

Energy Production by Biosynthetic Pathways

Aerobic respiration provides adenosine triphosphate (ATP) for cell biosynthetic reactions. Respiration involves the activity of glycolysis, the Krebs cycle, and the electron transport chain. Glycolysis is the starting point in the respiration pathway. This series of reactions converts glucose to pyruvate, which is the substrate for the Krebs cycle. The Krebs cycle produces reducing equivalents in the form of reduced flavin adenine dinucleotide ($FADH_2$) and reduced nicotinamide adenine dinucleotide (NADH). These reducing equivalents are utilized in the electron transport chain in the mitochondria to provide ATP for cellular reactions (Buchanan et al., 2000). In the initial steps of glycolysis, hexokinase (E.C. 2.7.1.1) converts glucose to glucose-6-phosphate or fructose to fructose-6-phosphate. Also, UDP-glucose pyrophosphorylase (E.C. 2.7.7.9) converts UDP-glucose to glucose-1-phosphate. These substrates cannot be utilized by glycolytic enzymes of plants until sucrose is hydrolyzed (Nelson and Cox, 2003). Sucrose can be converted to glycolytic substrates by either the isoforms of invertase or sucrose synthase (Sturm and Tang, 1999). Sucrose is cleaved to glucose and fructose by invertase. Sucrose can also be cleaved into fructose and UDP-glucose by sucrose synthase. Invertase under typical conditions essentially irreversibly hydrolyzes sucrose to glucose and fructose, while sucrose synthase reversibly converts sucrose and UDP to UDP-glucose and fructose (Taiz and Zeiger, 2002).

Physical Properties of the Invertase Protein

Invertase is a glycoprotein that has various physical properties depending on the plant species. In *Ricinus communis* L., the molecular weight was 22,000 Da (Prado et al., 1985) while in sugarcane *(Saccharum officinarum*. L.), the molecular weight was found to be 127,500 Da (Sampietro et al., 1980). The M_R has been shown to be 98,000 Da in rice (Isla et al., 1995), 74,000 in *Tropaeolum majus* L. (Isla et al., 1988), and 52,000 in *Carica papaya* (Lopez et al., 1988). The energy of activation was found to be 8,400 cal mol^{-1} in leaves of *Tropaeolum* sp. (Isla et al., 1988), while in rice the activation energy was 8,250 cal mol^{-1} at temperatures above 30˚C. When temperatures were below 30˚C, the activation energy was 24,000 cal mol^{-1} (Isla et al., 1995). The pH optimums have previously been shown to be about 3.0 and 8.0 for *T. majus* SAI and alkaline invertase (NI), respectively (Isla et al., 1988); 3.0 and 7.5 for rice SAI and NI, respectively (Isla et al., 1995); and pH 3.5 and 7.0 for *C. papaya* SAI and NI, respectively (Lopez et al., 1988).

There are three forms of invertase known; SAI, NI, and CWI. Soluble acid invertase is the soluble form that is found in the tonoplast/vacuole (Lyne and Ap Rees, 1971). This form was found to have a pH optimum of 3.5 to 3.7 in *R. communis* (Vattuone et al., 1983; and Prado et al., 1985), 4.8 in tomatoes (Chin and Weston, 1973), 5.0 in pea (*Pisum sativum* L.) roots (Lyne and Ap Rees, 1971), and pH 5.25 in *Tropaeolum* leaves (Isla et al., 1988). The SAI activity was found to be highest in a pea root segment that was 3 to 9 mm from the root apex (Lyne and Ap Rees, 1971). The root segment was an

area of the root that had a relatively low concentration of sucrose. In carrot (*Daucus carota* L.) roots, SAI activity was closely and inversely correlated to sucrose content (Ricardo and Ap Rees, 1970) indicating that SAI hydrolyzes sucrose whenever sucrose is available. This is supported by the K_m values of invertase for sucrose in rice with a value of 6.6×10^{-3} M (Isla et al., 1995). Raffinose was hydrolyzed by invertase with a K_m of 2.0×10^{-2} M and stachyose with a K_m of 6.6×10^{-2} M, so sucrose was the preferred substrate (Isla et al., 1995). Raffinose is a trisaccharide composed of one D-galactose, D-glucose, and D-fructose; stachyose is a tetrasaccharide with two D-galactose, one D-glucose, and one D-fructose (Berg et al., 2002).

Role of Invertase in Plants

A major function of invertase is during the unloading and utilization of sucrose where invertase and sucrose synthase are important enzymes in sink cells. Invertase activity has been shown to be most active in regions of cell elongation and lower in areas of mature plant parts. This hypothesis is supported by Maclachlan et al. (1970) who found that in pea epicotyl extracts, the youngest plant parts (0 to 10 mm) had the highest amount of sucrose catabolized (35.5 μg sucrose mg FW^{-1} hr^{-1}). This is also the region which demonstrated the most active growth. The plumule, of the same study, showed the lowest amount of sucrose metabolized, 2.0 μg sucrose mg FW^{-1} hr^{-1}.

Manipulation of Invertase Activity

My hypothesis is that inhibition of invertase activity in actively growing tissues, such as during grain development, may prevent the utilization of sucrose hydrolytic products by the ATP-dependent pathway thus leading to accumulation of sucrose in these regions. The products of the hydrolytic cleavage of sucrose by invertase inhibit the enzyme, but by different mechanisms. Glucose has been identified as a classical competitive inhibitor of invertase (Sampietro et al., 1980; Isla et al., 1988; Isla et al., 1995) while fructose is a classical non-competitive inhibitor (Sampietro et al., 1980; Isla et al., 1988; Lopez et al., 1988).

Enhancement of invertase under conditions of low invertase activity, through exogenous chemical application, has the potential to improve grain yield and biomass production due to the major role invertase has in the cleavage of sucrose in rapidly growing tissues. If invertase activity were increased during grain fill, this may lead to more products entering the glycolytic pathway and increased ATP production.

Lectins have been shown to activate hydrolytic enzymes. Invertase in rice was activated by lectins from *R. communis* (Isla et al., 1995). Lectins were shown to consistently increase the activity of invertase in *Ricinus* over activation by bovine serum albumin (Vattuone et al., 1991). Acid phosphatase (E.C. 3.1.3.2), another hydrolytic enzyme, was activated in rye (*Secale cereale* L.) germ by the lectins: wheat germ agglutinin (WGA), soybean agglutinin (SBA), lentil (*Lens culinaris* Medik) lectin (LL),

Concanavalin A (ConA), and *Solumom tuberosum* (L.) agglutinin (*St*A). This study indicated that the activation of invertase is through a mechanism that changes the reaction velocity (V_{max}) and not the K_M of the enzyme (Ferens and Morawiecka, 1985).

There are other activators of invertase other than proteins. Cations have been shown to activate the activity of invertase. In *C. papaya* fruits, the application of Mg^{2+}, Sr^{2+}, or Ba^{2+} provided a significant increase in invertase activity (Lopez et al., 1988). Activation by these cations is not associated with the divalent nature of the element. The metal cations Zn^{2+}, Cu^{2+}, Co^{2+}, and Mn^{2+} are inhibitors of invertase in *C. papaya* (Lopez et al., 1988). The apparent activation mechanism deals with properties of the alkali earth metal and not with the charge of the species.

The invertase activity can be inhibited by chemical application. Mercury inhibited the activity of invertase in castor bean (Vattuone et al., 1983) and sugarcane (Sampietro et al., 1980). The SAI activity was inhibited by ammonium heptamolybdate in yeast (S*accharomyces cerevisiae*), sugarcane, carrot roots, radish (*Raphinus sativus* L.), and turnip roots (*Brassica rapa* L.), while in sweet potato (*Ipomoea batatas* L.), NI was inhibited (Prado et al., 1979). The reversible inhibition of invertase activity was by the molybdate and not by the ionic strength or the ammonium ion.

Hypothesis/Objectives

Most studies involving invertase have been conducted under strict laboratory conditions to maximize the treatment effect on the activity of invertase. Furthermore, few if any studies have been done on plants in the field to evaluate the impact of environmental factors, the efficacy of chemical application, and their interactions on invertase activity and the carbohydrate profile, or in determining the influence of an effect on invertase activity on yield and yield characters.

The objectives for the early grain fill study are:

1. Determine if over-the-top applications of invertase effector chemicals influence the activity or rank of invertase in the penultimate leaf or panicle of field grown rice.
2. Determine if over-the-top applications of invertase effector chemicals influence the activity or rank of the carbohydrate pool in the penultimate leaf or panicle of field grown rice.
3. Determine how over-the-top applications of invertase effector chemicals influence yield and yield characters.
4. Determine how the applications of over-the-top effector chemicals of invertase fluctuate the invertase activity and carbohydrate pool in field grown rice during 0, 1, and 3 dat.
5. Identify any interactions between the above four objectives.

The objectives for the heat stress study are:

1. Determine the response to over-the-top applications of invertase effector chemicals on the activity of invertase in the penultimate leaf and the panicle.
2. Determine the response of over-the-top applications of invertase effector chemicals on the carbohydrate pool in a rice penultimate leaf and panicle.
3. Determine if an interaction is observable for the above two objectives with high nighttime temperature.

The objectives for the ratoon study are:

1. Determine if over-the-top applications of invertase effector chemicals influence the activity or rank of invertase in stem segments of the main crop where ratoon tiller initiation is occurring.
2. Determine if over-the-top applications of invertase effector chemicals influence the carbohydrate pool in main-crop stem segments of rice where ratoon tiller initiation is occurring.
3. Determine if over-the-top applications of invertase effector chemicals influence the yield and yield characters of rice.

CHAPTER III

MATERIALS AND METHODS

Experiments and Procedures

Plant Culture

The experiments were conducted at the Texas A&M University AgriLife Research and Extension Center located at Beaumont, TX (29° 57'N, 94° 21'W) during the 2004, 2005, and 2006 growing seasons. The early grain fill study experiment was performed during the 2004 and 2005 growing season. This experiment was done in the field. The ratoon study experiment was done during the 2004 and 2005 growing seasons. Two studies were conducted in the field while one study was done in the greenhouse. The heat stress study experiment was conducted in the greenhouse between 2004 and 2006.

In all field studies, seeds of 'Cocodrie' were planted into a Beaumont clay soil (fine, montmorillonitic, thermic, Entic Pelludert) (Rounds et al., 2007) to a depth of 25.4 mm using a Tye Pasture Pleaser 8-row drill (Lockney, TX) on 22 cm row-spacing. The seeding rate was between 67 to 78 kg ha^{-1}. Each research plot comprised an area of 8.4 m^2.

The greenhouse studies used soil from the field, as above. Seeds were planted into 20 cm pots to a depth of 25.4 mm and placed into waterproof boxes to maintain water levels between 25 mm and 51 mm above the soil surface of the pots.

Plants were maintained with water, pesticide, and fertilizer as recommended by the Texas Rice Production Guidelines (Texas Cooperative Extension Service, 2006).

Chemical Application

The chemical applications used in these experiments were consistent for all studies. In the field studies, thidiazuron (Sigma-Aldrich, St. Louis, MO) was applied at a concentration of 25 µM (0.5 g a.i. ha^{-1}) and ammonium molybdate (Sigma-Aldrich) was utilized at a concentration of 35 mM (4.0g a.i. ha^{-1}). The greenhouse studies used the same concentrations of thidiazuron and ammonium molybdate. Concanavalin A (Calbiochem 234567, San Diego, CA) was used in the greenhouse studies at a concentration of 25 µM (125 nmol $plant^{-1}$). The concanavalin A used in these experiments was from Jack Bean (*Canavalia ensiformis* L.).

The chemicals were measured to the chosen mass and mixed with diH_2O with 0.5% v/v Latron AG-98 Spreader Activator from Rohm and Haas (Philadelphia, PA). Application of thidiazuron and ammonium molybdate was done with a 4-nozzle backpack sprayer on 41 cm spacing. The nozzle tips used in the 2004 field studies and the 2005 greenhouse studies were TeeJet 8001VS with TeeJet Strainer and Check Valve with 100-mesh brass body. The tips used in the 2005 field studies were TeeJet 11001 with 100-mesh brass body Strainer and Check Valve (R&D Sprayer, Opelousas, LA). The sprayer was calibrated to deliver 93.5 L ha^{-1} with CO_2 propellant at 15 to 17 psi.

In the greenhouse studies, Concanavalin A was applied with a small atomizer or custom shaker that applied approximately 5 ± 1 mL plant^{-1}.

The control plants did not receive chemical applications. The Latron and diH_2O do not affect morphological characters. Both the Latron and diH_2O appear to be inert to the rice plants (A.R. Mohammed, Texas A&M University Agrilife Research and Extension Center at Beaumont, 2006, unpublished results).

Early Grain Fill Study. The chemical treatments were applied when the main-crop developmental stage was between the early- to mid-grain fill.

Heat Stress Study. Chemical treatments were applied when the main-crop developmental stage was between early- to mid-grain fill. After the plants were sprayed with the desired chemical, the plants were placed under a free-air controlled heating apparatus, containing continuously controlled infrared heating lamps customized to this experiment, based on the previous work of Tarpley et al. (2007). The air temperatures were regulated at the desired levels between 2000 h and 0600 h beginning on the day that the plants were chemically treated, and continuing for four nights.

The temperature under the high nighttime temperature (HNT) treatment was maintained at 30 ± 0.5°C while the ambient nighttime temperature (ANT) treatment did not receive temperature regulation. The set temperature for night (ambient) in the greenhouse was

25°C. If the thermocouple-monitored temperature in the HNT treatment fell below the set temperature, the temperature controller registered this difference and the infrared heating lamps were automatically adjusted in intensity at less than 1-second intervals.

Ratoon Study. The chemical applications were applied to the main-crop when the developmental stage was between mid- to late-grain fill.

Plant Sampling

Early Grain Fill. Plants were sampled at 0, 1, and 3 days after treatment (dat) from rows within the research plot, selected away from the edges to minimize border effects. For biochemical analysis, the penultimate leaf and the panicle were separated from the whole plant and wrapped in aluminum foil and plunged into liquid nitrogen within 2 min of sampling. The remaining plant material was discarded. Samples were stored in an ultracold freezer (-70°C) until enzyme and carbohydrate analyses were conducted.

The relative dry matter content was determined by 31 cm of drilled row of rice in field research plots taken in areas away from the plot edges to minimize the border effects. The sampling was done at 0, 1, and 3 dat. The samples were weighed to determine the fresh weight (FW) and placed into a drying oven (Stabi-THERM Laboratory Oven, Blue M-Electric Co., Blue Island, IL) for 5 to 6 days (d) at 70°C to remove moisture from the plant samples. The samples were again weighed to determine the DW. The relative dry matter content was determined by dividing the DW by FW and that value was multiplied

by 100. The relative dry matter content is expressed as a proportion of FW present as DW.

Heat Stress Study. Plants were sampled 4 d after the nighttime temperature treatment began. The plants were cut at the soil surface, below the water line. Plants were sampled for biochemical analysis. Biochemical analysis was conducted on the penultimate leaf and the panicle as for the early grain fill study.

Ratoon Study. Plants were sampled 8 dat. The plants were cut at the soil surface, below the water line, and 15 to 20 cm of stem segments were retained while the remainder of the plant material was discarded. The stem segments were wrapped in aluminum foil and plunged into liquid nitrogen within 2 min from cutting. Samples were stored in an ultracold freezer (-70°C) until enzyme and carbohydrate analyses were conducted.

The relative dry matter content was determined by a 31 cm of row sample in the field research plots taken in areas away from the plot edges to minimize the border effects. This sampling was done 8 dat. The samples were weighed as with the early grain fill study for FW and DW. The relative dry matter content is expressed as a proportion of FW present as DW.

In each field research plot, there were two separate 1.5 meter-row samples cut and the number of panicles counted. This panicle count provided the number of panicles m^{-2} based on 3 m of row. The samples were taken from the middle portions of the plot to remove the border effects.

The field research plots were harvested on the center 6-rows of each research plot for the main crop and the center 4-rows for the ratoon crop with a Massey Ferguson 8XP grain combine (Batavia, IL) equipped with a 1.5 m grain header.

Biochemical Analysis

Samples were removed from the ultracold freezer and ground with a chisel in a custom steel trough-shaped mortar under liquid nitrogen. Once the samples were ground to a fine powder, the samples were aliquoted into five subsamples of 50 mg each. Four subsamples were stored in the ultracold freezer until enzyme and carbohydrate analyses were conducted. One subsample was placed into a drying oven (Blue Island, IL) at 70°C for 3 to 4 d to remove all moisture for DW determination.

Invertase Purification

For invertase analysis, one 50 mg subsample was removed from the ultracold freezer and immediately dumped into a pre-chilled vial for homogenization in an ice bath (-4 to +3°C). Added to the subsample was 1000 µL homogenization medium [90 mM HEPES (N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) (Sigma-Aldrich), 10% v/v

glycerol (Acros Organics, Geel, Belgium), 5 mM $MgCl_2$ (Sigma-Aldrich), 10 mM L-Cysteine (Sigma-Aldrich), and 1 mM EDTA (ethylenediamine tetraacetic acid) (Alfa Aesar, Ward Hill, MA), adjusted to pH 7.0 with 4 M acetic acid or 4 M KOH], 2 mL of hydrochloric acid (HCl) washed PVPP (polyvinylpyrrolidone, cross-linked; pre-soaked in diH_2O) (Acros Organics), and 5 µL 100X Protease Inhibitor Cocktail [500 µM aminoethyl-benzene sulfonyl fluoride, hydrochloride; 150 nM aprotinin; 1 µM E-64 protease inhibitor; 0.5 mM EDTA; and 1 µM leupeptin hemisulfate] (Calbiochem 539131, San Diego, CA). The homogenization medium, PVPP, and Protease Inhibitor Cocktail was maintained in ice at 0 to +1°C until placed in the homogenization vial for homogenization. The sample was homogenized with a Tissue Tearor (Biospec Products, Inc, Bartlesville, OK) for 5 to 10 min, with chilling maintained, until a uniform slurry was formed.

The slurry was dumped directly onto spin columns for enzyme extraction. The spin columns were prepared with 4 mL of pre-swollen (excess diH_2O, overnight, 4°C) Sephadex G-25 (Sigma-Aldrich) in a 5 mL syringe barrel and spun in a Fisher Scientific Marathon 3200(R) centrifuge (Fisher Scientific, Pittsburg, PA) at 4°C for 5 min at 4000 rpm (2504 X g). Glass microfiber disks were cut to the size and placed at the base of the syringe barrel to hold the gel in place. The columns were pre-equilibrated with five passes of 1 mL homogenization medium excluding the PVPP and protease inhibitors. The enzyme preparation was aliquoted into 500 µL subsamples and stored in an ultracold freezer until the invertase reaction/assay was conducted.

A preliminary study was conducted to determine the optimal conditions for pH and temperature for acid invertase activity assay. The invertase reaction is nearly irreversible. Plant samples were selected from greenhouse plants which did not receive chemical or temperature treatments. These plant samples were prepared as before for grinding, homogenization, and purification.

Invertase pH Optimization

A pH curve was established from pH 4 to 8.5 in increments of pH 0.5 that used selected buffers. The buffer stock solutions were prepared at 250 mM concentration. Citric acid (Sigma-Aldrich) was used for the pH of 4.0 to 5.5. MES (morpholinoethanesulfonic acid) (Sigma-Aldrich) was used for the pH of 5.5 to 6.5. For the pH of 6.5 and 7.0, PIPES (Piperazine-1,4-bis(2-ethanesulfonic acid) (Sigma-Aldrich) was used. MOPSO (3-Morpholino-2-hydroxypropanesulfonic acid) (Sigma-Aldrich) was used for the pH of 7.0 and 7.5. Glycyl-glycine (Sigma-Aldrich) was used for the pH of 7.5 to 8.5.

The reaction for invertase activity in the pH response curve was preformed with 25 µL 500 mM sucrose (Sigma-Aldrich), 50 µL selected buffer, 30 µL enzyme preparation, and brought to a final volume of 150 µL with diH_2O.

The activity of invertase was measured across pH without concern for the buffer used. The activity was determined by the absorbance of NADH, as explained later. There was a peak of absorbance found at pH 5.5 (Fig 2, top panel).

There was a bump or plateau of activity found between pH 7.0 and pH 7.5 (Fig 2, top panel). This corresponds with the activity of neutral invertase (NI). In pea roots, the separation of activity for SAI and NI was identified at pH 5.0 and pH 7.0 with an optimum NI activity at pH 7.3 (Lyne and Ap Rees, 1971). The optimal activity in eggplant was found at pH 7.0 (Claussen et al., 1986).

The specific absorbance for invertase activity, by the selected buffer, shows the highest absorbance occurs at pH 5.5 (Fig 2, bottom panel). The citric acid and MES were the buffers corresponding to the highest absorbance at pH 5.5 (Fig 2, bottom panel). Therefore, citric acid and MES were used for the temperature optimization.

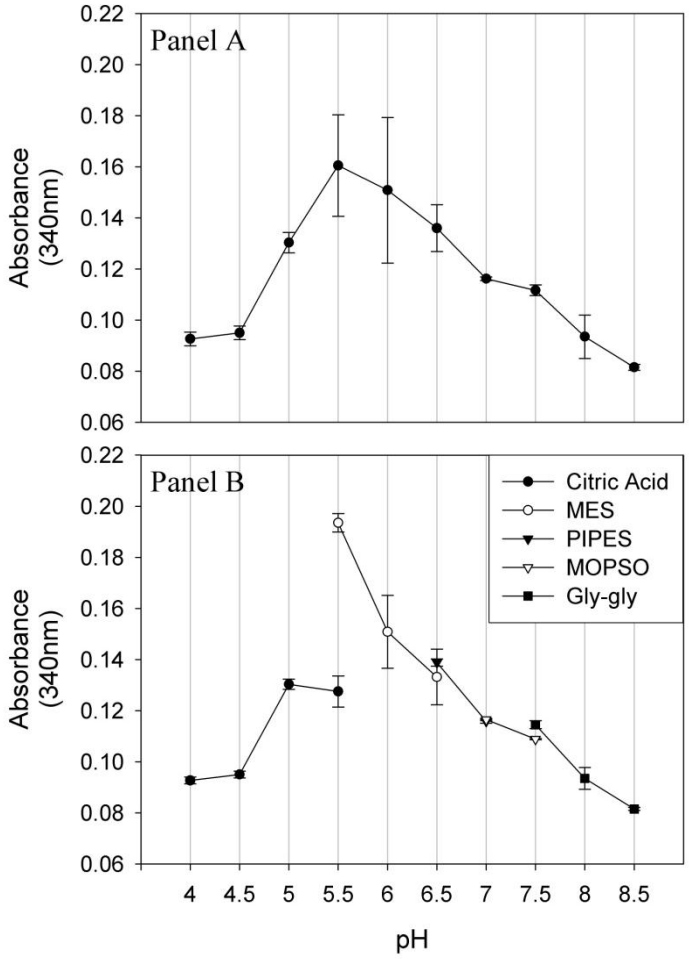

Figure 2. The pH optimization of invertase activity using buffering agents at selected pH for invertase activity. Panel A shows the absorbance of glucose produced as determined by the absorbance of NADH at 340 nm across the tested pH range. Panel B shows the activity of invertase based on the NADH absorbance at 340 nm for the buffering agents. Bars represent the standard error.

Invertase Temperature Optimization

The temperatures tested for invertase activity optimization were between 23°C and 48°C at 5°C increments. The temperature curve without regard to buffer showed an increase to 33°C and a plateau at 43°C. A dip was found at 38°C (Fig 3, top panel). This dip was

almost certainly due to the heating method. All the temperatures tested used a Dry Bath Incubator (Fisher Scientific) with the individual wells filled with water to provide good temperature transfer to the sample tubes except the 38°C. The 38°C portion of the temperature response study was conducted in a drying oven which uses blown heated air. The fluctuation that is consistent with this type of oven likely caused the reduction in the absorbance for invertase activity at 38°C.

The MES buffer consistently had higher absorbance compared to the citric acid (Fig 3, bottom panel). However, citric acid at pH 5.5 with temperature 37°C was decided to be suitable for the invertase reaction based on the previous work of Lingle and Dunlap (1987), Sung et al. (1989), Weersaooriyz and Yatawara (2002), and Kaur and Sharma (2005). These studies utilized an invertase reaction temperature of 37°C.

Invertase Analysis

The invertase reaction was performed in a Dry Bath Incubator (Fisher Scientific) with wells filled with water to improve temperature transfer with 25 µL 500 mM Sucrose (Sigma-Aldrich), 50 µL 250 mM citric acid (Sigma-Aldrich), 30 µL enzyme preparation, brought to a final volume of 150 µL with diH_2O. The samples were incubated for 15 min at 37 ± 1°C. The invertase reaction was stopped through a transfer to a hot bath (Thermolyne Cimaree 3 Hotplate, Dubuque, IA) at 90 to 95°C for 4 min. The stopped invertase reaction solution was stored in the ultracold freezer until the invertase assay.

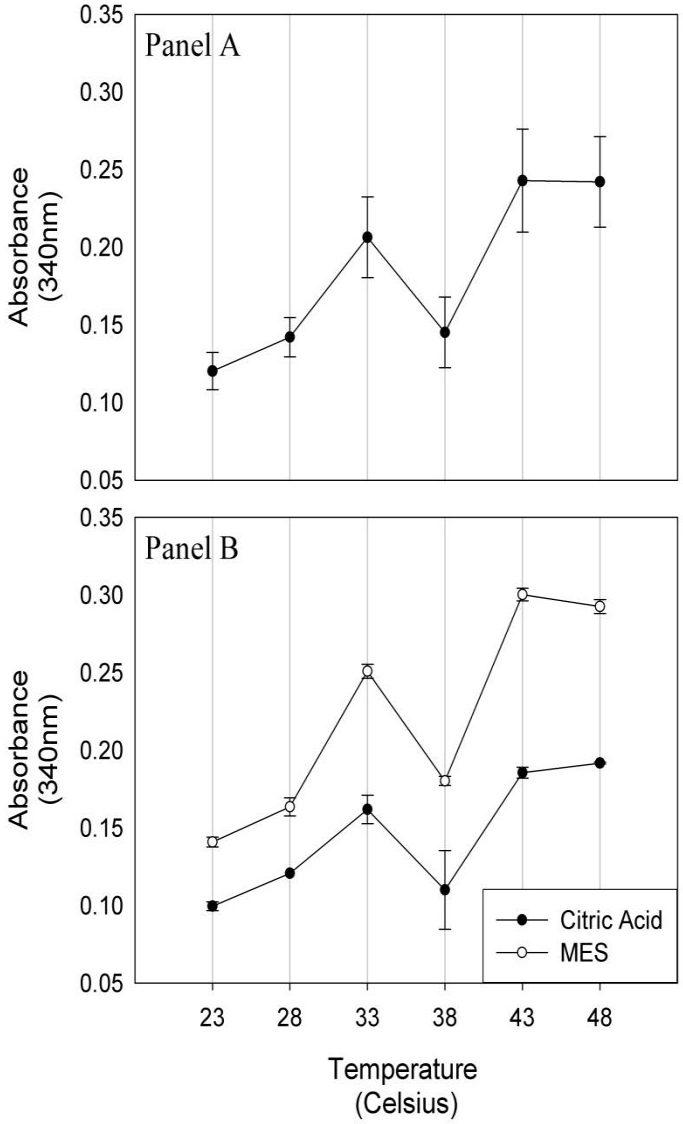

Figure 3. The temperature optimization for invertase activity using the buffers from the pH optimization at pH 5.5. The top panel indicates the response of invertase activity to temperature without regard to buffer. The bottom panel indicates the response of invertase activity to the selected buffers at pH 5.5. Bars represent the standard error.

The invertase assay (assay of product formed during the invertase reaction) was conducted by loading 100 µL ½X Fructose Assay Kit (Sigma-Aldrich), which contained glucose assay reagent (Sigma-Aldrich) and phosphoglucose isomerase (Sigma-Aldrich), into wells of a 96-well microtiter plate. The adequacy of the half-strength preparation of the sugar assay kits of Sigma-Aldrich was previously determined to be adequate for this range of produced hexose sugars (Tarpley and Sassenrath, 2006). A standard curve was established in triplicate using 20 µL providing 0.5, 1.5, and 5.0 µg of fructose (Sigma-Aldrich) per well. There were 20 µL stopped invertase reaction solution added to each well and the plates were incubated for 45 min at room temperature. The activity of invertase (amount of hexose sugar produced during the invertase reaction) was measured utilizing an enzyme-coupled reaction for the stoichiometric production of NADH. The NADH was measured using a PowerWave X microplate spectrophotometer (Bio-Tek Instruments, Inc., Winooski, VT) at 340 nm using the software provided by the manufacturer, KC4 2.7 Rev 8 (Bio-Tek, Inc.). The apparent activity of invertase is expressed as µmole hexose sugar produced (g DW tissue)$^{-1}$ hr^{-1}.

Carbohydrate Analysis

The soluble carbohydrate analysis began with the introduction of a 50 mg subsample into a vial with 3 mL 13.7 M (80% [v/v]) ethanol (Fisher Scientific, Waltham, MA) in diH_2O and placed in a hot bath at 70°C for 8 to 10 hr to extract the soluble carbohydrates, specifically glucose, fructose, and sucrose (Tarpley and Sassenrath, 2006). After each extraction, the 80% ethanol was replaced and the process repeated

three times. The extract was collected into a 15 mL vial fitted with a cap. The pooled extract was brought to a uniform volume of 10 mL for all samples with 80% ethanol. Activated charcoal (Darco G-60, Aldrich Chemical Company, St. Louis, MO) was added to the vials in excess to remove the phenols and other compounds that interfere with the enzymes used for the sugar analysis (Tarpley et al., 1993). The vials with the activated charcoal were placed overnight in the refrigerator (4°C), then 1500 µL soluble carbohydrate extract were removed and placed into deep-well 96-well storage plates at 4°C until the sugar assays were conducted.

The glucose assay was conducted by reconstitution of glucose assay reagent (Sigma-Aldrich) with 100 mL diH_2O (½X strength). The reagent was loaded into spectrophotometric-compatible 96-well microtiter plates at 100 µL per well. A glucose standard curve was established by loading 1, 2, 4, 7, and 10 µg glucose (Sigma-Aldrich) per well. For controls, 10 µg per well of fructose (Sigma-Aldrich) and sucrose (Sigma-Aldrich) were loaded in triplicate. The 5 µL sample aliquots were loaded into the remaining wells of each plate. The reaction was allowed 15 min at room temperature. The amount of glucose was calculated by the stoichiometric production of NADH measured at 340 nm with a PowerWave X Spectrophotometer (Tarpley et al., 1993). The amount of glucose is expressed as µg g DW^{-1}.

The fructose assay was conducted with a fructose assay mixture, which contained glucose assay reagent (Sigma-Aldrich) reconstituted with 100 mL diH_2O and phosphoglucose isomerase (from the fructose assay kit) (Sigma-Aldrich) for the conversion of fructose-6-phosphate to glucose-6-phosphate. Each well of the 96-well spectrophotometric plate was loaded with 100 µL of the fructose assay mixture. Fructose (Sigma-Aldrich) standards were loaded into the plate in triplicate at concentrations of 1, 2, 4, 7, and 10 µg fructose with assay controls of 10 µg sucrose (Sigma-Aldrich) and glucose (Sigma-Aldrich). Into each remaining well, 5 µL soluble carbohydrate extraction was loaded and the reaction was allowed 30 min at room temperature. The amount of fructose was calculated by measuring the stoichiometric production of NADH at 340 nm using the PowerWave X spectrophotometer (Tarpley et al., 1993). This method measures total glucose, including that converted from fructose, present in each well. Therefore, the amount of fructose was determined by subtracting the amount of glucose that was measured from the glucose assay. The amount of fructose is expressed as µg g DW^{-1}.

The sucrose assay was conducted by preparation and use of a sucrose assay reagent. The sucrose assay was made by first preparing an invertase stock solution. The invertase stock solution contained 100 mM MES (Sigma-Aldrich) adjusted to pH 5.5 with 4 M acetic acid or 4 M KOH, and contained 1350 ± 10 units invertase mL^{-1} stock solution. The invertase used was from baker's yeast (*Saccharomyces cerevisiae*) (Sigma-Aldrich, I4504). The sucrose assay reagent was prepared by reconstituting glucose assay reagent

(Sigma-Aldrich) with 98 mL diH_2O and adding 2 mL prepared invertase stock solution for a final concentration of 27 ± 1 invertase units mL^{-1} sucrose assay reagent. Each well of the 96-well microtiter plates was loaded with 100 µL sucrose assay reagent. Sucrose (Sigma-Aldrich) standards were loaded in triplicate at contents of 1, 2, 4, 7, and 10 µg per well. Controls were loaded into wells as 10 µg glucose (Sigma-Aldrich) and fructose (Sigma-Aldrich). There were 5 µL of sample soluble-carbohydrate extract loaded in triplicate into the remaining wells. The reaction was allowed 45 min at room temperature. The amount of sucrose was determined by measuring the stoichiometric production of NADH at 340 nm with a PowerWave X Spectrophotometer (Tarpley et al., 1993). This method measures total glucose present; therefore, glucose present from the cleavage of sucrose was determined by subtracting the glucose content determined by the glucose assay from the glucose measured in the sucrose assay. The amount of sucrose in the sample is equimolar with the amount of net glucose determined in the sucrose assay. The amount of sucrose is expressed as µg g DW^{-1}.

The starch analysis began by placing the pellet remaining from the soluble carbohydrate extraction into a hot bath at 80°C for 2 to 3 hr to evaporate the remaining ethanol. The pellet was then gelatinized in 1000 µL diH_2O for 8 to 10 hr in an 80°C hot bath (Tarpley and Sassenrath, 2006).

After cooling the gelatinized pellet/suspension, the starch degradation procedure was initiated. A 125 µL spike of 200 ± 5 units of purified amyloglucosidase (from *Aspergillus niger*, Sigma-Aldrich, A3042) in 360 mM HEPES (Sigma-Aldrich), pH 6.9, was added, and the tube placed into a 55°C hot bath for 2 to 3 hr. A second spike of 125 µL amyloglucosidase was added and the tube returned to the hot bath at 55°C for 2 to 3 hr. Once the starch degradation was complete, approximately 750 ± 100 µL was removed and placed into deep well plates and stored in a freezer at -20°C. The starch was quantitatively determined by the glucose assay procedure. Due to large differences in starch content in leaf and panicle samples, the leaf sample used 10 µL of starch degradate for the glucose assay. The panicle samples used 0.5 µL in the glucose assay. The apparent amount of starch is expressed as glucose equivalents of starch in µg g DW^{-1}.

Statistical Analysis

The early grain fill study and ratoon study field experiments were conducted in a completely randomized design; the research plots were assigned chemical applications based on the randomization procedure of Gomez and Gomez (1984).

Early Grain Fill Study. In the 2004 and 2005 field experiments, the ammonium molybdate, thidiazuron and non-chemical treated control (untreated) treatments were replicated six times.

Heat Stress Study. This experiment was conducted as a completely randomized design. In the HNT treatment, a minimum of 4 plants for each replication were placed under the free-air controlled heating apparatus for exposure followed by eventual sampling for biochemical analyses. In the ANT treatment, 4 plants in each replication were placed in the greenhouse and did not receive nighttime temperature regulation.

The measured amount of carbohydrates and invertase activity in the leaf and panicle were analyzed separately using the PROC UNIVARIATE procedure in SAS v 9.1.3 (SAS Institute, Cary, NC) for each dependent variable to check for normality. These dependent variables did not meet the requirement for normality because systematic variation was observed between replications, which were conducted at different times of the year. Within each replication and temperature treatment, the measured amount of the dependent variable was therefore ranked from lowest measured value to highest measured value using the methods described by Iman and Conover (1983). The lowest measured value received a value of one while the highest measured value received a value of four. This was done using a replication by heat treatment rank.

A paired t-test of the difference between HNT and ANT treatments was also conducted on the untreated (non-chemical treated) portion of the study to identify changes in biochemistry caused by the temperature treatment only, using the procedures described by Ott and Longnecker (2001).

A paired t-test of HNT and ANT for each chemical treatment and temperature treatment was conducted. The interaction between temperature and chemical would indicate how the chemical treatments are influenced by temperature during short-term heat stress. The test was conducted by comparing the ranked data for each replication, and analyzed by the PROC MEANS procedure in SAS.

Ratoon Study. In the 2004 field experiment, there were six replications for the untreated and the thidiazuron treatments and four replications for the ammonium molybdate treatment. The 2005 field study had six replications for each treatment.

For the 2005 greenhouse study, plants were planted and maintained until the desired developmental stage, and plants were then randomly selected for treatment. For each treatment, seven plants were selected. There were four plants used for the biochemical analysis and three plants for the FW and DW determinations.

The biochemical variables analyzed were glucose concentration, fructose concentration, sucrose concentration, total soluble carbohydrate (TSC) concentration, starch concentration, total nonstructural carbohydrate (TNC) concentration, and invertase activity. The agronomic variables analyzed were panicles m^{-2}, main-crop grain yield, ratoon-crop grain yield, total grain yield, and relative dry matter content.

The biochemical and agronomic data were analyzed for normality using the PROC UNIVARIATE procedure in SAS (SAS Institute, Cary, N.C.). The assumptions for normality were not met for the biochemical data. Therefore, each biochemical variable was ranked with the lowest measured value receiving a one and the highest measured value receiving a three or four, depending on if it was the greenhouse study or the field studies. Identical values were assigned the average rank for the range of represented ranks (Iman and Conover, 1983). The biochemical variables from the field studies and the greenhouse study were ranked independently.

The ranked data was analyzed using the PROC ANOVA procedure in SAS to identify differences, in each study, due to chemical applications. The ranks from both field studies and the greenhouse study were also combined and analyzed by PROC ANOVA to determine if differences observed within a specific study-year were retained throughout the ratoon experiment.

The medians were ranked within the field studies and the greenhouse study for each biochemical variable, and the median rank was analyzed using the PROC ANOVA procedure. Further, the medians were analyzed using a paired t-test of the chemical treatment compared to the untreated using the methods described by Ott and Longnecker (2001). The paired t-test was analyzed using the PROC MEANS procedure in SAS.

The agronomic data was analyzed using the PROC UNIVARIATE procedure in SAS to check for normality. The assumptions for normality were met using the measured variables for the field and greenhouse studies, thus no transformations of the agronomic data were performed.

The agronomic data was analyzed using PROC ANOVA in SAS for the field studies and the greenhouse study independently and also combined across the ratoon study. Further, the medians were determined for each agronomic variable and a paired t-test was conducted with the chemical treatment value compared to the untreated value (Ott and Longnecker, 2001).

When significant differences were observed from ANOVA, then means were separated using Duncan's multiple-range test at an α=0.05 level of significance. In the figures, levels with different letters are significantly different.

CHAPTER IV

EARLY GRAIN FILL STUDY

Results

Invertase Activity

The activity of SAI was not influenced by the chemical treatments at either 1 or 3 dat sample dates when analyzed in the leaf and panicle independently at the α=0.05 level of significance. However, a paired t-test of the median invertase activity at both 1 and 3 dat sample dates compared to the median invertase activity at 0 dat indicated an increase in the invertase activity (*p*=0.009) (Table 1).

The paired t-test indicates that the chemical application increases the activity of SAI by 11.01 µmol hexose sugar produced (g DW tissue)$^{-1}$ hr^{-1} compared to the 0 dat sample date for the 1 and 3 dat sample dates. The 95% confidence interval was 3.07 to 18.95 µmol hexose sugar produced (g DW tissue)$^{-1}$ hr^{-1}. This increase was not dependent on chemical treatment or tissue type.

Soluble Carbohydrate Concentrations

The average glucose concentration rank was influenced by the chemical application at the 3 dat sample date in the 2004 field study for the leaf samples (*p*=0.023) (data not shown). The thidiazuron treatment had higher average glucose concentration rank compared to the ammonium molybdate treatment; the untreated control was not different

than the thidiazuron or ammonium molybdate treatments. The thidiazuron treatment increased the mean rank of glucose concentration by 7.667 compared to the ammonium molybdate treatment.

When the glucose concentration rank was combined for the leaf samples in both the 2004 and 2005 field studies, the increase in average glucose concentration rank, due to the thidiazuron treatment, was higher than due to the ammonium molybdate (p=0.016) (Fig. 4). The untreated control was not different than the thidiazuron or ammonium molybdate treatments. The average increase in glucose concentration rank of the thidiazuron treatment was 3.8 above the ammonium molybdate treatment.

The median rank for glucose concentration was influenced by chemical application in the panicle when the study years were combined (p<0.0001) (Fig. 5). The thidiazuron treatment was higher than the untreated control and the ammonium molybdate treatments. The ammonium molybdate treatment was lower than the untreated control treatment. The thidiazuron treatment had a mean rank for the median glucose concentration of 3. The untreated control showed a mean rank for the median for glucose concentration of 2, while the ammonium molybdate treatment had a mean rank for the median glucose concentration of 1.

Table 1. The average difference of the treatment median compared to the 0 dat* median in both leaf and panicle samples combined of the 2004 and 2005 field studies. Carbohydrates are expressed in µg selected carbohydrate g DW^{-1} while the invertase activity is expressed as µmol hexose sugar produced g DW $tissue^{-1}$ hr^{-1}.

Biochemical variable	Average difference	95% CI lower	95% CI upper	*P*-value
Invertase activity	11.01	3.07	18.95	0.008
Sucrose	4.51	0.17	8.86	0.04
TSC †	7.25	2.58	11.91	0.004
Starch	14995.96	2802.63	27189.28	0.02

* dat is the days after treatment. The number of days after chemical application.
† TSC is the total soluble carbohydrate concentration. The TSC is the sum of glucose concentration, fructose concentration, and sucrose concentration.

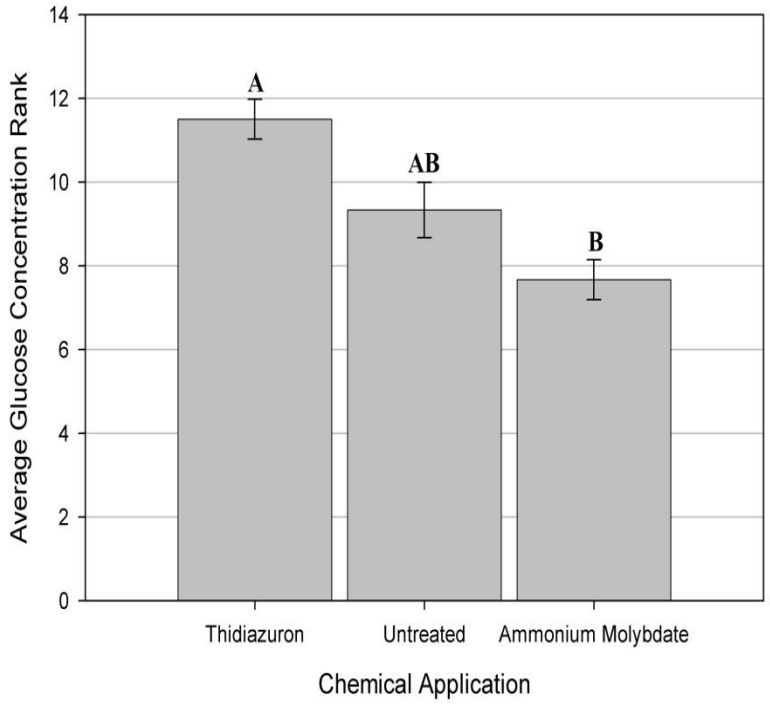

Figure 4. The average rank for glucose concentration in the leaf samples in the 2004 and 2005 field studies at the 3 dat sample date only. Bars represent the standard error.

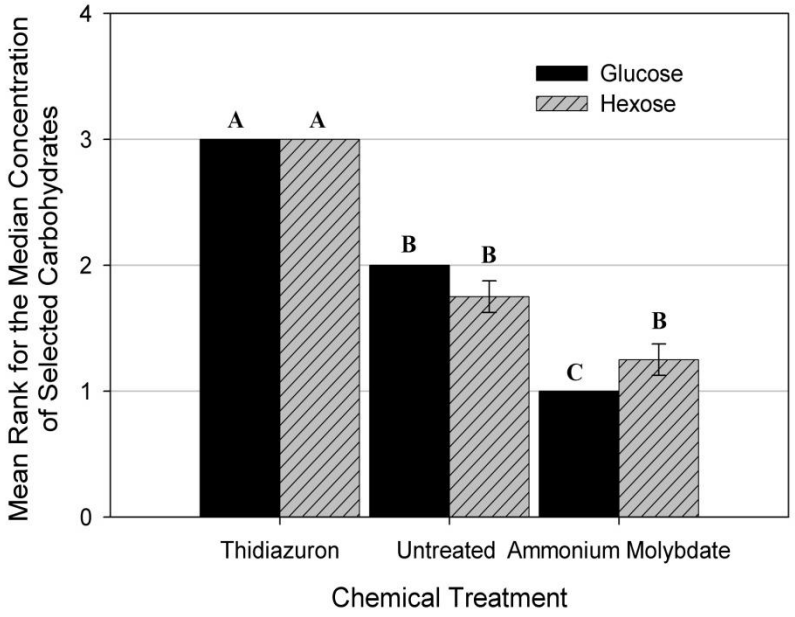

Figure 5. The mean rank for the median glucose and hexose sugar concentration in the panicle for the 2004 and 2005 field studies. Different letters for each dependent variable are significantly different for the chemical treatment. Bars represent the standard error.

The paired t-test of the median glucose concentration in the leaf at 1 and 3 dat sample dates for the 2004 and 2005 field studies were combined and compared to the median glucose concentration at the 0 dat treatment for each study-year. This showed an increase for the 1 and 3 dat sample date relative to 0 dat (p=0.016) (Table 2). The combination of the 1 and 3 dat median difference showed that the average of the median glucose concentration was increased by 1.64 µg glucose g DW tissue^{-1} with a 95% confidence interval between 0.38 to 2.91 µg glucose g DW tissue^{-1}.

Table 2. The average difference of the treatment median compared to the 0 dat* median of the selected carbohydrates in the early grain fill leaf samples from the 2004 and 2005 field studies.

Selected carbohydrate	dat	Average difference	95% CI lower	95% CI upper	*P*-value
Glucose	1 & 3	1.64	0.38	2.91	0.02
Hexose	1 & 3	1.87	0.19	3.54	0.03
Sucrose	1 & 3	10.86	3.79	17.92	0.006
TSC †	3	21.33	10.81	31.85	0.003
TSC	1 & 3	15.32	8.95	21.72	0.0003
Starch	1	727.27	122.48	1332.06	0.03
Starch	1 & 3	2428.72	1244.62	3612.83	0.0009

* dat is the days after treatment. The number of days after chemical application.
† TSC is the total soluble carbohydrate concentration. The TSC is the sum of glucose concentration, fructose concentration, and sucrose concentration.

The median for hexose sugar concentration was ranked and found to be altered by chemical application in the panicle for each study-year when the data was combined (p=0.01) (Fig. 5). The thidiazuron treatment had a higher median rank compared to the untreated control or the ammonium molybdate treatment. For the panicle, the thidiazuron treatment had a higher median rank for hexose sugar concentration compared to the untreated control or the ammonium molybdate treatment.

Median hexose sugar concentration was increased in the leaf for the 2004 and 2005 field studies when 1 and 3 dat sample dates were combined at each study year (p=0.03) (Table 2); the average increase was 1.87 µg g hexose sugar g DW tissue^{-1} and the 95% confidence interval was 0.19 to 3.54 µg hexose sugar g DW tissue^{-1}.

The proportion of hexose sugar to sucrose sugar in the tissue samples was analyzed; the thidiazuron treatment had a higher proportion compared to the ammonium molybdate, untreated control, and the 0 dat (Fig. 6).

The leaf had very low (≈5%) hexose compared to sucrose concentration. This may indicate that the hexose sugar is utilized in biochemical reactions and the sucrose is accumulating in the leaf. The panicle had a much higher proportion (≈50%) of hexose sugar compared to sucrose concentration.

The paired t-test of median sucrose concentration in the leaf for each study year at both sample dates compared to 0 dat indicated that the average increase was 10.86 µg sucrose g DW tissue^{-1}. The 95% confidence interval was 3.79 to 17.92 µg sucrose g DW tissue^{-1} (p=0.0061) (Table 2).

Furthermore, the sucrose concentration was increased based on the paired t-test of median sucrose concentration in the leaf and panicle at each sample date compared to the 0 dat concentration (p=0.04) (Table 1). The increase was 4.51 µg sucrose g DW tissue^{-1} regardless of tissue type, chemical application, or sample date. The 95% confidence interval was 0.17 to 8.86 µg sucrose g DW tissue^{-1}.

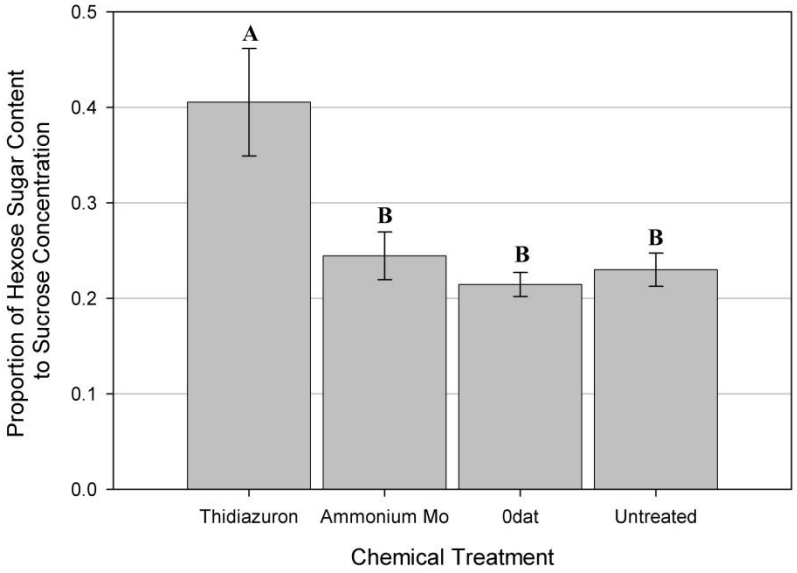

Figure 6. Hexose:sucrose content in leaf and panicle tissue samples combined. Bars represent the standard error.

Total soluble carbohydrate concentration is the sum of the glucose concentration, fructose concentration, and sucrose concentration for each tissue sample. The difference for median TSC concentration in the leaf and panicle compared to 0 dat was analyzed by ANOVA to determine the effects of chemical treatment, tissue type, and sample date. This analysis indicated the TSC difference to be affected by the above classes (p=0.006). However, tissue and tissue by sample date interaction were the only independent variables significant at the α=0.05 level, with p-values of <0.0001, and 0.01, respectively (Fig. 7). The difference in TSC concentration of the leaf increased over the sample date period while the difference in the panicle decreased over the sample date period. The

average difference for TSC concentration over the sample date period in the leaf was higher than in the panicle.

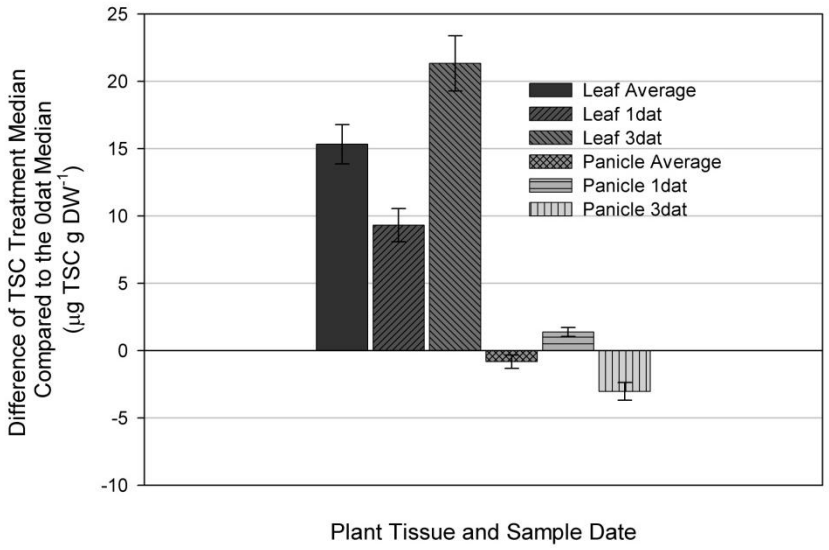

Figure 7. The difference of the total soluble carbohydrate concentration medians between sample date and tissue type compared to the 0 dat median as analyzed by the paired t-test. The TSC difference is the combination of the chemical treatments. Bars represent the standard error.

In the leaf, the median difference in TSC concentration at 3 dat compared to the 0 dat treatment was higher, regardless of chemical application (p=0.003) (Table 2). The average increase in TSC concentration was 21.33 µg TSC g DW tissue^{-1}. The 95% confidence interval was 10.81 to 31.85 µg TSC g DW tissue^{-1}.

When the median TSC concentrations in the leaf for each sample date were combined and compared to 0 dat, the increase was 15.32 µg TSC g DW tissue^{-1} (p=0.0003) (Table 2). The 95% confidence interval was 8.95 to 21.72 µg TSC g DW tissue^{-1}.

In the leaf and panicle samples, the median difference in TSC at both sample dates were combined and compared to 0 dat. The TSC concentration difference was significant (p=0.004) (Table 1); the average increase across tissue type, chemical treatment, and sample date was 7.25 µg TSC g DW tissue^{-1}. The 95% confidence interval was 2.58 to 11.91 µg TSC g DW tissue^{-1}.

The sucrose concentration was a large contributor (93%) to TSC in the leaf (Fig. 8), which may explain the TSC accumulation in the leaf. The TSC components were affected by chemical applications, specifically the glucose and hexose in both plant tissues yet chemical applications had no observable effect on TSC. This is likely because the chemicals did not affect the sucrose content.

Starch Levels

The starch concentration was increased over the sample date period in the leaf samples in the 2004 field study (p=0.02). The chemical treatments did not influence the starch concentration (data not shown). The samples from the 3 dat sample date had higher starch concentration compared to the 0 or 1 dat leaf samples. The increase in starch concentration in the 3 dat samples was 2,842.35 µg glucose equivalents g DW tissue^{-1}

above the 0 and 1 dat sample date average starch concentration. Similarly, the 3 dat leaf samples had higher starch concentration compared to the 0 or 1 dat leaf samples for the 2004 and 2005 field studies combined (p=0.02). The leaf samples in the 2004 and 2005 field studies had an increase in the starch medians at the 1 dat sample date compared to 0 dat (p=0.03) (Table 2) of 727.27 µg glucose equivalents g DW tissue^{-1} with a 95% confidence interval 122.48 to 1,332.06 µg glucose equivalents g DW tissue^{-1}. The increase in the starch at 1 dat was not affected by chemical application. The starch concentration of the 1 and 3 dat sample dates of leaf samples from the 2004 and 2005 field studies was higher (2,428.72 µg of glucose equivalents g DW tissue^{-1}) than the 0 dat samples (p=0.0009) (Table 2). The average increase in the difference was, well as for leaf and panicle samples combined (14,995.96 µg glucose equivalents g DW tissue^{-1}). Total nonstructural carbohydrates are the sum of the glucose concentration, fructose concentration, sucrose concentration, and starch concentration. The analyses of TNC found that starch concentration contributed over 90% to the TNC values, so specific TNC discussion will be omitted.

Grain Yield

The main-crop grain yield in both field studies was influenced by chemical application (p<0.0001) (Fig. 9). The grain yield in the untreated control was higher than for the thidiazuron treatment by over 700 kg ha^{-1}. The ammonium molybdate treatments were not different than either the untreated control or the thidiazuron treatment. The ratoon-

crop and total-crop grain yield was not affected by chemical treatment with *p*-values of 0.8 each.

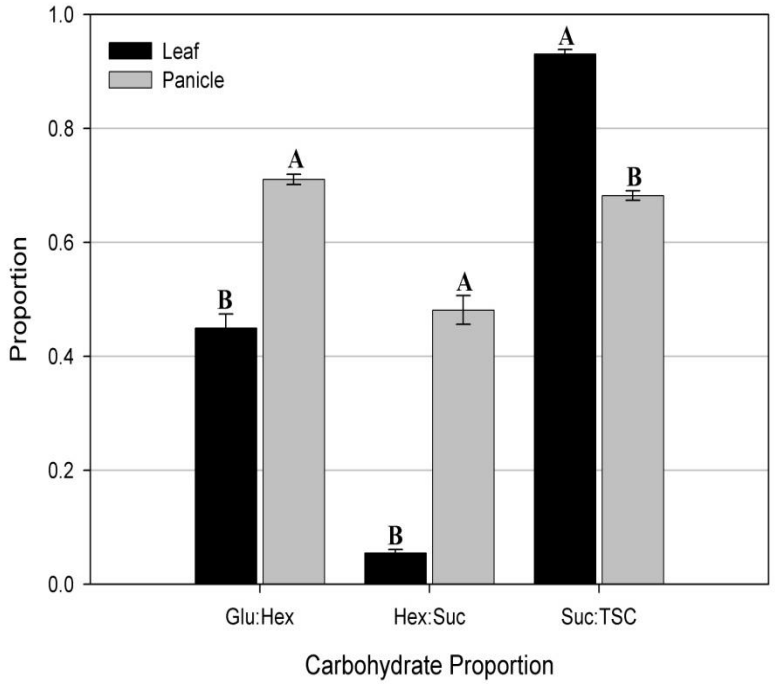

Figure 8. The proportion of selected carbohydrates without concern for chemical treatment for tissue types. Glu:Hex is the glucose to hexose proportion. Hex:Suc is the hexose to sucrose proportion. Suc:TSC is the sucrose to TSC proportion. Bars represent the standard error.

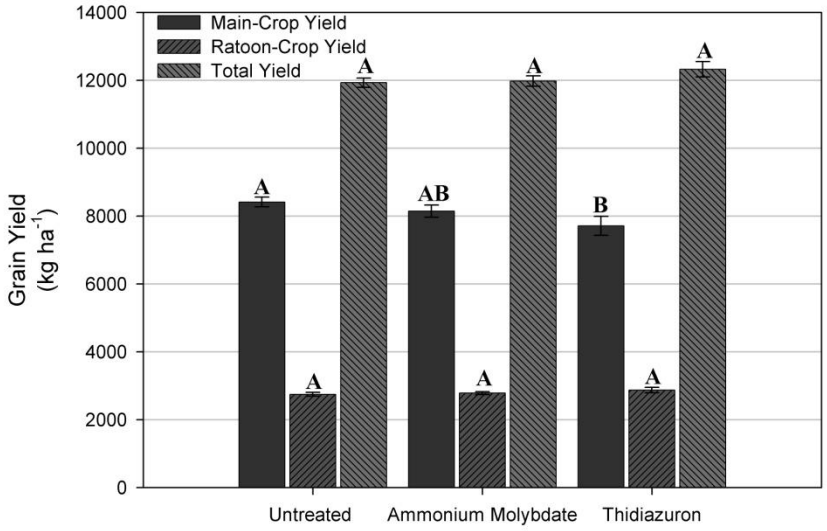

Figure 9. The grain yield parameters of the 2004 and 2005 field studies. Different letters are significantly different for each dependent variable. The bars represent the standard error.

Discussion

Invertase Activity

The activity of SAI or the rank of SAI activity was not affected by over-the-top chemical applications in the leaf or panicle samples of the early grain fill study. The activity of SAI could possibly be affected by increased application rates of the tested chemicals.

The activity of SAI was influenced by sample date, i.e., when the median activity of SAI for each tissue type and sample date was compared to the corresponding tissue of median invertase activity from the 0 dat sample date (Table 1). The activity of invertase was increased compared to the 0 dat. However, the increase in activity was not tissue or chemical specific, thus is probably developmental or environmental instead. The ability to detect differences in activity among sampling dates indicates the assay was conducted with reasonable precision.

The activity of SAI in the leaf was low compared to other studies that looked at the leaf. The activity of invertase in the leaves of rice was much lower (10 times lower) than those reported in soybeans during early reproductive growth (Liu et al., 2004), and was more than 20 times lower compared to the invertase activity in leaves of peas during early growth (Bruskova et al., 2004). The presence of differences among sampling dates, which were presumably due to developmental or environmental factors, suggests

the possibility of other dynamic regulation of invertase in the rice plants that were not considered in my study.

In this experiment, the invertase activity in the panicle of rice was over 20 times lower compared to soybean pods during early reproductive growth (Liu et al., 2004). However, the activity of rice panicle invertase was closer to those values reported by Tarpley et al. (1994) for sorghum (*Sorghum bicolor* L. Moench) panicle during mid anthesis. The values were 4 times lower than those for sorghum.

Soluble Carbohydrate Pool

The application of over-the-top chemicals that influence the activity or rank of SAI activity does affect selected carbohydrates in tissues of field grown rice. The evaluated carbohydrates that were influenced were the glucose rank and starch concentration, as well as the median concentration difference compared to the 0dat median concentration of several of the carbohydrates.

In the leaf, the glucose concentration rank was higher in the thidiazuron treatment compared to the ammonium molybdate treatment (Fig. 4). In the panicle, based on the ranked median analysis of the glucose concentration, the thidiazuron had a higher rank compared to the untreated control. Further, the ammonium molybdate was lower than the untreated control treatments (Fig. 5). Collectively, these glucose concentration

observations in the leaf and panicle tissue suggest that the glucose concentration can be affected by chemical application.

Thidiazuron is a hormonal activator of invertase activity (Abdelghani et al., 1991). Therefore, there would be increased sucrose cleavage in the system that would lead to increased glucose and fructose flux into the system. However, this hypothesis was not fully supported based on the observed activity of SAI and the concentrations of fructose and sucrose which were not influenced by chemical application.

Similarly, the hexose median rank was affected by chemical treatment in the panicle (Fig. 5); the thidiazuron treatment increased the median rank compared to the untreated control or the ammonium molybdate treatments. A potentially related result is the presence of a higher hexose-to-sucrose ratio in the panicle, which could be due to increased cleavage of sucrose providing substrates for biochemical reactions. However, the hexose sugar may be accumulating due to an inability of enzymes to keep pace with increased supply of substrates. An accumulation of hexose in a tissue can, however, also possibly indicate an unidentified stress event (Lohaus et al., 2000; Bishop et al., 2002). In this study, the thidiazuron may have caused a stress condition in the rice plants which reduced the main-crop yield (Fig. 9) while increasing the glucose and hexose concentrations in the leaf and panicle, and leaf, respectively.

Yield and Yield Components

The main-crop yield was influenced by the applications of chemicals which alter the activity of SAI. The untreated control treatment had higher grain yield compared to the thidiazuron treatment. The ammonium molybdate treatment was not different than either the untreated control or the thidiazuron treatment for main-crop grain yield (Fig. 9).

The thidiazuron treatment may be causing a stress event in rice, at the tested rates in this experiment. Thidiazuron is the active ingredient used in the cotton defoliant Dropp® SC. This defoliant is used at rates of 112 to 224 g a.i. ha^{-1}. Although the tested rates in this study were much lower (0.5 g a.i. ha^{-1}), I cannot rule out the possibility that the rates were triggering defoliation-related biochemical events. Regulation of these events, however, is complex. For example, leaf senescence is regulated by cytokinins and ethylene and there are some responses of cytokinins that are dependent on ethylene (Maxwell and Kieber, 2004). An inverse correlation was identified between leaf senescence progression and cytokinin levels in the leaf (Gan, 2004).

In support of thidiazuron causing stress, it has been reported that the ash residue of Regal geranium (*Pelargonium domesticum* L.) treated with thidiazuron had lower mineral content in shoots, but higher in roots. There was an increase in the accumulation of minerals, in the root thus allowing the geranium plants to be susceptible to mineral stress. Further, an increase in the stress-related markers of proline, ABA, and 4-

aminobutyrate and an increase in the levels of metabolic energy and reducing power with the thidiazuron application were observed (Murch et al., 1997). In rice, cytokinins have been identified as stress signals which originate in the root and transported via the xylem to the shoots. When rice is under water stress, the leaf senescence is controlled by levels of ABA and cytokinin (Yang et al., 2002). Although the mechanisms of the effect of thidiazuron on grain yield were not examined in this study, the effects on glucose and hexose concentration support the possibility that a stress effect due to the application of thidiazuron at these low concentrations as involved. Although an effect on invertase was not observed, it is likely that invertase activity or other hexose metabolism was affected by thidiazuron based on the observed changes in the sugars.

Since the main-crop yield is reduced by chemical application compared to the untreated control treatment, the use of thidiazuron at these rates for manipulating SAI activity is unlikely to be used in rice production fields for increases in grain yield.

CHAPTER V

HEAT STRESS STUDY

Results

Invertase Activity

For SAI activity in the leaf under the ambient nighttime temperature (ANT) treatment, the concanavalin A treatment rank for SAI activity was higher than the ammonium molybdate treatment and the untreated control rank. The thidiazuron treatment; however, was not different than the concanavalin A, ammonium molybdate, or the untreated control treatments (Fig. 10, Panel A). The average rank for SAI activity in the leaf under high nighttime temperature (HNT) was not altered by chemical application at the α=0.05 level of significance (Fig. 10, Panel B).

The chemical treatments influenced the SAI activity rank in the panicle under the ANT treatments (p=0.03). The ammonium molybdate treatment increased the mean rank for SAI activity compared to the thidiazuron or concanavalin A treatments while the untreated was not different than the ammonium molybdate, thidiazuron, or concanavalin A treatments (Fig. 10, Panel C). In the panicle under the HNT treatment, there were no observable differences in the rank of SAI activity at the α=0.05 level of significance among the chemical applications (Fig. 10, panel D).

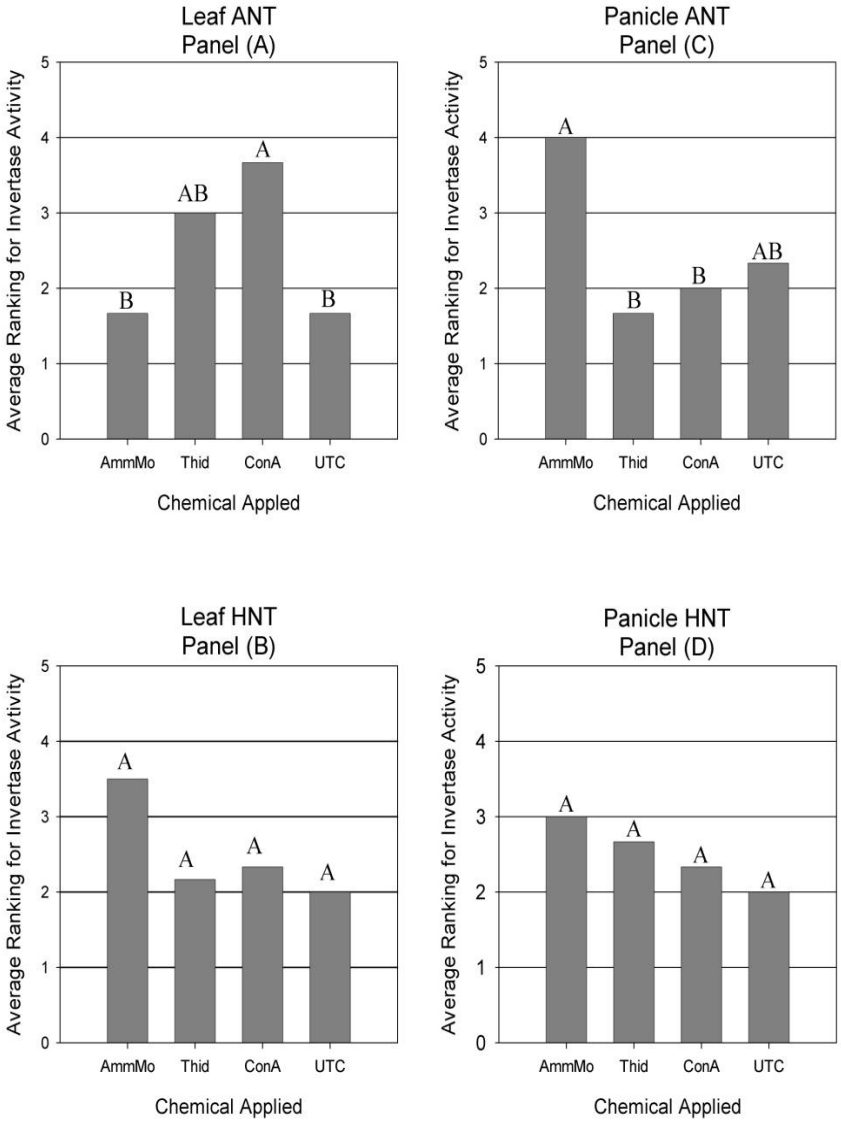

Figure 10. The average invertase activity rank in the leaf and panicle samples under either ambient nighttime temperature (ANT) or high nighttime temperature (HNT). AmmMo is the ammonium molybdate treatment, Thid is the thidiazuron, ConA is the concanavalin A treatment, and the UTC is the untreated control treatment.

The HNT treatment, without regard to chemical application, did not influence the activity of invertase in either the leaf or panicle at the α=0.05 level of significance. This was confirmed using a paired t-test for the difference of the untreated rank for the ANT compared to the HNT.

The SAI activity rank difference between HNT and ANT treatment in the leaf was increased (p=0.003) in the ammonium molybdate treatment by an average 2.1667 with a 95% confidence interval of 1.1346 to 3.1985 (data not shown). In the panicle, the SAI activity ranked difference between HNT and ANT was reduced (p=0.04) by an average 1.0000 in the ammonium molybdate treatment with a 95% confidence interval of 0.0614 to 1.9386 (Fig. 11).

Soluble Carbohydrate Levels

The ranks for glucose, fructose, hexose, sucrose, and TSC concentrations in the leaf and panicle were not influenced by chemical application (Table 3). Temperature also did not affect the rank for these in either the panicle or leaf (Table 4).

The HNT treatment of the panicle reduced (p=0.04) the TSC rank by 1.0000 in the ammonium molybdate treatment with a 95% confidence interval of 0.0614 to 1.9386. The HNT of the panicle also showed an average increase (p=0.01) of 1.8333 in the TSC rank for the thidiazuron treatment with a 95% confidence interval of 0.6065 to 3.0157 (Fig. 11).

Starch Levels

The starch concentration rank in the leaf and panicle was not influenced by heat treatment or chemical application at the α=0.05 level of significance (data not shown). The difference between the HNT and ANT treatment, as indicated by the paired t-test, showed the starch concentration rank was decreased (p=0.03) in the panicle with ammonium molybdate treatment by an average of 1.6000 with a 95% confidence interval of 0.1843 to 3.0157 (Fig. 11). The leaf was not influenced by HNT treatment for starch concentration rank.

Table 3. The average rank for selected soluble carbohydrates in the penultimate leaf and panicle of rice plants under ambient nighttime temperature for 4 d.

Carbohydrate	Tissue	*P*-value	Chemical treatment			
			Ammonium molybdate	Concanavalin A	Thidiazuron	Untreated
Glucose	Panicle	0.5	3.00	3.00	1.67	2.33
	Leaf	0.4	2.00	2.33	2.33	3.33
Fructose	Panicle	0.8	3.00	2.67	2.00	2.33
	Leaf	0.6	1.83	3.00	2.67	2.50
Hexose	Panicle	0.8	3.00	2.67	2.00	2.33
	Leaf	0.5	1.83	2.67	2.33	3.17
Sucrose	Panicle	0.08	3.67	2.67	1.33	2.33
	Leaf	0.9	2.67	2.67	2.67	2.00
TSC	Panicle	0.08	3.67	2.67	1.33	2.33
	Leaf	0.9	2.67	2.67	2.67	2.00

Total Nonstructural Carbohydrates

Total nonstructural carbohydrate is the sum of the glucose, fructose, sucrose, and the starch concentrations. Levels of TNC were not influenced in either HNT or ANT treatments in the leaf or the panicle. In the panicle, the paired t-test of the ranked

difference between HNT and ANT treatments found a reduction (p=0.04) in the TNC rank by an average of 1.3333 in the ammonium molybdate treatment with a 95% confidence interval of 0.0624 to 2.6043 (Fig. 11).

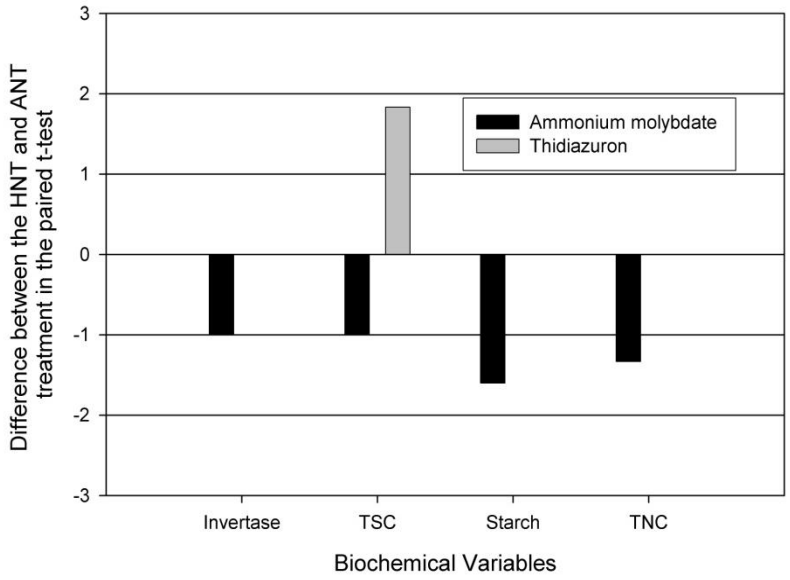

Figure 11. The ranked difference between the HNT and the ANT with the paired t-test for selected biochemical variables from the panicle in plants from the heat stress study. The differences are presented if significant at the α=0.05 level. TSC is the total soluble carbohydrates, and TNC is total nonstructural carbohydrates.

Table 4. The average rank for selected soluble carbohydrates in the penultimate leaf and panicle of rice plants exposed to short-term high nighttime temperature stress of 30°C for 4 d.

Carbohydrate	Tissue	*P*-value	Chemical treatment			
			Ammonium molybdate	Concanavalin A	Thidiazuron	Untreated
Glucose	Panicle	0.8	2.17	2.33	2.83	2.67
	Leaf	0.5	2.67	2.33	2.33	2.67
Fructose	Panicle	0.6	2.50	2.00	2.83	2.67
	Leaf	0.17	2.58	1.92	2.42	3.08
Hexose	Panicle	0.97	2.50	2.33	2.67	2.50
	Leaf	0.17	2.58	1.92	2.42	3.08
Sucrose	Panicle	0.3	2.67	2.00	3.17	2.17
	Leaf	0.5	2.50	2.50	2.00	3.00
TSC	Panicle	0.6	2.50	2.17	3.00	2.33
	Leaf	0.5	2.50	2.50	2.00	3.00

Discussion

Invertase Activity

The measured activity of SAI in this study was lower than those previously reported. In the leaves of rice plants in the ANT treatment without chemical treatment, the activity of SAI was 22 times lower than those reported in soybeans during early reproductive growth (Liu et al., 2004), and 15 times lower than reported in early growth of peas (Bruskova et al., 2004). In the panicle of rice, the activity was 12 times lower than soybean pods in early reproductive growth (Liu et al., 2004) and 4 times lower than sorghum panicles at mid-anthesis (Tarpley et al., 1994). The SAI assay was optimized, and it is not apparent from this study what the physiological differences would be between rice and these other species explaining the differences in enzyme activities.

Over-the-top application of chemicals that influence the activity of invertase affected the rank of SAI activity in the penultimate leaf and panicle of rice plants during the early grain-fill developmental period (Fig. 10, Panels A and C). In the leaf, the concanavalin A treatment increased the rank of SAI activity over the untreated control and the ammonium molybdate treated plants (Fig. 10, Panel A). Concanavalin A is a lectin and lectins enhance the activity of invertase in rice (Isla et al, 1995) and in *Ricinus* (Vattuone, et al, 1991). This experiment supports the previous work of Ferens and Morawiecka (1985) that concanavalin A increases invertase activity in the leaves of rice plants. Concanavalin A, however, is too expensive for field use, but was used in this

study to examine the concept of the use of over-the-top applications of chemicals to influence invertase activity.

In the thidiazuron treatment, SAI activity rank was not significantly different than the untreated control, ammonium molybdate, or concanavalin A treatments (Fig. 10, Panel A). Thidiazuron is a hormonal activator of invertase activity (Abdelghani et al., 1991). Since thidiazuron is a plant hormone analog in the cytokinin family of hormones, thidiazuron is likely affecting other plant processes and reactions not measured in this study. Ammonium molybdate is a chemical inhibitor of invertase activity (Prado et al., 1979). In this study, the rank of invertase activity in the leaf was not different than the untreated control. This response suggests, in the leaves of rice, ammonium molybdate does not alter the activity of invertase during early-grain development.

In the panicle, the chemical inhibitor of invertase activity significantly increased the invertase activity rank compared to chemicals that stimulate invertase activity (Fig. 10, Panel C). The ammonium molybdate treatment significantly increases activity rank over the concanavalin A and thidiazuron treatments. The mechanism of the observed SAI activity rank increase with a chemical that has been reported to reduce the activity of SAI requires additional investigation (Prado et al., 1979). The differential response to the ammonium molybdate at ANT in the leaf and panicle in invertase activity rank may be due to the surface area application difference between the leaf and panicle. In this study, when the treatments were applied during early- to mid-grain fill of the main crop,

the leaves had a much higher surface area than the panicle. Theoretically, the leaves would receive more active ingredient per unit mass compared to the panicle. This hypothesis was not tested in this experiment, but should be addressed in future work. Another possibility for the differential response is the diverse metabolic activity in the leaves compared to the panicle. During the tested developmental stage, the leaf is primarily undergoing plant maintenance metabolism while the panicle is in reproductive reactions, which require metabolic activity in support of seed filling and tissue maintenance. The ammonium ion is not likely to have an effect on the invertase activity rank, such as one possibly mimicking salt stress, because the inhibition of invertase activity by ammonium molybdate was caused by the molybdate ion and not the ionic strength or the ammonium ion which was fairly low at 4 g ammonium molybdate ha^{-1} (Prado, et al., 1979).

Carbohydrate Pool

The applications of over-the-top chemicals that influence the activity of invertase do not affect the carbohydrate profile in the leaf or panicle of rice plants (Table 3). This study indicates that the carbohydrate profile in rice plants is partially buffered against environmental stimuli, such as nighttime temperature. The static hexose concentration rank indicates that the plants coordinated their metabolism in response to the short-term nighttime temperature stress of 30°C for the 4 d duration. If the hexose rank differed, it would indicate a buildup (or decline) of glucose and fructose that did not enter glycolysis or other biochemical reactions and would further indicate stress due to the

temperature or chemical application (Lohaus et al., 2000; Bishop et al., 2002). This hypothesis should be tested by measuring daytime and nighttime respiration and evaluation of other 'respiration' enzymes, such as phosphofructokinase (PFK) (E.C. 2.7.1.11) or pyruvate kinase (E.C. 2.7.1.40).

Temperature Effect on Invertase Activity and Carbohydrate Pool

The temperature treatment in this study did not alter the SAI activity rank in the panicle (Fig. 10, Panel B) or leaf (Fig. 10, Panel D). The paired t-test of the difference between the untreated control in the ANT from the HNT showed no differences for SAI activity. Furthermore, the temperature did not affect the rank for the level of selected carbohydrates in either the leaf or panicle (Table 4). This was confirmed using the paired t-test, as before. The results from this study show that rice plants exposed to short-term elevated nighttime temperatures of 30°C for 4 d do not exhibit altered activity of SAI or the carbohydrate profile in the leaves or the panicles. A high nighttime temperature of 30°C does not appear to have an effect on the biochemistry of the rice plants, at least for the variables measured in this experiment. The rice plants are able to adjust their metabolism to the environment to maintain homeostasis within this range of nighttime temperatures.

Interaction between Temperature and Invertase Activity and between Temperature and Carbohydrate Pool

There was an interaction observed for the rank of SAI activity in the ammonium molybdate treatment under HNT. In the panicle, the activity rank was reduced by 1.0000 (Fig. 11), but in the leaf the activity rank was increased by 2.1667 (data not shown). This was the only interaction observed for the rank of invertase activity with chemical application and temperature treatment. The mechanism of the interaction was not identified, and should be investigated in future work.

The carbohydrate profile in the panicle demonstrated an interaction between chemical application and temperature treatment. The ammonium molybdate treatment reduced the rank of TSC concentration, starch concentration, and TNC concentration (Fig. 11). It was found that sucrose contributes 64% to the total measured amount of TSC, while starch contributed 97% to the total measured TNC. Since the starch contribution to TNC is very high, TNC discussion will be omitted. Starch is the primary component found in rice grain (Nakamura and Yuki, 1992; Umemoto et al., 1994). As the grain matures, glucose, fructose, and sucrose concentrations decline as the starch concentration increases. The chemical applications occurred late in the development of rice, specifically early- to mid-grain fill, and the treatments likely did not influence the starch concentration or production.

This experiment did identify an interaction between HNT and the ammonium molybdate treatment for starch concentration rank. The starch rank was decreased with this treatment interaction. The mechanism of this interaction was not identified in this experiment. Future work should look at other biochemical reactions in the starch production and starch degradation pathways. This approach would identify what processes are most susceptible to HNT and ammonium molybdate. Also, grain characteristics, such as filled versus unfilled grain, grain length and width, and 100-seed weight, would indicate if the starch reduction observed in this experiment could have an economic impact on rice production.

High nighttime temperature in conjunction with ammonium molybdate reduces the SAI activity rank, TSC concentration rank, and the starch concentration rank in the panicle. The ammonium molybdate treatment appears to inhibit the activity of SAI while increasing the carbohydrate degradation within the panicle. The mechanism for this requires further investigation.

There was an interaction observed for TSC in the panicle with chemical application and temperature treatment. The thidiazuron treatment increased the TSC concentration rank by 1.8333 in the HNT compared to the ANT treatment (Fig. 11). Thidiazuron is a cytokinin hormone and could possibly affect other process and reactions that may inhibit the TSC component degradation or increase the storage of the TSC components. Future research should address the process that affects the rank of TSC and its components.

The heat-stress study demonstrated the ability to affect invertase activity through over-the-top applications of putative invertase-activity effector chemicals. The results from the ammonium molybdate application were unexpected in that SAI activity was increased in the panicle relative to the other treatments while it was used as a chemical inhibitor of invertase activity. Additional research would be needed to establish if the variable response between organs is a transient occurrence or reflects a condition of the study.

CHAPTER VI

RATOON TILLER BUD DEVELOPMENT STUDY

Results

Invertase Activity

The SAI activity in main-crop stem segments during ratoon tiller bud expansion was not influenced by chemical application as determined at the α=0.05 level of significance.

Soluble Carbohydrate Levels

Based on analysis of the median rank for fructose concentration in the 2005 greenhouse study, the concanavalin A treatment increased the fructose median rank compared to the ammonium molybdate, untreated control, or thidiazuron treatments (p=0.03) (Fig. 12).

The analysis of the median rank indicated the sucrose concentration was affected by chemical application when the concanavalin A treatment was included in the analysis (p=0.02) (Fig. 12). The ammonium molybdate treatment had higher sucrose rank than the untreated control or the thidiazuron treatments. The concanavalin A treatment was higher than the thidiazuron treatment, but not different than the ammonium molybdate or the untreated control.

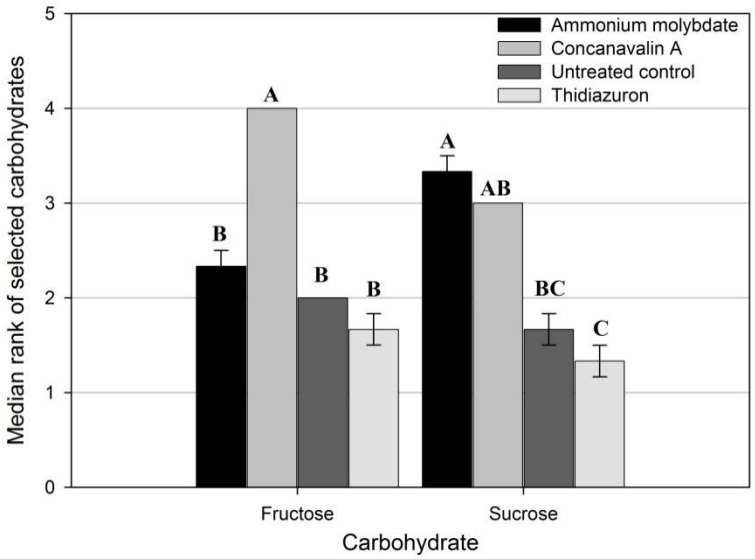

Figure 12. The median rank for the fructose and sucrose concentrations in the ratoon study experiments. Bars represent the standard error.

The concanavalin A treatment was used in the greenhouse study only. Therefore, the sucrose median rank data was also analyzed without the inclusion of the concanavalin A treatment. This method indicated that the ammonium molybdate had higher median sucrose rank compared to the untreated control or thidiazuron treatments (p=0.01) (Fig. 13).

The 2004 field study showed that the mean rank for the TSC was influenced by chemical application (p=0.004). The mean rank observed in the ammonium molybdate treatment was higher than the thidiazuron or the untreated control treatments (Fig. 14). The thidiazuron and untreated control treatments were not different.

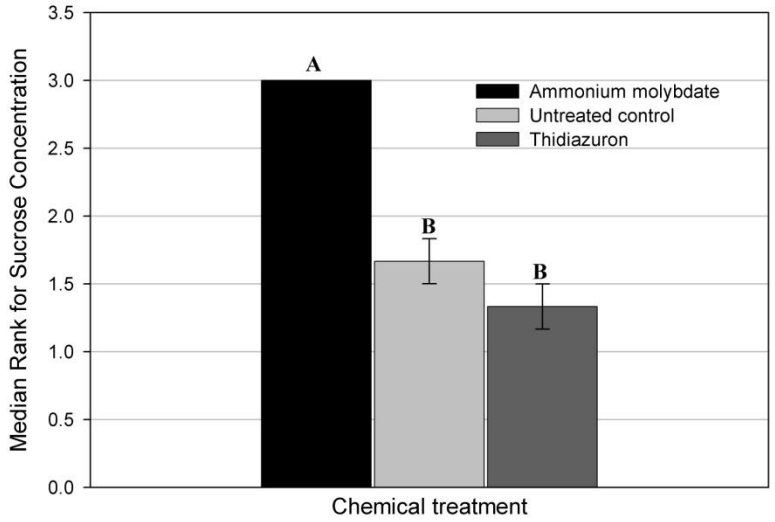

Figure 13. The median rank for sucrose concentration in the 2004 and 2005 ratoon study field experiments. The field studies did not have concanavalin A as a treatment. The bars represent the standard error.

The soluble carbohydrates that contribute to TSC are hexose sugar and sucrose. In rice, the hexose concentration is approximated by the sum of the glucose and fructose concentrations. About 84% of the TSC was attributed to sucrose while the difference was attributed to hexose sugar (Fig. 15).

Starch Levels

The starch concentration rank was not influenced by chemical treatment in the ratoon study experiments.

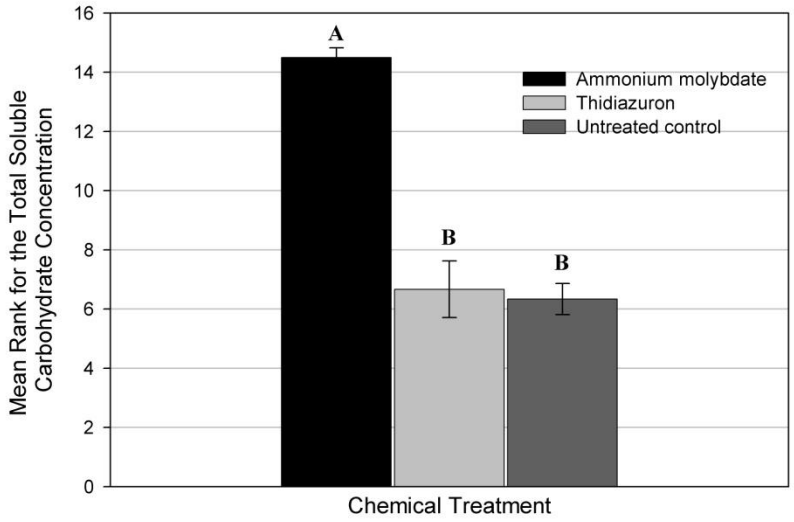

Figure 14. The mean rank for total soluble carbohydrate rank in the 2004 ratoon study field experiment. Total soluble carbohydrate is the sum of the glucose, fructose, and sucrose concentrations. Bars represent the standard error.

Agronomic Characteristics

The panicles m^{-2} of the ratoon crop in the 2004 and 2005 field studies were influenced by chemical application. The 2004 field experiment showed an increase in the panicles m^{-2} for the ammonium molybdate and thidiazuron treatment compared to the untreated control (p=0.01) (Fig. 15, top panel). However, in the 2005 field experiment, the response of panicles m^{-2} was opposite that of the 2004 field study. The untreated control had more panicles m^{-2} than the ammonium molybdate (p=0.02). The thidiazuron treatment was not different than either the untreated control or the ammonium molybdate treatments (Fig. 15, middle panel). The panicles m^{-2} of the ratoon crop in the 2005 greenhouse study was not affected by chemical application (Fig. 15, bottom panel).

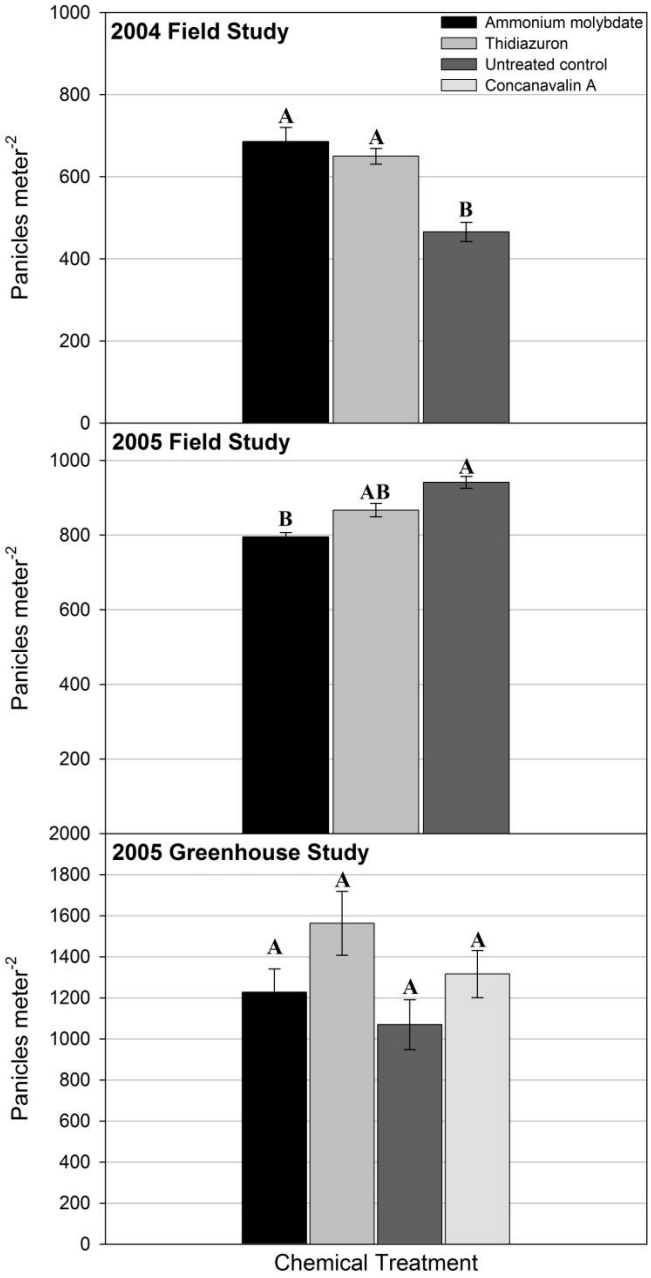

Figure 15. The panicles m^{-2} in the ratoon study experiments. Bars represent the standard error.

Analysis of grain yield in the 2005 field study showed that the rates and chemicals tested did not influence on main-crop yield, ratoon-crop yield, or the total grain yield at α=0.05 (Fig. 16).

The relative dry matter content was influenced in the 2005 field and greenhouse experiments by the chemical application when analyzed by the paired t-test of the median percent DW based on FW (p=0.01). The chemical treatments reduced the relative dry matter content compared to the untreated control (Fig. 17).

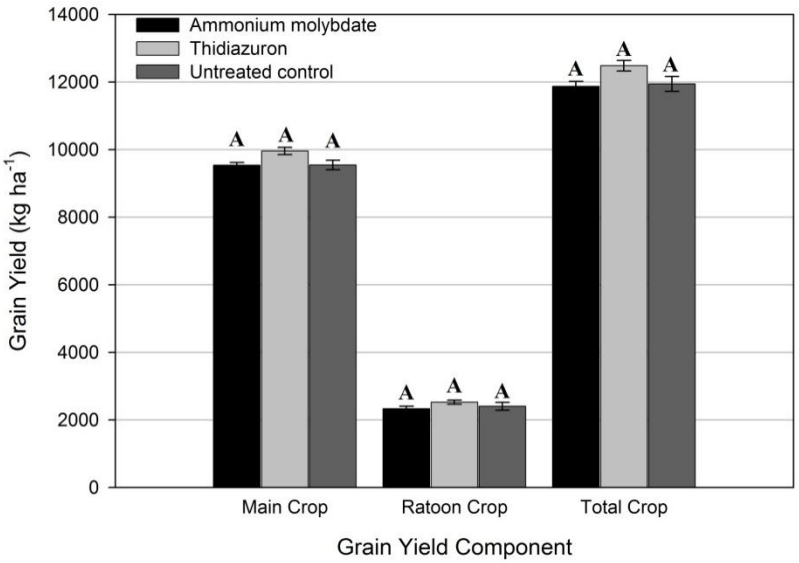

Figure 16. The grain yield from the 2005 ratoon study field experiment. Comparisons are for each yield category independently of the others. Bars represent the standard error.

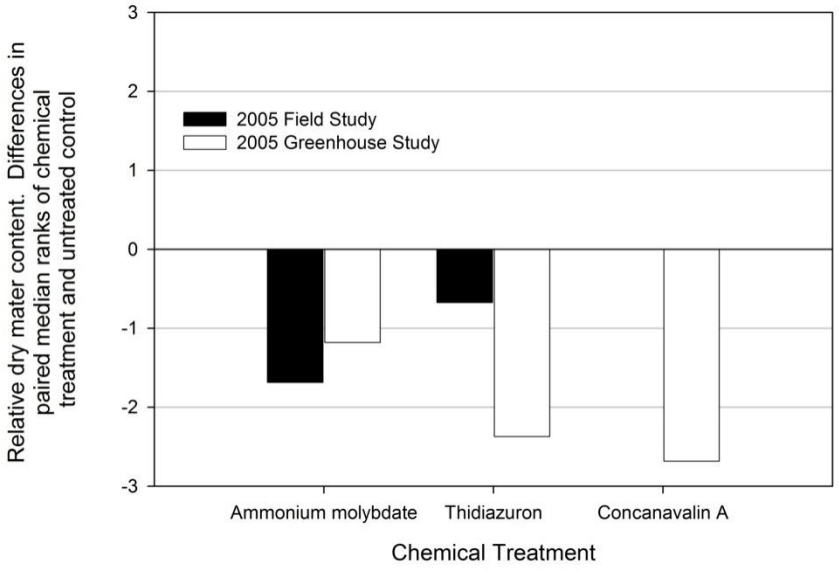

Figure 17. Relative dry matter content. Differences in paired median ranks of chemical treatment and untreated control. The relative dry matter content is the percentage of above-ground FW as DW.

Discussion

Invertase Activity

The activity of invertase in main crop stem segments during ratoon tiller bud expansion was not influenced by chemical treatment. These stem segments appear to be unaffected by exogenous manipulators of SAI activity at the tested application rates. The SAI activity in the main crop stem segments during ratoon tiller bud expansion was very low compared to pea seedlings (22 times lower) (Bruskova et al., 2004) and tulip (*Tulipa gesneriana* L.) internodes (8 times lower) (Lambrechts and Kollöffel, 1993). Both studies examined at young plants, up to 18d after germination when the activity of SAI is expected to be high due to early rapid growth. The SAI activity in the main crop stem segments in the 2004 field study was nearly identical to those previously reported in stem internodes of sorghum during grain development (Tarpley et al., 1994). However, the 2005 field and greenhouse studies had much higher SAI activity compared to sorghum (Tarpley et al., 1994). The ratoon tiller bud expansion period is the period in which ratoon tiller growth is started. The objective of this study was to examine the potential for the use of over-the-top chemical applications to manipulate the activity of invertase during this critical developmental period of tiller bud expansion and early growth as a possible technology to prevent invertase-associated stress effects on yield components. However, the invertase activity was not altered under the conditions of this study.

Soluble Carbohydrate Pool

The carbohydrate profile in the stem segments of the main crop during ratoon tiller bud initiation was influenced by chemical treatments. The concanavalin A treatment had a greater rank for fructose level than did the ammonium molybdate, thidiazuron, or untreated control treatment (Fig. 12). Concanavalin A is a lectin activator of hydrolase enzymes. Lectins have been shown to increase the activity of invertase in rice (Vattuone et al., 1991; and Isla et al., 1995) and concanavalin A increases the activity of acid phosphatase in rye (Ferens and Morawiecka, 1985). Concanavalin A and thidiazuron increase the activity of SAI and therefore the cleavage of sucrose. One possible result of increased SAI activity is the presence of higher hexose sugar in the affected tissue system. In this experiment, the fructose rank of the measured median was higher with the concanavalin A treatment but not the thidiazuron treatment. The lack of change in the fructose median rank due to thidiazuron could be that thidiazuron treatment affect processes other than SAI activity. Thidiazuron, hormone analog that belongs to the cytokinin family of hormones, is known to influence cell division, leaf expansion, shoot initiation, and growth of lateral buds, to name a few processes affected by cytokinin hormones (Mok and Mok, 2001; Taiz and Zeiger, 2002; Davis, 2004).

The transcript levels of mRNA for the genes of SAI have been reported to be increased with cytokinin application (Abdelghani et al., 1991; Ehneb and Roitsch, 1997). The mRNA levels were not measured in this study; however, there were no observable differences in the SAI activity. Alternatively, the lack of response observed in this study may have been due to a low application rate of thidiazuron (0.5 g a.i. ha^{-1}).

The concanavalin A results are tentative for the median fructose ranks because the concanavalin A was only used in the 2005 greenhouse study; thus, these results were not validated through an independent study. In the field experiments, fructose was not influenced by chemical application in 2004 and 2005. Further, the measured fructose levels in the 2005 field and greenhouse experiments were nearly all zero. In the 2005 field experiment, there were no readings greater than zero while only 7 readings in the 2005 greenhouse experiment had positive values. The fructose levels do not appear to significantly contribute to the total nonstructural carbohydrate concentration due to the low or near zero levels. Therefore, additional fructose discussion is omitted.

The sucrose concentration rank was influenced by chemical treatment (Fig. 12). The ammonium molybdate treatment increased the median rank compared to the untreated control or the thidiazuron treatments. This observation was further supported in the field studies where concanavalin A was omitted from the treatment list; the sucrose median rank was higher for the ammonium molybdate treatment compared to the untreated control or thidiazuron treatments (Fig. 13).

Ammonium molybdate is an inhibitor of SAI activity, so a possible outcome from application would be an observed level of sucrose higher than the untreated control or treatments that increase the activity of SAI. This was observed in both field studies and the greenhouse study. However, the measured activity of SAI does not support or contradict this observation.

In the 2004 field study, the TSC rank was higher in the ammonium molybdate treatment compared to the thidiazuron or untreated control treatments (Fig. 14). To further investigate this observation, the TSC components of glucose, fructose, hexose, and sucrose were analyzed to determine the relative amount of TSC as a particular soluble carbohydrate or group (e.g. hexose) (data not shown). Sucrose comprised a greater proportion of TSC than glucose or fructose with nearly 60% of the TSC content explained by the sucrose concentration. However, these ratios among sucrose, glucose, fructose (and hexose) were not influenced by chemical application.

Although, the ammonium molybdate treatment increased the TSC average rank (Fig. 14), it did not alter the ratios among the component sugars. Further the ammonium molybdate treatment did not affect the SAI activity, so the change in carbohydrate profile did not appear to be caused by a response of SAI activity influenced by chemical application.

In all the ratoon study experiments, sucrose was a greater proportion of TSC than the other sugars were (Fig. 18). Since the activity of SAI was not influenced, there may be another enzyme responsible for the increase in the sucrose proportion to TSC. The activities of sucrose synthase may have been reduced by the chemical treatments causing the larger proportion of sucrose to TSC. Further, the stem segments may have increased levels of sucrose-phosphate synthase (E.C. 2.4.1.14), which would potentially increase the amount of sucrose in the system. This experiment did not study other sucrose metabolic enzymes in the stem segments of the main crop during ratoon tiller bud expansion.

The starch levels and the TNC levels were not influenced by the application of chemicals that influence the activity of invertase. The TNC levels were found to be around 1% of the tissue DW.

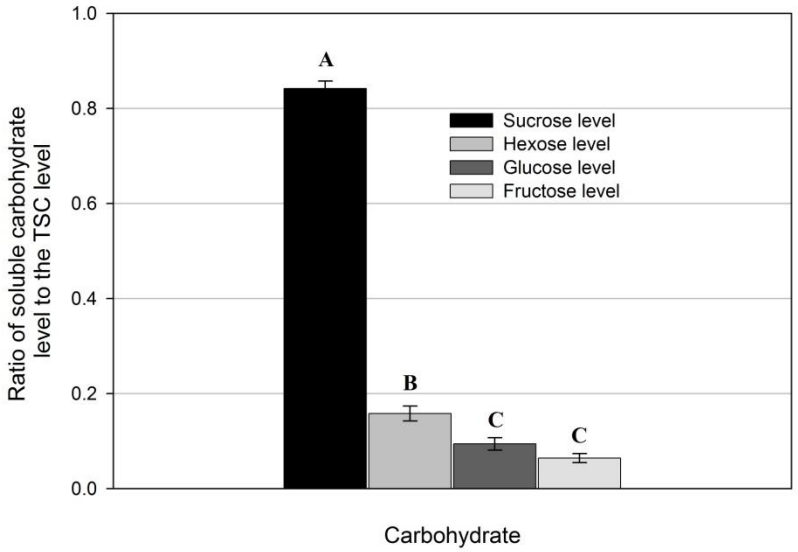

Figure 18. The proportion of TSC as sugar species for the combined ratoon study experiments. The hexose concentration is the sum of the glucose and fructose concentrations. Bars represent the standard error.

Agronomic Characters

The panicles m^{-2} was increased with each chemical application in the 2004 field study compared to the untreated control. The increase in ratoon tiller panicles m^{-2} was nearly 20% more than the untreated control. However, in the 2005 field study, the results were opposite. In the 2005 field experiment, the untreated control had greater panicles m^{-2} compared to the ammonium molybdate treatment. Further, in the 2005 greenhouse study, the panicles m^{-2} was not influenced by chemical treatment (Fig. 15). The differential response of panicles m^{-2} in the 2004 and 2005 field experiments is unclear. It was possibly caused by an interaction with environmental factors not measured in the

field experiments. The 2005 field study consistently had higher panicles m^{-2} compared to the 2004 field study.

In the 2005 field study, grain yield was collected for the main crop, ratoon crop and total crop. The chemical treatments had no effect on these grain yield components (Fig. 16). The grain yield studies should be replicated to determine if the variation in grain yield components are significant. Further, the ratoon study should include a rate study with the chemicals. The thidiazuron treatment was applied at 0.5 g a.i. ha^{-1} and therefore, in future studies the rate should be applied at rates of 0.5, 1.0, and 2.0 g a.i. ha^{-1}. The ammonium molybdate treatment was applied at 4.0 g a.i. ha^{-1} and the rates should be applied at 4.0, 8.0, and 16.0 g a.i. ha^{-1} in future experiments.

The absolute DM content was not affected by chemical treatment. However, the relative dry matter content was reduced due to chemical application in the 2005 field and greenhouse experiments when the chemical treatments were compared to the untreated. The paired t-test of the median relative dry matter content indicates that chemicals reduce the relative dry matter content (Fig. 17). It is uncertain what caused this reduction.

CHAPTER VII

SUMMARY

The purpose of this study was to determine if exogenous agrochemical application had the potential to prevent, or reduce, the negative impact of abiotic stress on grain yield by manipulating carbohydrate metabolism, and the potential flow of carbon to developing tissues of economic importance to rice (panicles, penultimate leaves, and main-crop stem segments during ratoon tiller expansion) under abiotic stress conditions. I looked at the concentration of sucrose, other carbohydrates, and SAI activity during the stress periods.

These experiments tested three exogenous chemicals which, according to the literature, can manipulate the activity of SAI during stress events and important developmental stages. These included thidiazuron, a hormonal activator of invertase activity, concanavalin A which is a chemical activator of invertase activity and ammonium molybdate, a chemical inhibitor of invertase activity.

The effects of the exogenous chemicals on yield and yield components were variable. Main-crop grain yield of the early grain fill study was reduced by exogenous chemical application. The yield from the plots receiving the thidiazuron treatment was lower than the untreated. Thidiazuron likely caused stress in the rice plants. Thidiazuron applications have been reported to cause mineral stress in geranium (Murch et al., 1997) and leaf senescence (Gan, 2004). However, the ratoon-crop grain yield and total grain

yield were not affected by the chemical treatments. This suggests the chemical effects are short-term in nature. The agrochemicals applied for the ratoon study did not have a measurable effect on grain yield. However, there was a chemical response with panicles m^{-2}, but the responses were not consistent across experiments and no meaningful conclusions can be drawn.

The methods used in this study appear to be adequate to detect differences among the chemical and environmental treatments if present. For example, the enzyme-coupled method of starch determination utilized in this experiment was able to detect changes in the starch concentration in the penultimate leaf and panicle of the early grain fill and the heat stress studies. The change in the starch concentration was detectable during the sample date duration of the early grain fill study. It was found that the 3 dat starch concentration in the leaf was higher than the 0 and 1 dat. The ability to detect minor changes in starch concentration of the leaf supports this method for starch assays. In addition, the enzyme-coupled method of SAI activity determination was appropriate for rice penultimate leaves and panicles of the early grain fill study and the heat stress study. However, the activity of SAI in stem segments of developing ratoon tiller buds (ratoon study) did not show effects by chemical treatment. This may be due to two possible reasons: 1) the rates tested were inadequate to alter the SAI activity in these tissues, or 2) the detection level of the assay was inadequate to precisely quantify minor differences in SAI activity during ratoon tiller expansion. It is likely that the agrochemical rates were

the reason for lack of SAI activity in the stem segments because the method worked in the penultimate leaves and panicles.

The SAI activity in the penultimate leaf samples of the early grain fill study and the heat stress study responded as expected, based on published results. The SAI activity in the leaf of the ANT treatment was higher with the activators of invertase, i.e. concanavalin A and thidiazuron. Further, the SAI activity was reduced, or lower with the agrochemical that lowers invertase activity, i.e. the ammonium molybdate treatment.

The glucose concentration rank of the leaf samples in the thidiazuron treatment of the early grain fill study was higher than in the ammonium molybdate treatment. This observation suggests that SAI activity may be enhanced by the thidiazuron treatment thus leading to increased sucrose cleavage and higher hexose sugars in the system. However, there were no observable differences in SAI activity or fructose concentration in the thidiazuron treatment.

The application of agrochemicals to rice plants exposed to 30°C nighttime temperatures for a 4 d duration were observed to have an interaction effect on selected biochemical variables in the panicle. The ammonium molybdate treatment in conjunction with HNT reduced the ranked difference for SAI, TSC, Starch, and TNC compared to the ammonium molybdate in the ANT treatment. However, in the penultimate leaf, the ranked difference of SAI activity was increased by HNT compared to the ammonium

molybdate in the ANT treatment. These results indicate that there are physiological responses of rice plants under various stresses that are related to the role in the source/sink relationship. The data suggests that leaves are exporting carbohydrates (source tissue) to the panicle (sink tissue). The utilization of the carbohydrates can be manipulated by exogenous application of chemicals in all tested developmental stages and stress events of this experiment.

These experiments did not determine if the carbohydrate transport mechanism was influenced by the chemical application. However, the results suggest that the tested agrochemicals and rates do influence the transport of carbohydrates from source tissues to sink tissues because sugar compositions and invertase activities were manipulated in the sink tissues. This hypothesis could be tested using radiolabeled sucrose and tracking its concentration throughout important organs and sugars (e.g. Tarpley and Vietor, 2007).

The lack of consistent SAI activity in response to the exogenous applications of agrochemicals could likely be due to the chemical responses affecting sugars that regulate the activity of invertase, i.e. glucose and fructose. Glucose and fructose levels have inhibitory effects on invertase activity. The application of chemicals that manipulate the SAI activity may indirectly affect SAI activity. An increase in the SAI activity by exogenous chemical application which increases sucrose cleavage also increases the concentration of inhibitory carbohydrates (glucose and fructose) and

further lowers SAI activity. Because multiple factors influence invertase activity in the plant, sugar concentrations or other variables might be at least as useful for determining the effects of these exogenous chemicals.

The hypothesis of the thesis study is difficult to test or predict in whole plant systems when cells, organs, and tissues remain intact during the chemical treatment. The literature proposed that these agrochemicals could affect invertase activity. Those experiments have primarily been done in the laboratory, under strict environmental conditions which maximized the invertase response to chemical treatments. This study took those results and attempted to replicate them under field conditions using the whole plant systems under normal rice growing conditions and management practices. These experiments were able to mimic some of the reported response, but not all. This observation supports that field grown rice plants are dynamic systems which can buffer against environmental stresses through many physiological processes. Those physiological processes that buffer the system are not present during protein and cell experiments as they are in whole plant response experiments because important tissues for signal transduction are not present.

Abiotic stresses can severely impact yield of the cereal crops, including rice. The activity of invertase, one of the key sucrose metabolizing enzymes, has been implicated in some abiotic stress effects. Studies have also shown that disruption of the carbohydrate flow to the sink tissues is often involved in yield loss due to abiotic stress.

The study tested the concept that exogenous chemicals known to positively or negatively affect invertase activity could be exogenously applied to maintain invertase within a window of activity for a short period during a stress event, might have potential as plant growth regulators as preventatives. The results were inconclusive, probably due to the complexity of the plant system. A number of the results were, however, encouraging. Given the huge magnitude of the effects of abiotic stresses on rice crop yield, additional research is warranted.

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