Cool Temperature Effects on Productivity and Photosynthesis of Two Biomass Fuel Species: Switchgrass (*Panicum virgatum*) and Miscanthus (*Miscanthus* × *giganteus*)

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ABSTRACT

The world's highest yielding crops are C_4 plants due to their higher water use efficiency, nitrogen use efficiency, and productivity compared with C_3 plants. With an increasing demand for renewable resources as a result of the decreasing global supplies of fossil fuels, we need to improve our understanding of the limitations of biomass fuel feedstock to improve yields and better satisfy energy requirements. The ability to attain the goal feedstock production in the US is limited by available arable land and cool temperatures. This study investigates the effects of cool temperatures on the productivity and photosynthesis of the two species with the highest potential for feedstock production in the US: switchgrass (*Panicum virgatum*) cv. Alamo and miscanthus (*Miscanthus × giganteus*). At 14/12°C and a 14/10 hour light/dark photoperiod, switchgrass showed lower productivity and light saturated photosynthetic rates (A_{max}=10.3 µmol $m^{-2}s^{-1}$) compared with 28/25°C and the same photoperiod (A_{max}=18.8 µmol $m^{-2}s^{-1}$). Miscanthus has demonstrated cold tolerance in previous studies, and here showed no significant decrease in the productivity or photosynthetic rates in cool, compared with warm, growing conditions (A_{max}=8.2 µmol $m^{-2}s^{-1}$ and 7.0 µmol $m^{-2}s^{-1}$ for warm and cool conditions, respectively). Also, this study examines the potential limitations of C_4 photosynthesis by the enzyme pyruvate phosphate dikinase (PPDK) under the same cool conditions, transgenic switchgrass cv. Alamo were created with the insertion of the miscanthus PPDK gene. Productivity and photosynthetic

responses of the transgenic plants were evaluated in cool and warm growth temperatures. Of the two transgenic events tested here, line S(1) displayed cold tolerance, as seen in no loss of both carboxylation efficiency and the ratio of CO_2 assimilation to electron transport (A_{sat}/J_{max}). These results indicate that PPDK may pose a significant limitation to C_4 photosynthesis in cool conditions and there is a possibility that cold season photosynthesis of switchgrass cv. Alamo could be improved.

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Introduction

Limitations to Biomass Fuel production by C_4 plants in the US

The C_4 photosynthetic pathway concentrates CO_2 around ribulose bisphosphate carboxylase oxygenase (Rubisco) in the bundle sheath cells; here O_2 concentration is considerably lower and photorespiration is essentially eliminated. This concentration of CO_2 in bundle sheath cells is achieved by the initial capture of CO_2 by phosphoenol pyruvate carboxylase (PEPc) in the mesophyll cells and transporting it via a 4-carbon molecule into the bundle sheath cells (Figure 1). This photosynthetic pathway offers higher nitrogen use efficiency (NUE) and water use efficiency (WUE) than the C_3 photosynthetic pathway by reducing the amount of Rubisco, and therefore nitrogen, and gas exchange, respectively, required for photosynthesis (Long 1999). These adaptations have given a significant advantage to C_4 species in warm arid environments; however, C_4 photosynthesis has been shown to typically decrease significantly at lower temperatures (Long 1999). Temperature effects on Rubisco activity, phosphoenol pyruvate (PEP) regeneration via pyruvate phosphate dikinase (PPDK), and PEPc efficiency have been associated with a decrease in light saturated carbon assimilation at cold temperatures in plants with C_4 photosynthesis (Pittermann and Sage 2000).

Several of the most productive agricultural crops are C_4 species, some of which are used as feedstock for biomass fuel production. In fact, maize (a C_4 grass) is currently the leading biomass fuel crop in the U.S. The arable land for maize production is restricted to areas of relatively high growth temperature, because maize productivity is inhibited by cool temperatures early and late in the growing season (Naidu, Moose et al.

2003). If all the maize produced in the U.S. was used for biomass fuel production, only 12% of the gasoline requirement for the U.S. would be satisfied (Hill, Nelson et al. 2006). If the biomass fuel needs of the U.S. are to be met, an increase in the cold tolerance of maize or other biomass fuel species that are not inhibited by cold need to be identified, so that a larger area of appropriate crop land would be available for biomass fuel production.

Several other C_4 species have become leading candidates for feedstock in the United States, namely switchgrass (*Panicum virgatum* L.) and miscanthus (*Miscanthus* x *giganteus*). Both species exhibit the desired characteristics of C_4 photosynthesis, high WUE, NUE and productivity. Switchgrass has been the prime candidate, and most thoroughly studied in the United States for use as a feedstock for biomass fuel production (Sanderson, Reed et al. 1996; McLaughlin and Adams Kszos 2005; Gesch and Johnson 2010), while miscanthus has been the most studied potential feedstock in Europe (Beale and Long 1995; Lewandowski, Clifton-Brown et al. 2000).

Switchgrass is native to the United States, east of the Rocky Mountains with ecotypes adapted to the different climates and stress associated throughout this range (Casler, Vogel et al. 2007); this gives it an advantage over some other potential feedstock fuels species which are not native to the U.S. and may have the potential to become invasive. Also, the inherent properties of C_4 plants will allow switchgrass to be cultivated on marginal lands. This tolerance of marginal land is critical because it reduces the amount of agricultural land needed for feedstock, decreasing the impact of biomass fuel production on food production. Switchgrass has been subjected to long-term analysis of varietal performance and management practices to maximize production (Sanderson, Reed et al. 1996; McLaughlin and Adams Kszos 2005; Schmer, Vogel et al.

2008). These studies have yielded region specific varieties, harvesting strategies and fertilization methods, which have significantly increased production (McLaughlin and Adams Kszos 2005). A comparison of four leading switchgrass cultivars (two lowland and two upland) has shown higher light saturated photosynthetic rates (A_{max}) rates in lowland ecotypes than the upland in warm conditions; however, when transferred to cold conditions one upland cultivar (Cave-in-Rock) showed less A_{sat} inhibition (measurements made at growth temperature) (Gesch and Johnson 2010).

Miscanthus is an exception among C_4 plants because there is little to no difference in photosynthesis when grown at 14°C and 25°C and measured over a temperature range of 5-38°C (Naidu, Moose et al. 2003). Also, when miscanthus was grown at 14 and 25°C, and photosynthesis was measured at 10 and 20°C for each treatment, there was little difference in A_{max} between growth temperatures (Farage, Blowers et al. 2006). This could indicate that the temperature at which the measurements are made may have a stronger influence on photosynthetic rates than the growth temperature. Miscanthus has been thoroughly studied as the primary candidate for feedstock in Europe (Beale and Long 1995; Beale, Bint et al. 1996; Venturi, Huisman et al. 1998). Those studies have shown that miscanthus is able to produce more leaves in the beginning of the growing season and maintain them later in comparison to other C_4 grasses. Dohleman and Long (2009) found that producing more leaves earlier in the year extended the growing season by up to 60%, without the use of fertilizer, in comparison with fertilized maize. Miscanthus displayed significantly higher productivity than switchgrass in side-by-side field trials in Illinois, possibly because it was able to sustain a higher leaf area index (LAI) than switchgrass from late May through senescence (Heaton, Dohleman et al.

2008). In addition, miscanthus is able to conserve the majority of its nitrogen by translocating it belowground before senescence (Beale and Long 1997). Therefore, it is important to time the harvest of miscanthus after the nutrients have been translocated in order to minimize nutrient supplements the following season. However, there are restrictions on using miscanthus as a feedstock species, because miscanthus reproduces vegetatively, consisting of a single clone, which increases the cost of initial establishment and susceptibility to disease. Conversely, vegetative reproduction can be an advantage because genetically modified lines would not be transmitted outside the cultivated land via pollen or seeds. Heaton, Voigt et al. (2004) reviewed the prior literature showing that: both miscanthus and switchgrass show a significant positive response to both water and nitrogen, with miscanthus responding more to water and switchgrass more to N. Finally, while miscanthus does show higher rates of productivity compared with switchgrass, drought presents a significant limitation on the high rates of productivity seen in miscanthus (Clifton-Brown, Lewandowski et al. 2002).

Mechanisms of low temperature inhibition

There is an increasing need for biomass fuel feedstock in the United States which maize alone cannot fulfill. Other potential feedstock species must be developed in order to meet these needs; however, the colder temperatures at the beginning and end of the growing season inhibit production and photosynthesis in switchgrass and most other C_4 species over a large portion of arable land in the U.S. Therefore, it is important to identify and understand the mechanisms by which cool temperatures inhibit C_4

photosynthesis and productivity. Several possible mechanisms may lead to this cool air temperature induced decrease in C_4 photosynthesis.

There may be an inherent disadvantage to C_4 photosynthesis at cold temperatures due to its anatomy. It has been suggested that the anatomical restriction of Rubisco to the bundle sheath cells limits its potential concentration, thereby imposing a limit on the maximum rate of assimilation (Pittermann and Sage 2000). It has been shown in several C_4 species, but not miscanthus, that the catalytic efficiency (k_{cat}) of Rubisco decreases at lower temperatures (Sage 2002). The activation energy of Rubisco has been linked to decreased photosynthetic rates at low temperatures. It has been demonstrated that the activation energy required for photosynthesis and Rubisco are similar, *in vitro*, showing control over photosynthesis; although, control has also been shown to be shared with PPDK, at temperatures below 17°C in *Bouteloua gracilis* (Pittermann and Sage 2000) and 22°C in *Muhlenbergia montana* (Pittermann and Sage 2001). Furbank, Chitty et al. (1997) showed that Rubisco and PPDK both impose limitations on the photosynthetic rate under saturating light conditions, but Rubisco exhibited the majority of the control (70%). At lower temperatures, the control Rubisco exerts on photosynthesis can be seen by increased CO_2 leakage from the bundle sheath cells and by the similarity between the *in vitro* activity of Rubisco and the *in vivo* CO_2 assimilation rate (Kubien and Sage 2004).

PPDK may also limit photosynthetic rates in C_4 plants (Figure 1). Maize exhibited a 57% reduction in PPDK levels under 14°C growing conditions (Naidu, Moose et al. 2003), and approximately 70% reduction after 14 days of 14°C treatment after being transferred from 25°C (Wang, Portis et al. 2008). The activity of PPDK *in vitro* for switchgrass is similar to the observed rates of photosynthesis in maize (Edwards,

Nakamoto et al. 1985; Usuda, Ku et al. 1985). These results suggest that PPDK is a rate limiting step in the C_4 photosynthetic pathway. Miscanthus, which has unusually high cold tolerance for a C_4 plant, has displayed an increase of 2.1 times the PPDK content after fourteen days of 14°C treatment (Wang, Portis et al. 2008). Also, Nogueira, De Rosa et al. (2003) showed that the production of PPDK increases in low temperature stress in sugarcane (*Saccharum* spp.). Du, Nose et al. (1999), also using sugarcane, demonstrated an increase in PPDK for cold tolerant species and a decrease for cold sensitive species. Thus, there is a general pattern that PPDK activity can regulate C_4 photosynthetic response to cool temperature.

As a result of the potential for PPDK's limiting role in C_4 photosynthesis, the effect of overexpressing this gene has shown improvements in the photosynthetic rates, which have even been reflected by increased productivity. Ohta, Ishida et al. (2006), using the *Flaveria brownii*, a cold tolerant C_4 species, PPDK gene as a model, introduced point mutations into the maize PPDK gene to mimic the C-terminus of the *F. brownii* PPDK gene. The transformed maize demonstrated cold tolerance of PPDK similar to that of *F. brownii*, and increased photosynthetic rates of 23% on average over the non-transformed line (Ohta, Ishida et al. 2006). Using the Ohta, Ishida et al. (2006) results as a guideline, it is reasonable to hypothesize that inserting or mimicking the miscanthus PPDK gene in switchgrass will increase cold tolerance, which may result in increased productivity of switchgrass when grown under cool conditions.

Objectives

C_4 plants have been selected as the leading potential feedstock species for biomass fuel production due to their relatively high rates of productivity, water use efficiency and nitrogen use efficiency; however, the productivity and fitness of C_4 plants is typically inhibited by cool growing conditions. This research program will concentrate on the mechanisms inhibiting photosynthesis in switchgrass at cool temperature growing conditions. The PEP regeneration rate, which is dependent on the amount of PPDK, PEP and pyruvate, offers an explanation of the reduced photosynthetic rates of switchgrass at cool temperatures. Also, the fact that Rubisco is located strictly in the bundle sheath cells of C_4 plants and its activity is reduced at cold temperatures brings on inherent limitations to the rate of photosynthesis by limiting the total content of Rubisco in the plant. This research aims to assess the effects of cold growing conditions on C_4 plants by:

- Determining any inherent differences between the photosynthetic traits of switchgrass, and miscanthus at each temperature treatment, such as the maximum PEP carboxylation rate (V_{cmax}), dark respiration rate (R_d), and electron transport rate (ETR).
- Measure the same photosynthetic properties on transgenic switchgrass, with the miscanthus PPDK gene inserted, to determine if PPDK is a major limiting factor of C_4 photosynthesis and if this limitation can be overcome.
- Analyze the aboveground biomass under each treatment to determine the rate of growth for each species, and possibly realize an increase in production at cool temperatures with the overexpressed miscanthus PPDK in switchgrass.

Effects of Low Temperature on Switchgrass and Miscanthus

Introduction

The decreasing supply of fossils fuels, which is relied upon by most of the world, necessitates our shift toward renewable resources to satisfy our increasing demand. The United States (US) and the European Union have employed policies which order these nations to produce 16 billion gallons of cellulosic ethanol and comprise 10% of all transport fuel, for the US and European nations respectively (Robertson, Dale et al. 2008). Currently, the major source for U.S. ethanol production is maize, which is restricted to a warm climate and requires considerable inputs in the form of fertilizers, pesticides and annual establishment.

C_4 feedstock plants, like maize, have developed an efficient photosynthetic pathway by concentrating CO_2 in bundle sheath cells around ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). This concentration mechanism reduces the nitrogen requirement and duration that stomata are open to give these species a higher nitrogen and water use efficiency than their C_3 counterparts (Long 1999). Also, these adaptations allow C_4 plants to achieve high production rates on a wider diversity of arable land. Utilizing these marginal lands for feedstock production will be essential for controlling the effect of biofuel production on food crop production. These adaptive traits give C_4 species a significant advantage in warm arid climates; however, C_4 photosynthesis typically decreases under cooler conditions (Long 1999). Enhancing productivity of C_4 plants on marginal lands in cooler climates will greatly improve the potential of these species to produce feedstock for biomass fuel production.

Two perennial C_4 grasses, *Panicum virgatum* L. (switchgrass) and *Miscanthus* × *giganteus* Greef et Deu ex. Hodkinson et Renvoize (hereafter referred to as miscanthus) have

recently proven to be the leading candidates to produce feedstock for biomass fuel production in the US and Europe due to their high yields and low input requirements, compared with crops such as maize (Lewandowski, Clifton-Brown et al. 2000; Heaton, Voigt et al. 2004; McLaughlin and Adams Kszos 2005).

Switchgrass is native to the US east of the Rocky Mountains and has developed upland and lowland ecotypes, of which the Alamo cultivar has shown to be overall the most productive of either ecotype. (Sanderson, Reed et al. 1996; McLaughlin and Adams Kszos 2005). While Alamo has shown overall to be the most productive, studies repeatedly show that other cultivars may be more site-specifically adapted, possibly due to different environmental stresses. A variety of cultivars should be screened for optimal performance at each location (Wullschleger, Sanderson et al. 1996; McLaughlin and Adams Kszos 2005; Fike, Parrish et al. 2006; Casler, Vogel et al. 2007). One of the major limitations to the performance and production of switchgrass, and many other C_4 species, is the inhibition of photosynthesis at cool temperatures (Gesch and Johnson 2010).

This restriction of photosynthesis at cool temperatures has been overcome by some C_4 species which have adapted to higher elevation or latitudinal habitats where temperatures can be substantially cooler (Beale, Bint et al. 1996; Du, Nose et al. 1999; Pittermann and Sage 2001), especially early and late in the growing season. Most notably among these exceptions is miscanthus, a sterile hybrid of *Miscanthus sinensis* Anderson and *Miscanthus sacchariflorus* (Maxim.) Franch., which demonstrates little to no differences in efficiencies of photosynthesis at cool (12-14°C) or warm conditions (Naidu, Moose et al. 2003; Farage, Blowers et al. 2006). The ability to maintain high photosynthetic rates allows miscanthus to produce high levels of biomass in cool climates, by extending its growing season earlier and later in the year as tested in

comparison with maize, yielding a 59% longer growing season and approximately 50% more biomass produced (Dohleman and Long 2009). A comparison of miscanthus and maize has revealed that one possible mechanism miscanthus employs to adapt to cold conditions is by increasing the levels of pyruvate phosphate dikinase (PPDK) at cold temperatures, which could imply a C_4 limitation in the carboxylation efficiency in cool conditions (Naidu, Moose et al. 2003; Wang, Portis et al. 2008). Also, these advantages have been shown in studies comparing miscanthus to switchgrass in field trials; in Illinois miscanthus was able to produce significantly higher amounts of biomass, possibly due to a higher leaf area index than switchgrass from May through senescence (Heaton, Dohleman et al. 2008). This study compared miscanthus to the Cave-In-Rock switchgrass cultivar, which has been shown to be more cold tolerant (and appropriate for Illinois trials) but less productive than the Alamo cultivar at many test sites (Lemus, Brummer et al. 2002; McLaughlin and Adams Kszos 2005).

The need for a better understanding of the mechanisms by which cold temperatures inhibit photosynthesis and productivity of switchgrass is imperative to developing this species as a source of feedstock for biomass fuel production. Also, understanding the adaptations of miscanthus to cool climatic conditions will yield insight into the potential for C_4 species to thrive in cool growing conditions. Therefore, this study will explore the following hypotheses:

- Productivity will significantly decrease in switchgrass and show no significant decrease in miscanthus when grown at 14/12°C compared to 28/25°C.
- Miscanthus will not show cold temperature inhibition of photosynthesis when grown and measured at 14/12°C compared with 28/25°C; while there will be a significant reduction in switchgrass.

- These cold temperature limitations will lie in the initial portion of the CO_2 response curve, where PPDK (PEPcase regeneration) play a limiting role, and will be interpreted by the carboxylation efficiency.

Methods

Plant Materials

Ten individuals of both switchgrass (*Panicum virgatum* L., Alamo cultivar) and Miscanthus (*Miscanthus* × *giganteus*) were received from the University of Tennessee, Department of Plant Sciences in July 2010. These plants were provided 3g of four month slow-release fertilizer (Osmocote 19-6-12) and maintained in their original pots in the BIOL/VBI Plant Growth Facility at Virginia Tech. Each species was propagated by dividing individuals and replanting in one gallon pots with Pro-Mix BX (Premier Tech Horticulture, Quebec, Canada) to achieve the desired sample size. Transplants were fertilized and grown in the greenhouse for two weeks before being transferred into growth chambers (E8 and CMP4030, CONVIRON, Winnipeg, Canada). All plants were watered every other daily to avoid a water limitation. Of four total growth chambers, two were set to warm (28/24°C; day/night) and two to cool (14/12°C), all with a 14 hour light/10 hour dark cycle and roughly 200 μmol m^{-2} s^{-1} of photosynthetically active radiation (PAR). Eight individuals of switchgrass were acclimated in each of the growth chambers for two weeks. After equilibration in growth chambers each individual plant was cut back to five remaining shoots. Once all measurements were made on switchgrass plants, the same methods were used in screening miscanthus.

Productivity Measurements

Newly formed shoots were counted once a week throughout the experiment. Once all gas exchange and chlorophyll fluorescence measurements were taken the shoots produced in the growth chambers were harvested and dried to a consistent weight. Final weights per shoot were used with weekly shoot counts to calculate an average dry weight per plant for each individual and used as an indication of temporal changes in aboveground biomass during the experiment.

Gas Exchange & Chlorophyll Fluorescence

All measurements were made on the third or fourth leaf of newly formed shoots using an open path gas-exchange system (LI-6400; Li-Cor Inc., Nebraska, USA) with relative humidity between 55-75% and leaf temperature maintained at the respective growth temperature. Leaves were allowed to acclimate to cuvette conditions for five minutes prior to initiating photosynthetic response programs. Initially, the photosynthetic response to light (A/Q curve) (with light levels of 2000, 1500, 1000, 800, 500, 300, 200, 100, 80, 50, 20, 10, 0 PAR) at 400 μmol/mol CO_2 was measured on at least 4 individuals from each growth chamber to determine the light saturation points for species and temperature treatments, calculated using photosynthetic response curve fitting software (Li-Cor Inc., Nebraska, USA). The photosynthetic response to C_i (A/C_i curve) (with CO_2 levels of 400, 300, 200, 100, 50, 30, 400, 400, 600, 800, 1000, 1200, 1400, 1600 μmol/mol CO_2) was then measured on all eight plants per chamber at saturating light, which varied depending on species and growth temperature (about 1000 PAR for 14°C and 1500-2000

PAR for 28°C conditions). Gas exchange results were calculated using PhotosynAssitant (Dundee Scientific Ltd, Scotland, UK).

To determine the response of photoinhibition (F_v/F_m), electron transport rate (ETR), and nonphotochemical quenching (qN) to the growth conditions, chlorophyll fluorescence was measured on three dark-adapted leaves similar to those used for gas exchange measurements from all individuals. Leaves were dark-adapted for at least 10 minutes prior to measurement using a pulse-modulated fluorometer (OS-500, Opti-Sciences, New Hampshire, US)

Statistical Analysis

We combined the observations within growth chamber of productivity, A/Q, and A/C_i response curve variables, yielding results of n=2 for each temperature treatment. These results were then analyzed using a Wilcoxon rank sum test (α=0.05) (JMP Pro 10, SAS Institute, Cary, NC, USA).

Results

Productivity

Switchgrass developed leaves, notably the third mature leaf, more quickly than miscanthus, and therefore chlorophyll fluorescence and gas exchange measurements were able to be taken earlier in the study and resulted in a much shorter experiment for switchgrass (Figure 2.1). Over a period of 102 days, switchgrass produced 23.46 g aboveground biomass per plant in

the warm treatment (28/25°C), 22.5 shoots individual^{-1}, and 10.94g plant^{-1} in the cool treatment (14/12°C), and an average of 13.8 shoots individual^{-1}, a decrease in biomass of about 53% at lower temperatures. Miscanthus showed a warm treatment aboveground biomass per plant of 5.60g, with 5.6 shoots individual^{-1}, and a cool treatment biomass of 1.65 g, for 1.7 shoots individual^{-1}, over the 193 day experiment, a 70% decrease in the cool conditions.

Chlorophyll Fluorescence

Each species, for both treatment groups, showed some degree of photoinhibition, as indicated by F_v/F_m, (Table 2.1), however, the level of photoinhibition in switchgrass was lower in the cool treatment group compared with the warm and miscanthus showed lower photoinhibition in the warm treatment group. A similar relationship was observed in the electron transport rates (ETR) of each species with switchgrass showing higher rates under warm conditions and miscanthus demonstrating higher rates in the cool growing condition. There were no differences observed in the nonphotochemical quenching (qN) for either species between growing temperatures. Both species showed higher quanta yields from photosystem II (PSII) in the cool than the warm growing conditions.

Gas Exchange

For switchgrass grown under cool conditions, the light saturation point was 964.37±82.09 µmol $m^{-2}s^{-1}$ and 1061.72±196.42 µmol $m^{-2}s^{-1}$ in the warm treatment group. Miscanthus showed lower overall light saturation points of 554.13±31.13 µmol $m^{-2}s^{-1}$ at 14/12°C and 472.04±7.38

μmol $m^{-2}s^{-1}$ at 28/25°C. The light compensation points measured are typical for C_4 plants, 10-15 μmol $m^{-2}s^{-1}$ light and did not differ between temperature treatments of either species. Also, there was no difference between the apparent quantum efficiencies (AQE) of switchgrass or miscanthus between treatment groups. Based on the data in Table 2.2 and the need to minimize photoinhibition, the switchgrass CO_2 response curves were conducted at 2000 μmol $m^{-2}s^{-1}$ and 1500 μmol $m^{-2}s^{-1}$ for the warm and cool treatments, respectively; the miscanthus CO_2 response curves were performed at 1200 μmol $m^{-2}s^{-1}$ in both the warm and cool conditions.

When measured at 200 μmol $m^{-2}s^{-1}$ light, the level of light in the growth chambers, switchgrass photosynthetic rates were inhibited in the cool conditions; 10.0 μmol $m^{-2}s^{-1}$ and 5.8 μmol $m^{-2}s^{-1}$ at 14/12°C and 28/25°C respectively. However, miscanthus photosynthetic rates were similar between temperature treatments at 200 μmol m^{-2} s^{-1} light: 5.9 μmol $m^{-2}s^{-1}$ and 5.0 μmol $m^{-2}s^{-1}$ at 14/12°C and 28/25°C respectively.

The CO_2 response curves showed inhibition of photosynthesis for switchgrass under cool growing conditions, but not for miscanthus (Table 2.3). Switchgrass' CO_2 saturated assimilation rates (A_{sat}) decreased from 25.7 μmol $m^{-2}s^{-1}$ at 28°C to 12.6 μmol $m^{-2}s^{-1}$ at 14°C, and there was a corresponding reduction in the carboxylation efficiency (CE), triose phosphate use (TPU), and the maximum carboxylation rates of Rubisco (V_{cmax}). Also, higher respiration rates were observed in the warm treatment, as expected with higher CO_2 assimilation rates. However, there was no observed difference in the maximum electron transport rates (J_{max}) measured for switchgrass in the two temperature treatments. In contrast, photosynthetic parameters for miscanthus growing in the two temperature regimes were not substantially reduced in the cold, except for V_{cmax} and J_{max}. A_{sat}/J_{max} shows the rate of CO_2 fixation via the C_4 pathway per

electron produced by PSII; switchgrass showed 50% decrease in A_{sat}/J_{max} in the cool temperature treatment, while miscanthus demonstrated no difference between warm and cool conditions.

Discussion

Productivity

While there was a substantial difference in the productivity of switchgrass and miscanthus (Figure 2.1), miscanthus did not show inhibition of photosynthetic potential due to the cool growing conditions (Table 2.3). The lower light saturation points of miscanthus in both the warm and cool treatment groups indicates an acclimation to the low light of the growth chambers, which was not observed in either switchgrass treatment. The light saturation points and A_{max} of cool grown miscanthus presented here are similar to those of previous studies (Farage, Blowers et al. 2006), for miscanthus grown at 14°C and measured at 10°C. However, Farage, Blowers et al. (2006) showed much higher light saturation points and A_{max} for miscanthus grown and measured at 25°C than the data presented here for the same parameters at 28°C, possibly due to the light intensity at plant height of 700 µmol $m^{-2}s^{-1}$ compared with 200 µmol $m^{-2}s^{-1}$ used in our experiment. The low light saturation point of miscanthus at 28°C in this study is not a product of photoinhibition of PSII as indicated by the F_v/F_m values, which are similar to those of switchgrass which showed higher light saturation points. These low light saturation points and A_{max} are attributed to the significantly lower photon flux density of the growth chambers compared with field conditions. This may explain the discrepancy between the results of this study and that of Farage, Blowers et al. (2006).

A recent field study, comparing miscanthus and switchgrass cv. Cave-in-Rock, showed that miscanthus was able to produce significantly more biomass over a growing season and develop a closed canopy earlier and intercept more light over the entire growing season than switchgrass (Heaton *et al*. 2008). This was not found in the present study where switchgrass was able to develop leaves and obtain a higher biomass earlier than miscanthus in both temperature conditions. Switchgrass reached 90% of the total biomass produced in the study in about 20 days at the warm temperature and in about 80 days for the cool treatment, whereas miscanthus reached 90% total biomass in about 160 days at both temperatures treatments. Therefore, in our study, switchgrass was much more affected by temperature treatment than that of miscanthus. This supports the reduced or lack of low temperature inhibition on photosynthesis and productivity for miscanthus. However, the generally slower productivity could be a result of miscanthus' acclimation to the low light of the growth chambers. Switchgrass, according to the results presented here, had a much higher rate of productivity in both the warm and cool treatments compared with miscanthus. Yet, in other research, field grown miscanthus can obtain significantly higher rates of productivity than switchgrass (Heaton, Dohleman et al. 2008; Dohleman and Long 2009). These conflicting results between field grown and growth chamber plants may be attributed to the low light intensity of the growth chambers. It is also possible that miscanthus allocates a higher proportion of biomass below-ground (when grown at low light intensity than does switchgrass), which Beale and Long (1995) showed to be about 40% of its biomass.

Chlorophyll Fluorescence

While switchgrass experienced higher levels of photoinhibition in the warm treatment group and miscanthus in the cool treatment group, these differences were small and may not reflect biological differences. These data may be a result of a limited amount of water stress and relatively low light conditions experienced by each species. Nevertheless, these results indicate that both species exhibit minimal differences in photoinhibition due to growing temperature under these growth chamber conditions.

The higher ETR of switchgrass in the warm treatment group is concurrent with the higher A_{sat} and A_{max} rates observed in this study, as with higher rates of CO_2 assimilation, higher rates of electron turnover are required. Miscanthus showed lower ETR in the warm treatment group compared with the cool treatment group, however, did not experience a loss of photosynthetic rates. The overall lower ETR, of miscanthus compared with switchgrass further suggests that miscanthus acclimated to the low light conditions of the growth chambers in both temperature treatments. These results suggest that photosynthesis is not limited by ETR in miscanthus, but because switchgrass demonstrates lower ETR in the cool treatment group, along with lower A_{max}, ETR may potentially limit photosynthetic rates in switchgrass under cool conditions. There was no difference in the nonphotochemical quenching between treatment groups for either species, though the qN values were lower in the cool growing conditions for each species and much lower for miscanthus than switchgrass. These results suggest a lower need to quench excitation energy through nonphotochemical means under cool conditions for miscanthus compared with switchgrass, and therefore more energy is diverted to photochemical pathways such as photosynthesis in miscanthus.

Gas Exchange

Light saturated photosynthetic rates of miscanthus have been shown not to be affected under cool conditions, compared with warm temperatures (Naidu, Moose et al. 2003). Our data agrees with this earlier study in that miscanthus showed no reduction in A_{sat} when grown at 14/12°C conditions. However, miscanthus had much lower rates of CO_2 uptake at both temperature treatments than switchgrass in our study. Miscanthus light saturated photosynthesis was constrained under warm temperature conditions because of the low light acclimation, as supported by the low electron transport rates under both temperature treatments.

Cold temperature inhibition of switchgrass photosynthesis was supported by reductions in the carboxylation efficiency (CE), maximum carboxylation rate (V_{cmax}), A_{sat}/J_{max}, and triose use efficiency (TPU) compared with warm growth conditions. The carboxylation efficiency of switchgrass suggests that the carboxylation of phosphoenol pyruvate (PEP) may be a significantly limiting factor of photosynthesis in the cool conditions in our study. Kingston-Smith, Harbinson et al. (1997) showed lower rates of PEPcase activity in chilled maize, leading to the conclusion that PEP carboxylation is a main low temperature limitation on C_4 photosynthesis, and the results here support this hypothesis. The lower V_{cmax} of switchgrass under cool growth conditions also reflects the lower carboxylation capacity. Also, the substantial decrease of A_{sat}/J_{max} in switchgrass indicates a possible rubisco limitation in cool conditions, possibly due to increased CO_2 leakage from the bundle sheath cells (Naidu and Long 2004).

Conversely, miscanthus did not show a decline in A_{sat}/J_{max}, CE, or TPU at 14/12°C, but did show a decrease in V_{cmax} compared to warm conditions. This decrease in V_{cmax} at low growth temperatures indicates a possible Rubisco limitation on photosynthesis and may explain

the slightly lower CO_2 assimilation rates observed here. Kubien, Sage et al. (2003), using genetically modified *Flaveria bidentis* with reduced amounts of Rubisco, showed nearly equivalent rates of photosynthesis and k_{cat} of Rubisco, *in vitro*, below 20°C, demonstrating a strong limitation on CO_2 assimilation. A similar restriction on photosynthesis by the activity of Rubisco below 17°C was shown in *Bouteloua gracilis* (Pittermann and Sage 2000), another cold tolerant C_4 species of the Rocky Mountains. Thus, while miscanthus may experience Rubisco limitations at lower temperatures, this has little to no effect on the overall rate of photosynthesis in this study. It has been reported that at lower temperatures miscanthus increases the expression of pyruvate phosphate dikinase (PPDK), and PPDK also demonstrates lower activation energy in miscanthus at cold temperatures (Wang, Portis et al. 2008). In addition, Ohta, Ishida et al. (2006) introduced point mutations into the maize PPDK gene to mimic that of the cold-tolerant *F. bidentis* and showed a 23% increase in photosynthetic rates over the nontransformed lines at 8°C, and no difference at warmer temperatures (13,20, 30°C), supporting the limiting role PPDK plays in C_4 photosynthesis. This mechanism of cold temperature acclimation may be supported by this study, where miscanthus shows a slight increase in the A_{sat}/J_{max} at 14/12°C. The lack of a decrease in the A_{sat}/J_{max}, from the warm treatment, indicates that the electron transport chain and CO_2 fixation pathway are coordinated, as where an increase would possibly indicate that the CO_2 assimilation rate has slowed in relation to the electron transport. Because these systems are coordinated, it indicates that there is no increased CO_2 leakage from the bundle sheath cells and both rubisco and PPDK may be limiting under these conditions.

Effects of Low Temperature on Transgenic Switchgrass

Introduction

The need for increased renewable energy production has become increasingly evident in recent decades with the decreasing availability of fossil fuel supplies. This demand for alternative energy sources has induced governmental policies which require the respective nations to produce significantly greater amounts of renewable, more specifically here biomass fuel, sources of energy. For instance, the European Union and the United States (US) have initiated policies requiring renewable energy sources to comprise 10% of all transport fuel by 2020 and to produce 16 billion tons of cellulosic ethanol by 2022, respectively (Robertson, Dale et al. 2008). Most of the biomass fuel feedstock species being considered are C_4 species, like most of the world's highest yielding crops, due to their high rates of productivity and high water- and nitrogen-use efficiencies (Long 1999). Most notable of these species is maize, which is currently the major source of ethanol globally and requires substantial inputs for establishment and development, but like many other potential feedstock species is significantly inhibited by cool growing conditions.

C_4 species have achieved higher productivity and water- and nitrogen-use efficiencies by developing a photosynthetic pathway which concentrates CO_2 around ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), reducing the oxygenase activity to negligible levels and therefore the amount of Rubisco required by the plant, which makes up a significant amount of the leaf-nitrogen, and also reduces the duration stomata are open (Long 1999). These adaptations give C_4 species an advantage over C_3 species under warm arid conditions; however,

C_4 photosynthesis typically declines significantly under cool growing conditions (Long 1999). The reduction of photosynthesis and productivity in C_4 species at cool temperatures has been studied extensively (Furbank, Chitty et al. 1997; Du, Nose et al. 1999; Pittermann and Sage 2000; Wang, Naidu et al. 2008; Wang, Portis et al. 2008). Several potential mechanisms of this inhibition have been identified, namely phosphoenol pyruvate carboxylase (PEPc), pyruvate phosphate dikinase (PPDK), and Rubisco, the enzymes associated with CO_2 fixation (Sage and Kubien 2007). Cool temperature inhibition of photosynthesis reduces the growing season early and late in the year, therefore reducing the productivity and capability of many C_4 species as biomass fuel feedstock species.

Rubisco may be one potential limitation to C_4 photosynthesis at cold temperatures. Rubisco is restricted to the bundle sheath cells of C_4 plants and therefore may impose an inherent disadvantage on these species by reducing the potential concentration of Rubisco and therefore possibly limiting the maximum rate of assimilation (Pittermann and Sage 2000). It has also been demonstrated that the catalytic efficiency (k_{cat}) of Rubisco decreases in some C_4 species at lower temperatures (Sage 2002), reducing the rates of photosynthesis. The activity of Rubisco has been shown to be nearly equivalent to the CO_2 assimilation rate at temperatures below 17°C in *Bouteloua gracilis* Lag. grown at 26/16°C day/night temperatures and acclimated to 14/7°C for 17 days (Pittermann and Sage 2000), further demonstrating Rubisco's possible inhibition of photosynthesis at cool temperatures. Additionally, the control that Rubisco has over photosynthesis at low temperatures is exhibited by the similarities in the activation energies of each and as indicated by increased CO_2 leakage from the bundle sheath cells (Furbank, Chitty et al. 1997). Also, there may be an inherent limitation on C_4 photosynthesis due to Rubisco's localization within the bundle sheath cells, limiting its potential concentration and therefore the

potential maximum assimilation rates (Pittermann and Sage 2000). Kubien and Sage (2004) showed that PPDK and Rubisco both impose photosynthetic limitations at cool temperatures under light saturating conditions, but with Rubisco exerting the majority of control (70%).

Additionally, PEPc regeneration via PPDK may be a limiting factor in C_4 photosynthesis at low temperatures. Significant reductions of 57% in the PPDK content of maize has been shown under 14/11°C growing conditions (Naidu, Moose et al. 2003) and nearly a 70% reduction following 14 days of 14°C conditions, after being transferred from 25°C treatment (Wang, Naidu et al. 2008). *Miscanthus* × *giganteus* Greef et Deu ex. Hodkinson et Renvoize (hereafter referred to as miscanthus) which demonstrates a high cold tolerance for C_4 plants, has displayed an increase of 2.1 times the PPDK content following transfer from warm conditions to 14°C conditions after fourteen days (Wang, Naidu et al. 2008). Miscanthus also demonstrated no loss in the amount of Rubisco and a slight increase in PPDK under cool growing conditions (14/11°C, compared with 25/20°C), and also showed no difference in photosynthetic rates between growth temperature treatments at the same measuring temperature (Naidu, Moose et al. 2003). Also, increases in the PPDK content has been shown in three sugarcane species (*Saccharum* spp.) under low temperature stress (Nogueira, De Rosa et al. 2003). Sugarcane species, of different cold tolerances, have shown changes in the PPDK activity with cold stress; hybrids of cold tolerant and cold sensitive sugarcane species showed an increase in the PPDK content while cold sensitive species demonstrated a decrease in the PPDK activity, along with a decrease in the malate dehydrogenase (NADP-MDH) (Du, Nose et al. 1999). These results suggest that PPDK content may be an underlying factor in the ability to tolerate cold temperatures in C_4 species.

Given PPDK's role as the possible limiting factor in C_4 photosynthesis, overexpressing this gene may result in increased rates of assimilation and possibly in turn, productivity. Ohta, Ishida et al. (2006), using the *Flaveria brownii* A. Powell PPDK gene as a model, introduced point mutations into the maize PPDK gene to mimic that of the C-terminus of the *F. brownii* PPDK gene. The resulting transformed maize displayed photosynthetic cold tolerance similar to *F. brownii* and increased photosynthetic rates of 23% over the nontransformed line (Ohta, Ishida et al. 2006). Thus, in this study we test the hypothesis that switchgrass, with the miscanthus PPDK gene inserted into the genome, will demonstrate increased cold tolerance in the C_4 pathway and an increased productivity under cool growing conditions.

Methods

Plant Materials

Callus of ST1 genotype, switchgrass cv. Alamo was produced from immature inflorescence, transformed with the miscanthus PPDK (MgPPDK) gene and ubiquitin promoter via *Agrobacterium*, and confirmed with red fluorescent protein reporters by the University of Tennessee, Department of Plant Sciences (Stewart Lab). We received two transgenic events plus a nontransgenic control, of eight to ten individuals each, for photosynthetic and productivity analysis. These plants were provided 3g of four month slow-release fertilizer (Osmocote 19-6-12) and maintained in their original pots in the BIOL/VBI Plant Growth Facility at Virginia Tech. To achieve the desired sample size of at least 16 individuals for each line, individuals

were propagated by dividing and replanting in one gallon pots with Pro-Mix BX (Premier Tech Horticulture, Quebec, Canada) two weeks prior to being transferred into the growth chambers.

Four individuals from each of two transgenic events and two nontransgenic individuals were transferred into each growth chamber (E8, CONVIRON, Winnipeg, Canada). Two of the growth chambers were programmed for warm conditions (28/24°C; day/night) reflecting summer temperatures of the mid-Atlantic states, and two were programmed to reflect spring temperatures (14/12°C; day/night), all with a 14/10 (light/dark) hour cycle and roughly 200µmol $m^{-2}s^{-1}$ of photosynthetically active radiation (PAR). The same photoperiod was used in both temperature treatments to isolate the effects of temperature from photoperiod. Following two weeks of acclimation, all plants were cut back to five remaining shoots. After all productivity, gas exchange, and chlorophyll fluorescence measurements had been made, the two untested transgenic events were tested in the same manner as the first two.

In order to verify successful transformation, *MgPPDK* expression levels were measured by quantitative real-time PCR relative to *PvUbi1* expression levels (Figure 3.1) using SYBR Green (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) as the reporter dye using the Applied Biosystems 7900 HT Fast Real-Time PCR System. Primers were designed to amplify *MgPPDK* transcripts from the AcV-5 epitope tag at the 3' end of the ORF (reverse: 5'-CAGCCGCTCGCATCTTTC-3') and approximately 100 base pairs upstream (forward: 5'-AGGCTAGCTGCAGCTCAGGT-3'). *PvUbi1* transcript levels were amplified using the forward primer (5'-TTGGTGCTCCGCCTGAGA-3') and reverse primer (5'-CCTGGATCTTGGCCTTCACA-3').

Productivity Measurements

Newly formed shoots were counted once a week throughout the experiment. Once all gas exchange and chlorophyll fluorescence measurements were taken the shoots produced in the growth chambers were harvested and dried to a consistent weight. Final weights per shoot were applied to weekly shoot counts to calculate an average dry weight per plant for each individual and used as an indication of temporal changes in aboveground biomass during the experiment.

Gas Exchange & Chlorophyll Fluorescence

All measurements were made on the third or fourth mature leaf of newly formed shoots using an open path gas-exchange system (LI-6400; Li-Cor Inc., Nebraska, USA) with relative humidity between 55-75% and leaf temperature maintained at the respective growth temperature. Leaves were allowed to acclimate to cuvette conditions for five minutes prior to initiating photosynthetic response programs. Initially, the photosynthetic response to light (A/Q curve) at 400 μmol/ml CO_2 was measured on all individuals from each growth chamber to determine the light saturation points, calculated using photosynthetic response curve fitting software (Li-Cor Inc., Nebraska, USA) for event and temperature treatments. The photosynthetic response to C_i (A/C_i curve) was then measured on all plants per chamber at saturating light (1000 PAR). Gas exchange results were interpreted using PhotosynAssitant (Dundee Scientific Ltd, Scotland, UK).

To determine the response of photoinhibition (F_v/F_m), electron transport rate (ETR), and nonphotochemical quenching (qN) to the growth conditions, the chlorophyll fluorescence was measured on three dark-adapted leaves, similar to those used for gas exchange measurements,

from all individuals. Leaves were dark-adapted for 10 minutes prior to measuring using a pulse-modulated fluorometer (OS-500, Opti-Sciences, New Hampshire, US)

Statistical Analysis

Initially, we tested if the mean of productivity, A/Q, and A/C_i response curves variables were significantly different between the two growth chambers for each temperature treatment (n=2-4 plants per chamber) using a student's t-test (α=0.05) (SigmaStat 3.0, Jandel Corporation, Illinois, USA). Where there was no significant difference between growth chambers results, from within the same temperature treatment, results were pooled when testing the differences of the mean between the two temperature treatments (student's t-test (α=0.05), with n= 4-8). In cases when there was a significant chamber effect within a temperature treatment data were not pooled and means were compared among the chambers individually. Also, when treatment group comparisons failed normality or equal variance assumptions of the student's t-test, possibly due to low sample sizes, Whitney's Rank Sum Test was used to determine significant effects.

Results

Productivity

Significant differences were found between temperature treatments in all groups ($p<0.01$), with the exception of transgenic line S(1) tested in the warm growth chamber D, after a

period of 113 days (Table 3.1). The control group showed a decrease of nearly 50% productivity in cool conditions from 53.1±4.92g to 27.3±6.78g. Transgenic Line A(1) produced less in the warm condition, 36.9±7.23g and 26.4±3.97g in chambers B and D respectively, than the control group and also showed significant cool temperature inhibition of productivity with 13.7±7.31g produced at 14°C. Transgenic Line S(1) experienced a significant chamber effect, producing 50.7±5.77g and 16.2±6.95g in chambers B and D respectively. There was also a significant decrease between chamber B and the cool growing conditions which produced 13.7±7.31g, however, this was not different than the production in chamber D. The low productivity seen in chamber D, in comparison with chamber B, is attributed to the overall health and stature of two (of four) individuals which appeared stunted and fragile throughout the experiment.

Chlorophyll Fluorescence

Each line and treatment group showed some degree of photoinhibition (Table 3.2). The amount of photoinhibition (as indicated by F_v/F_m) experienced in the cool growing temperature was significantly higher than in the warm treatment for transgenic line A(1) and the control group in chamber C ($p<0.01$). Transgenic line S(1) showed no pattern in the photoinhibition and growth temperature. The electron transport rate (ETR) did not show significant decreases due to cold temperatures, with the exception of the control group in the cool and warm chambers, A and D respectively. The level of nonphotochemical quenching (qN) also increased for each of the cold treatment individuals compared with those in the warm environment. There was very little change in the quanta yield of PSII (Y) from high to low temperature in any group.

Gas Exchange

The light saturation point (Q_{sat}) of the transgenic lines was significantly lower in the 14/12°C growing conditions than the 28/25°C ($p<0.01$), and there was also a substantial decrease in the control Q_{sat} at lower growing temperatures (Table 3.3). The light saturated rates of photosynthesis (A_{max}) decreased significantly in both transgenic lines ($p<0.01$) and the nontransgenic control ($p<0.05$) under cool growing conditions. The control group had slightly higher rates of A_{max}, in both the warm and cool conditions, than both transgenic lines. There was no apparent difference in the dark respiration rates (R_d) between temperature treatments for any experimental groups, and the rates were similar between all groups. No apparent difference in the apparent quantum efficiencies were observed between temperature treatments for any groups and light compensation points for all groups and temperature treatments did not vary from those typical of C_4 plants, about 10-15 µmol $m^{-2}s^{-1}$ light.

The assimilation response to internal CO_2 concentration (A/C_i) for control groups in growth chambers C and D had a sample size of one and were therefore not included in the statistical comparisons between temperature treatments. The transgenic line A(1) showed a significant decrease in the CO_2 assimilation rates (A_{sat}) from 10.9±3.90 µmol $m^{-2}s^{-1}$ and 17.7±2.32 µmol $m^{-2}s^{-1}$ in the warm conditions to 5.0±0.71 µmol $m^{-2}s^{-1}$ in the cool environment with a corresponding significant decrease in carboxylation efficiency (CE), respiration (R), maximum rubisco carboxylation rate (V_{cmax}), and triose phosphate use (TPU) (Table 3.4). The light saturated electron transport rate (J_{max}) was also significantly less in the cool condition compared with one warm growth chamber (D) and substantially less than the other warm growth chamber (B). Line S(1) did not show a decrease in A_{sat} or A_{max}/J_{max} between the temperature conditions, however, a significant decrease was observed in each of the other photosynthetic

parameters in the cool growing conditions compared with the warm. Due to the low sample size of the control group, the statistical analysis was limited to Whitney's Rank Sum test with regards to all parameters calculated from the A/C_i response curves, and no significant differences were observed. A substantial decrease was observed in the control group's A_{sat} from 21.4±0.64 µmol $m^{-2}s^{-1}$ at 28/25°C to 4.6±0.35 µmol $m^{-2}s^{-1}$ at 14/12°C. Comparable decreases under cool conditions were observed in all other A/C_i response curve results for the control group.

Discussion

Productivity

The transgenic switchgrass showed productivity at 28/25°C that was comparable to the nontransgenic control group, with the exception of line S(1) in growth chamber D. This exception is likely the result of two individuals which appeared unhealthy and fragile throughout the experiment. Overall, the transgenic groups displayed a shorter and more fragile appearing shoots than the control group. The average productivity of line S(1) in this chamber without the two smaller individuals is 22.19±1.69g, which is still significantly less than those individuals of line S(1) in the other warm growth chamber (B), but is significantly higher than the cold treatment's productivity. Line S(1) also showed productivity, in growth chamber B, that was nearly equivalent to the nontransgenic control group, while line A(1) produced substantially less than the control group in both growth chambers. Also, under cool growing conditions, the transgenic lines showed much lower productivity than the nontransgenic controls.

These results suggest that the transformation process has a negative effect on the productivity of ST1 genotype, switchgrass cv. Alamo in the growth chamber environments. The

insertion of the *Miscanthus* × *giganteus* PPDK (MgPPDK) gene does not confer cold tolerance in terms of productivity to switchgrass according to the results presented here. This could be a result of the insertion gene disrupting other genetic processes. Also, the negative effect of transformation on plant productivity could be due to the high resource requirement of the overproduction of PPDK throughout the plant (Figure 3.1).

Chlorophyll Fluorescence

All groups experienced higher levels of photoinhibition (F_v/F_m decreased) under the cooler temperatures, as would be expected with higher stress conditions. However, while the photoinhibition was significantly higher in the cool treatment groups, the differences and more importantly the variations were small and may not reflect biologically significant differences. This may be due to the length of the study allowing more than sufficient time for the plants to acclimate to lower temperatures and not suffer from substantially more photoinhibition in comparison with the warm treatment group. The electron transport rate (ETR) did not vary between the temperature treatments for either of the transgenic lines tested, and was only significantly lower in one of the growth chambers for the control group. It would be expected that the ETR and the quanta yield from PSII (Y) would be inhibited by the cold temperature environment where lower rates of assimilation were observed, but the lack of inhibition by cold suggests that the limitation on photosynthesis under low temperatures is in the CO_2 fixation pathway and not in the electron transport chain. The higher than expected rates of electron transport and Y at cool temperatures that aren't coordinated with CO_2 fixation results in an excess of free electrons, which must be dissipated via nonphotochemical quenching (qN). The

higher rates of qN observed in the cool conditions are in accordance with the higher electron transport rate. All groups show approximately the same rates of qN, ETR, Y, and photoinhibition in the warm and cool treatments which indicates that the transformation process had little to no effect on the light harvesting apparatus.

Gas Exchange

The control group showed the highest light saturation point (Q_{sat}) among the groups tested in both the warm and cool treatment, and the difference between warm and cold Q_{sat} was significant for both transgenic lines, and substantially different for the control. These low light saturation points of the transgenic lines are not a product of photoinhibition of PSII as indicated by the F_v/F_m values which are relatively the same as the nontransgenic control. The low Q_{sat} values can be attributed to the low levels of photosynthetically active radiation in the growth chambers, and these low saturation points may be a factor responsible for the low productivity of the transgenic groups. The same significant differences between warm and cool A_{max} rates in the transgenic groups as well as the control suggests that there is no effect of the MgPPDK gene insertion on light saturated photosynthetic rates in relation to the temperature treatments used here. Miscanthus was shown not to experience any significant decrease in A_{max} (Naidu, Moose et al. 2003). Similarly, there were no differences in the AQE response to temperature either. This lack of difference in AQE further suggests that the limitation to C_4 photosynthesis is not in the electron transport chain, but is located in the CO_2 fixation pathway, because it would be expected that if the limitation was in the electron transport under cool conditions then there would be a substantially lower AQE at cold temperatures.

The CO_2 saturated rates of photosynthesis (A_{sat}) decreased substantially in cold temperatures for the nontransgenic controls and transgenic line A(1), and the other parameters derived from the A/C_i response curve show similar trends. There was no significant decrease in A_{sat} at cold temperature for transgenic line S(1), with the lowest A_{sat} at 28/25°C and the highest at 14/12°C. This similarity between temperatures is not observed in the rest of the A/C_i response variables. From the parameters derived from the A/C_i response curves, carboxylation efficiency (CE) is indirectly indicative of PPDK response to temperature, due to this region of the response curve being limited by PEPc activity (Sage and Kubien 2007). CE, however, is unable to distinguish between limitations due to PEPc activity and Rubisco activity. Kubien and Sage (2003) demonstrated the rubisco's limiting effect on CO_2 assimilation, with C_4 *Flaveria bidentis* transformants with reduced rubisco content and showed that the *in vitro k*cat matched the *in vivo k*cat at, and below 15°C. Similar results were shown by Sage (2002) where rubisco activity in vivo matched the rate of CO_2 assimilation at temperatures below 20°C. Both transgenic lines and the control group showed a significant decrease in CE in the cool growing conditions, indicating that the MgPPDK transformation did not enhance PPDK performance at 14/12°C, and the CE of the transgenic lines was substantially less than the control group suggesting that the transformation may have had negative effects on the CO_2 fixation apparatus, most likely PPDK and/or rubisco.

The ratio of A_{sat}/J_{max} gives the amount of CO_2 fixed per electron from PSII, giving a relative measure of assimilation. The nontransgenic control demonstrated values, in both treatment groups, similar to those found in the previous experiment (switchgrass A_{sat}/J_{max}= 0.37±0.155 at 14/12°C and 0.84±0.332 at 28/25°C) while the transgenic lines showed A_{sat}/J_{max} values substantially lower. However, line S(1) showed very similar values as miscanthus in the

previous experiment (Ch. 1) (0.44±0.187 at 14/12°C; 0.42±0.172 at 28/25°C) also with no significant difference between temperatures. Transgenic line A(1) showed a significantly lower A_{sat}/J_{max} in the cool conditions than in the warm. These results may indicate that the transformation event in line S(1) has decreased the inhibition of assimilation by cool temperatures. Along with the lack of temperature effect on the A_{sat}, the MgPPDK transformation supports the hypothesis that PPDK is a significantly limiting factor in the assimilation rates of switchgrass with regards to cool temperature for the S(1) line only.

Conclusion

In this first study, miscanthus demonstrated acclimation to the low light of the growth chambers and the photosynthetic traits were unaffected by the temperature conditions. Switchgrass, however, did not acclimate to the growth chamber light and showed trends of cool temperature inhibition of photosynthetic traits. Low temperatures decreased the carboxylation efficiency and ratio of CO_2 assimilation to electron transport rate in switchgrass, but not in miscanthus, which supports the cold tolerance of carboxylation in miscanthus. The productivity of miscanthus was limited in both temperature treatments by the low light conditions of the growth chambers. This study examined the switchgrass cultivar Alamo, a lowland ecotype. Upland ecotypes, such as the cultivar Cave-in-Rock, may be able to retain higher photosynthetic rates via a greater capacity to maintain high carboxylation efficiency at cool temperatures. Thus, a direct comparison of switchgrass (Cave-in-Rock) and miscanthus photosynthesis and productivity at cool temperatures would provide substantial insight into the most efficient biomass fuel species for cold regions.

This second experiment showed acclimation of both transgenic groups and the control group to the low light conditions of the growth chamber, and similar to the first experiment, the control group and line A(1) showed significant cold inhibition of CO_2 saturated photosynthesis (A_{sat}). Line S(1) did not demonstrate cold inhibition with regards to A_{sat}, which may be attributed to its unhealthy, fragile appearance in the warm growth conditions. Low temperature also decreased the carboxylation efficiency of the transgenic lines, similarly to the decrease seen in the control group and switchgrass from the first experiment. However, the ratio of CO_2 assimilation to electron transport of the transgenic line S(1) demonstrated cold temperature

effects more similar to that of miscanthus than switchgrass from the first experiment, which suggests that the miscanthus PPDK gene insertion conferred a degree of coordination between PSII and the carbon fixation pathway. These results may indicate that cold tolerance was transferred to some extent via the gene insertion. The full potential of this insertion may be realized in field studies, where the transgenic lines may potentially emerge and form closed canopies earlier in the season, similar to miscanthus, and achieve higher yields both in warm and cool climates than nontransformed switchgrass.

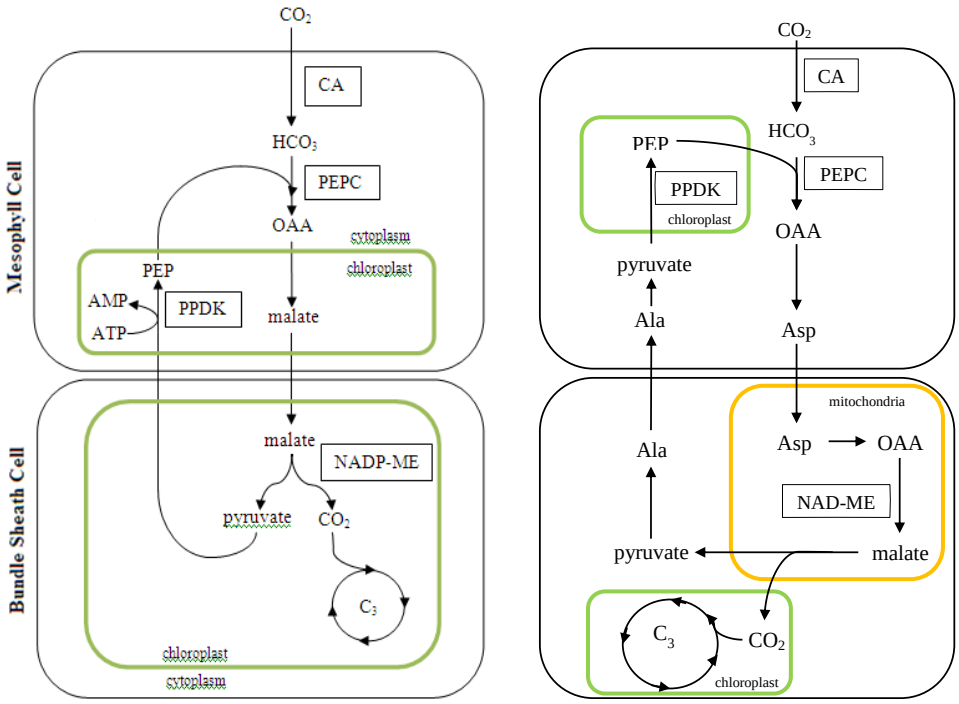

Figure 1.1. C_4 NADP-ME and NAD-ME Pathway – highlighting carbonic anhydrase (CA), phosphoenol pyruvate carboxylase (PEPC), oxaloacetate (OAA), the Calvin Benson cycle (C_3), phosphoenol pyruvate (PEP) and pyruvate phosphate dikinase (PPDK), alanine (Ala), aspartate (Asp). Switchgrass utilizes the NAD-ME pathway and miscanthus the NADP-ME pathway.

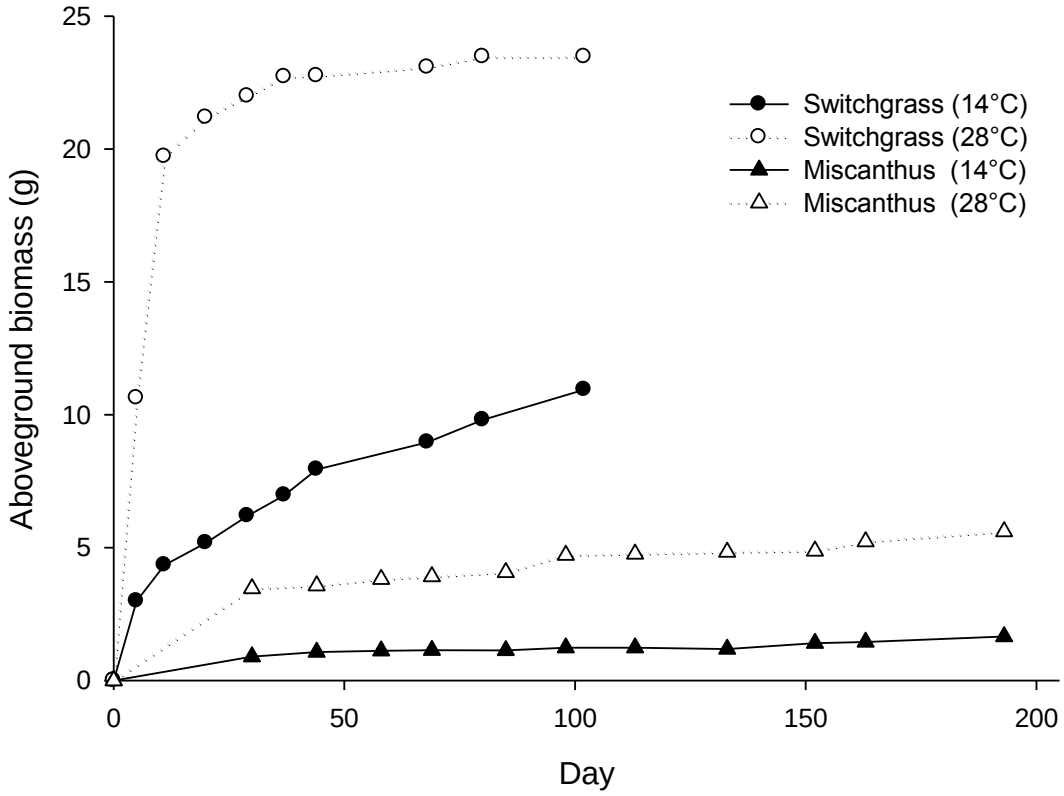

Figure 2.1. Switchgrass and miscanthus average aboveground productivity. Productivity rates of above-ground biomass per individual for *Panicum virgatum* L. (Switchgrass) and *Miscanthus* × *giganteus* (miscanthus). Measurements of the number of newly formed shoots taken throughout the experiment multiplied by the final dry weight yielded estimates for the above-ground biomass produced throughout the study. . 14°C = 14/12°C day/night; 28°C = 28/25°C day/night. (n=16).

Table 2.1. *Panicum virgatum* L. (Switchgrass) and *Miscanthus* × *giganteus* (Miscanthus) chlorophyll fluorescence response. Means (one SE) for leaves developed at either of two temperature treatments. 14°C = 14/12°C day/night; 28°C = 28/25°C day/night; F_v/F_m = variable fluorescence/maximum fluorescence, which is used to indicate photoinhibition; ETR = electron transport rate; qN = nonphotochemical quenching; Y = yield. (n = 2).

	Switchgrass		**Miscanthus**	
	14°C	**28°C**	**14°C**	**28°C**
F_v/F_m	0.741 (0.0010)	0.723 (0.0026)	0.767 (0.0001)	0.780 (0.0017)
ETR $\mu mol\ m^{-2}s^{-1}$	331.027(0.725)	412.137 (9.469)	253.728 (0.502)	216.048 (7.169)
qN	0.264 (0.0142)	0.278 (0.017)	0.205 (0.039)	0.272 (0.013)
Y	0.525 (0.017)	0.491 (0.011)	0.503 (0.001)	0.429 (0.014)

Table 2.2: *Panicum virgatum* L. (Switchgrass) and *Miscanthus* × *giganteus* (Miscanthus) response of assimilation to light (A/Q). Means (one SE) of leaves developed, and measured, at either of two temperature treatments. 14°C = 14/12°C day/night; 28°C = 28/25°C day/night; Q_{sat} = light saturation point; A_{max} = light saturated rate of photosynthesis; Q_{comp} = light compensation point; AQE = apparent quantum efficiency; R_d = dark respiration rate. (n=2)

	Switchgrass		**Miscanthus**	
	14°C	**28°C**	**14°C**	**28°C**
Q_{sat} $\mu mol\ m^{-2}s^{-1}$	964.37 (82.09)	1061.72 (196.42)	554.13 (31.125)	472.04 (7.375)
A_{max} $\mu mol\ m^{-2}s^{-1}$	10.11 (0.253)	18.51 (1.494)	7.05 (0.864)	8.342 (0.942)
Q_{comp} $\mu mol\ m^{-2}s^{-1}$	9.33 (1.335)	11.20 (0.798)	12.49 (1.499)	13.83 (0.833)
AQE	0.071 (0.0040)	0.066 (0.0023)	0.058 (0.0050)	0.050 (0.0041)
R_d $\mu mol\ m^{-2}s^{-1}$	-0.67 (0.117)	-0.75 (0.018)	0.71 (0.106)	0.69 (0.015)

Table 2.3: *Panicum virgatum* L. (Switchgrass) and *Miscanthus* × *giganteus* (Miscanthus) assimilation response to internal [CO_2] (A/C_i). Means (one SE) of leaves developed, and measured, at either a warm or cool temperature. 14°C = 14/12°C day/night; 28°C = 28/25°C day/night; CE = Carboxylation Efficiency; A_{sat} = Light and CO_2 -saturated assimilation rate, R = Light respiration rate; V_{cmax} = maximum carboxylation rate of phosphoenol pyruvate; J_{max} = maximum electron transport rate; TPU = Triose phosphate use. (n=2)

	Switchgrass		**Miscanthus**	
	14°C	**28°C**	**14°C**	**28°C**
CE $\mu mol\ m^{-2}s^{-1}Pa^{-1}$	3.46 (2.059)	10.97 (0.602)	5.49 (0.440)	3.21 (0.061)
A_{sat} $\mu mol\ m^{-2}s^{-1}$	12.62 (1.567)	25.65 (2.827)	13.74 (0.357)	18.01 (1.790)
Resp $\mu mol\ m^{-2}s^{-1}$	-1.44 (0.565)	-6.56 (1.767)	-0.88 (0.248)	-2.62 (1.616)
V_{cmax} $\mu mol\ m^{-2}s^{-1}$	15.73 (2.325)	127.56 (5.481)	13.23 (0.984)	48.94 (3.42)
J_{max} $\mu mol\ m^{-2}s^{-1}$	30.14 (2.179)	31.61 (7.727)	29.23 (2.313)	39.13 (6.730)
TPU $\mu mol\ \mu mol^{-1}$	4.99 (1.271)	25.28 (3.138)	4.64 (0.308)	9.18 (5.543)
A_{sat}/J_{max}	0.43 (0.083)	0.87 (0.119)	0.49 (0.031)	0.48 (0.043)

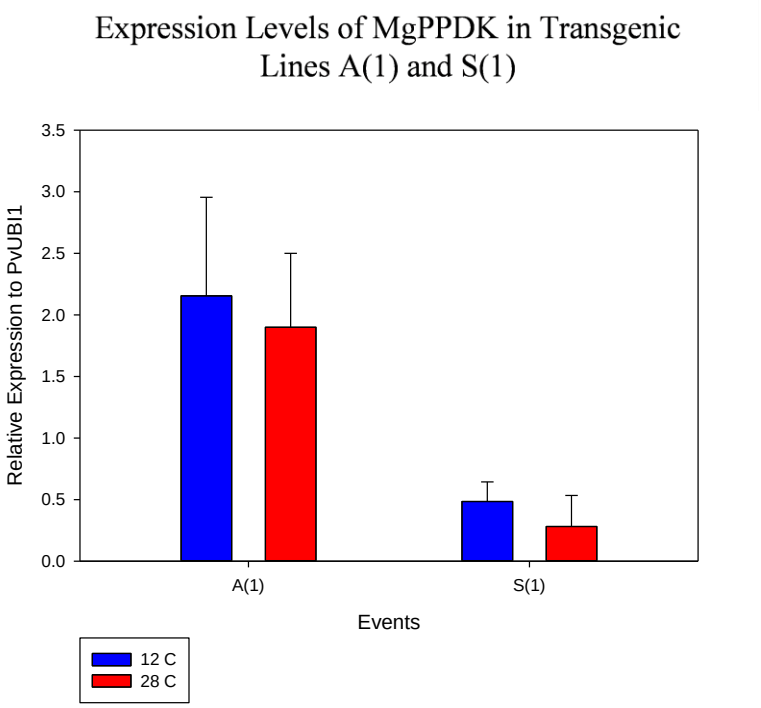

Figure 3.1 Expression levels of MgPPDK in transgenic switchgrass events A(1) and S(1) as measured by quantitative Real-Time polymerase chain reaction (qRT-PCR) and reported as expression relative to the ubiquitin transcript.

Table 3.1. Productivity rates of transgenic events and nontransgenic control. Means (one SD) for two transgenic lines and nontransgenic controls of ST1 genotype, switchgrass cv. Alamo. Results are aboveground biomass per plant (g). 14°C = 14/12°C day/night; 28°C = 28/25°C day/night. Significant differences between temperature treatments (Student's t-Test, Whitney's rank sum test was used when Student's t-Test normality and equal variance assumptions failed) denoted by ** ($\alpha<0.01$) (n= 4-8). Where a chamber effect was found, each chamber's mean (SD) is given and tested against opposite temperature treatment.

	14°C	28°C	
Chamber		B	D
Line A(1)	15.9 (3.56)	36.9** (7.23)	26.4** (3.97)
Line S(1)	13.7 (7.31)	50.7** (5.77)	16.2 (6.95)
Control	27.3[b] (6.78)	53.1** [a] (4.92)	

[a] failed normality test; [b] failed equal variance test

Table 3.2. Switchgrass, Transgenic Lines and Control, Chlorophyll Fluorescence Response. Means (SD) for two transgenic lines and nontransgenic control ST1 genotype, switchgrass cv. Alamo. 14°C = 14/12°C day/night; 28°C = 28/25°C day/night; F_v/F_m = variable fluorescence/maximum fluorescence, which is an indication of photoinhibition; ETR = electron transport rate µmol $m^{-2}s^{-1}$; qN = nonphotochemical quenching; Y = electron yield of PSII. Significant differences between temperature treatments (Student's t-Test, Whitney's rank sum test was used when Student's t-Test assumptions failed) denoted by ** ($\alpha<0.01$) (n= 2-8). Where a chamber effect was found, each chamber's mean (SD) is given and tested against opposite temperature treatment.

		14°C			28°C		
	Chamber	A	combined	C	B	combined	D
F_v/F_m	A(1)		0.723 **,**(0.009)		0.703(0.009)		0.712(0.007)
	S(1)	0.730**[a] (0.014)		0.696 **[a] (0.017)		0.718(0.008)	
	Control	0.721 (0.003)		0.737 **(0.006)		0.717(0.005)	
ETR	A(1)		205.0[a] (35.06)			207.9(21.44)	
µmol $m^{-2}s^{-1}$	S(1)		179.4[a] (38.23)			193.0(19.93)	
	Control	179.9[o],** (16.41)		203.9(12.71)	188.2(22.91)		218.5(12.91)
qN	A(1)		0.38** (0.101)			0.29(0.098)	
	S(1)		0.42**[a],**(0.092)		0.27(0.037)		0.19(0.065)
	Control	0.38 **(0.028)		0.45 **(0.026)		0.32(0.035)	
Y	A(1)		0.41 (0.063)			0.41 (0.036)	
	S(1)		0.36 (0.073)			0.38[a] (0.036)	
	Control		0.38[b] (0.033)			0.40[b] (0.035)	

[a] failed equal variance test; [b] failed equal variance test in chamber comparison; [o] no significant difference

Table 3.3. Switchgrass, Transgenic Lines and Control, Response of Assimilation to Light (A/Q). MeanS (one SD) for two transgenic lines and nontransgenic control ST1 genotype, switchgrass cv. Alamo. 14°C = 14/12°C day/night; 28°C = 28/25°C day/night; Q_{sat} = light saturation point; A_{max} = light saturated rate of photosynthesis; R_d = dark respiration rate; AQE = apparent quantum efficiency. Significant differences between temperature treatments (Student's t-Test, Whitney's rank sum test was used when Student's t-Test assumptions failed) denoted by ** ($\alpha<0.01$) (n= 2-8). Where a chamber effect was found, each chamber's mean (SD) is given and tested against opposite temperature treatment.

		14°C			28°C		
	Chamber	A	combined	C	B	combined	D
Q_{sat}	A(1)		284.4(106.62)		783.9** (117.49)		549.7** (52.24)
µmol $m^{-2}s^{-1}$	S(1)		206.4(67.84)			616.9** (217.48)	
	Control		404.9(60.39)			849.7[b] (347.46)	
A_{max}	A(1)		4.1(0.92)		9.8** (1.38)		8.1** (0.17)
µmol $m^{-2}s^{-1}$	S(1)		3.5(0.84)			7.6**[b] (2.43)	
	Control		4.7(0.69)			10.9*[b] (3.87)	
R_d	A(1)		-0.75(0.249)			-0.90 (0.245)	
µmol $m^{-2}s^{-1}$	S(1)		-0.81(0.584)			-0.82[a] (0.259)	
	Control		-0.82(0.335)			-0.75[b] (0.137)	
AQE	A(1)		0.061 (0.0290)		0.065 (0.0112)		0.059 (0.0078)
	S(1)		0.087 (0.0943)			0.076 (0.0293)	
	Control	0.071 (0.0339)		0.157 (0.0849)		0.066 (0.0082)	

[a] failed normality test; [b] failed equal variance test

Table 3.4. Results of the response of assimilation to internal CO_2 concentration (A/C_i) [mean (SD)] for two transgenic lines and nontransgenic control ST1 genotype, switchgrass cv. Alamo. 14°C = 14/12°C day/night; 28°C = 28/25°C day/night; CE = Carboxylation Efficiency; A_{sat} = CO_2 saturated photosynthesis; Resp = light respiration; V_{cmax} = maximum carboxylation rate of rubisco; J_{max} = maximum electron transport rate; TPU = triose phosphate use. Significant differences between temperature treatments (Student's t-Test, Whitney's rank sum test was used when Student's t-Test assumptions failed) denoted by ** (α<0.01) (n= 2-8). Where a chamber effect was found, each chamber's mean (SD) is given and tested against opposite temperature treatment.

		14°C			28°C		
	Chamber	A	combined	C	B	combined	D
CE	A(1)		0.145 (0.0712)			2.43**[a] (1.444)	
µmol $m^{-2}s^{-1}Pa^{-1}$	S(1)		0.088 (0.0662)			1.70**[a] (2.226)	
	Control	0.348 (0.310)		0.045[c]	8.09[b] (0.502)		3.45[c]
A_{sat}	A(1)		5.0 (0.71)		10.9*[a] (3.90)		17.7**[b] (2.32)
µmol $m^{-2}s^{-1}$	S(1)		7.2 (3.04)			9.8[a] (3.95)	
	Control	4.6 (0.35)		5.16[c]	21.4[b] (0.64)		15.3[c]
Resp	A(1)		-0.73 (0.277)			-3.27**[b] (1.369)	
µmol $m^{-2}s^{-1}$	S(1)		0.18 (0.296)			-1.57*[b] (1.263)	
	Control	-0.79 (0.320)		-0.60[c]	-8.68[b] (1.655)		-1.70[c]
V_{cmax}	A(1)		4.1 (0.64)			44.6**[a] (19.49)	
µmol $m^{-2}s^{-1}$	S(1)		3.9 (0.58)			22.4**[a] (12.01)	
	Control	4.5 (0.71)		2.78[c]	77.9[b] (7.78)		35.4[c]
J_{max}	A(1)		14.9 (1.23)		23.6[a] (7.02)		36.0**[b] (5.10)

µmol $m^{-2}s^{-1}$	S(1)		15.3 (2.05)			21.4** (5.51)	
	Control	13.9 (2.26)		10.6[c]	23.0[b] (0.21)		31.1 [c]
TPU	A(1)		1.88 (0.475)			9.56**[b] (2.833)	
µmol $µmol^{-1}$	S(1)		2.08 (1.212)			4.67** (2.062)	
	Control	1.58 (0.474)		0.85[c]	51.35[b] (15.344)		6.33[c]
A_{sat}/J_{max}	A(1)		0.34 (0.037)			0.48 **(0.082)	
	S(1)		0.40 (0.235)			0.40[a] (0.157)	
	Control		0.36 (0.090)			0.78 (0.254)	

[a] failed normality test; [b] failed equal variance test; [c] n=1

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