# LOW TEMPERATURE PERFORMANCE OF LEADING BIOENERGY CROPS UTILIZING THE $C_4$ PHOTOSYNTHETIC PATHWAY

by

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## Abstract

The use of bioenergy has been considered one of the most important solutions for the reduction in the dependence on fossil fuels and to mitigate global climate change. $C_4$ perennial grasses stands as the best alternative for biofuel feedstock and *Miscanthus x giganteus* has shown outstanding performance in cool climates. In addition, sugarcane has a history of high productivity and success in the biofuel industry of Brazil. The high photosynthetic rate at low temperature (14º to 20ºC) and radiation use efficiency of *M. x giganteus* allow this plant to build a vast canopy early in the spring, allowing it to intercept high amounts of solar radiation once temperature rise in summer, obtaining high yields of biomass by the end of growing season. It is unclear, however, how *Miscanthus* will tolerate the severe winter cold and frequent episodic frost in the spring of higher latitudes, like Canada. Here, I show that rhizomes of diploid *Miscanthus* can tolerate temperatures above -14ºC if sub-zero acclimation is allowed, while allopolyploid *Miscanthus* are tolerant to temperatures above -6.5ºC. Also, in contrary to *M. sinensis*, shoots of *Miscanthus* hybrids were killed when exposed to sunlight at temperatures below 10ºC in the spring. However, triploid *Miscanthus* showed great recovery and three weeks

later plants had a vast and closed canopy while *M. sinensis* plants had a poor canopy. Lastly, I show that upland and lowland Hawaiian sugarcane grown at two moderate temperatures have little variation in temperature response and acclimation to low temperature. Because of its high productivity, *Miscanthus* should be considered in areas where it can securely be grown. According to the results found here, rhizomes of diploid *Miscanthus* have the potential to be grown up to 60ºN, but the frequency of episodic chilling events should also be taken into consideration. Here, I show that the genepool of the *Miscanthus* genus has the potential to increase cold tolerance in the most productive lines, which will increase the range for this crop. Sugarcane is still restricted to tropical and subtropical zones, but higher cold tolerance can be achieved by hybridizing this plant with *Miscanthus*.

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# Chapter 1 - Introduction

Over the past decade, humans have been consuming about 12 terawatts per hour of energy, of which about 90% was provided by fossil fuels, the main source of $CO_2$ release to the atmosphere (Chow *et al.*, 2003). With the rapid increase in the world's population, the need for food and energy is expected to increase by 70% by 2050 (FAO, 2009; United Nations, 2012; Smith *et al.*, 2013). This demand for energy cannot be perpetually supplied with gas, coal and oil, which are currently the main sources of energy, because the reserves of these resources are in decline (International Energy Agency (IEA), 2013). Bioenergy stands as a long term solution because it is a renewable and sustainable source of energy produced from biological material (Chum *et al.*, 2011). Currently, bioenergy is mainly produced from sugarcane sucrose, maize grain, straw, wood, and agricultural byproducts. Energy crops are an important alternative for energy production, and can be directly used as a source of heat or transformed into liquid fuel (Tillman, 2000; Ragauskas *et al.*, 2006; Somerville, 2007). Plants are cost-effective and efficient in the capture of solar energy, but they are also important in the capture of $CO_2$ from the atmosphere, which can limit the greenhouse effects and thus global warming (Tilman *et al.*, 2006; Heaton *et al.*, 2008a; Vermerris, 2008; Zhu *et al.*, 2008).

Inhibiting $CO_2$ emissions have been a global priority since the first Kyoto protocol in 1997, and more recently the Copenhagen treaty of 2009. One suitable means of reducing $CO_2$ emissions is substituting the use of fossil fuel with biologically-produced energy (biofuel). In the USA in 2007, the Energy Independence and Security Act (EISA) mandated that by 2022, 136 billion liters of biofuel should be produced, where 44% (60 billion liters) should come from lignocellulose plant

material (EPA - United States Environmental Protection Agency). Lignocellulose is essentially the plant dry biomass and consists primarily of carbohydrate polymers cellulose and hemicellulose, as well as the aromatic polymer lignin. Producing fuel from biomass is more efficient in the use of resources than producing from sucrose and grains in terms of less energy input and crop area (

Table 1.1). According to the U.S. Department of Energy (DOE, 2006), 1 ton of dry biomass

| Crop | Bioenergy Generation | Max biomass yield (Mg $ha^{-1}$) | Location |
|---|---|---|---|
| *Miscanthus x giganteus* "Illinois" (3n) | Lignocellulosic Biomass G2 | 23[1] | Illinois,USA |
| *Panicum virgatum* "Cave-in-Rock" | Lignocellulosic Biomass G2 | 10[1] | Illinois,USA |
| *Miscanthus x giganteus* "Nagara" (3n) | Lignocellulosic Biomass G2 | 23[3] | Ontario, Canad |
| *Panicum virgatum* "Cave-in-Rock" | Lignocellulosic Biomass G2 | 9[3] | Ontario, Canad |
| *Spartina pectinata* "Red River" | Lignocellulosic Biomass G2 | 6.5[3] | Ontario, Canad |
| Sugarcane | Stem sucrose G1 | 85[4,5,6] | São Paulo, Braz |
| Sugarcane | Stem sucrose G1 + Lignocellulosic Biomass | 85[4,5,6] | São Paulo, Braz |
| Corn | Grains G1 | 10[8, 9] | Midwest USA |
| Corn | Grains G1 + corn stover G2 integrated | 18[8] | Midwest USA |
| | | | Total cropland in |
| | | | Total cropland |

can yield 380 liters of biofuel. Arundale *et al.* (2014a) estimates that to reach the 60 billion liters of cellulosic ethanol demanded by EISA would require 6.8 Mha of farmland of *Miscanthus x giganteus*. Thus, the substitution of corn grain ethanol by *Miscanthus x giganteus* ethanol from lignocellulose represents a reduction in 50% of the farmland used for bioenergy production.

Table 1.1 compares these numbers with other first generation (G1) biofuel production,

| Crop | Bioenergy Generation | Max biomass yield (Mg ha$^{-1}$) | Location |
|---|---|---|---|
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| *Panicum virgatum* "Cave-in-Rock" | Lignocellulosic Biomass G2 | 10[1] | Illinois,USA |
| *Miscanthus x giganteus* "Nagara" (3n) | Lignocellulosic Biomass G2 | 23[3] | Ontario, Canad |
| *Panicum virgatum* "Cave-in-Rock" | Lignocellulosic Biomass G2 | 9[3] | Ontario, Canad |
| *Spartina pectinata* "Red River" | Lignocellulosic Biomass G2 | 6.5[3] | Ontario, Canad |
| Sugarcane | Stem sucrose G1 | 85[4,5,6] | São Paulo, Braz |
| Sugarcane | Stem sucrose G1 + Lignocellulosic Biomass | 85[4,5,6] | São Paulo, Braz |
| Corn | Grains G1 | 10[8, 9] | Midwest USA |
| Corn | Grains G1 + corn stover G2 integrated | 18[8] | Midwest USA |
| | | | Total cropland in |
| | | | Total cropland |

which is based on the use of sucrose and starch, and second generation (G2) biofuel production based on lignocellulose from bioenergy feedstock from Canada, Brazil and the USA. Developing *Miscanthus* as a bioenergy crop allow us to reduce land required for fuel crops reducing the environmental impact and the competition with agriculture and livestock. This thesis aims to study the tolerance and acclimation to cold temperature of *Miscanthus* and sugarcane, analyzing the possibility for introduction of these crops to higher latitudes.

## 1.1 - Bioenergy

The energy crisis in the 1970s demonstrated to the world the need for alternative sources of energy and for efficient techniques for food production (Akins, 1973; Pimentel *et al.*, 1973). In Brazil, the

government created the “Proalcool”, a program of incentives for ethanol fuel production from sugarcane, which also included the production of cars that could be fueled exclusively from bioethanol (Macedo *et al.*, 2008; van den Wall Bake *et al.*, 2009). In the 1980’s, the increase in the price of sugar and decrease in the price of oil resulted in a big decline in bioethanol production, reducing the interest of the population in ethanol-fueled vehicles. Recently, flex fuel cars, which can use bioethanol or gasoline in different proportions, have been introduced in the Brazilian market, benefiting the bioethanol production in the country.

The higher prices of oil and the need for clean and renewable sources of energy has increased the need for biofuel, mostly blended with petroleum-based fuel. In the USA, most gasoline contains at least 10% bioethanol, and in 2011, the blend ratio was allowed to increase to 15% (US Department of Energy, website). In September 2014, the Brazilian government approved bill 13033 allowing an increase of ethanol in gasoline from 25% to 27.5%. In addition, the same law allows an increase from 5% to 7% biodiesel in diesel by November 2014. In Canada, ethanol content in gasoline varies from 5-8.5% depending on the province (Canadian Renewable Fuels Association, 2013). Currently, the US is the biggest ethanol producer, reaching 54.2 billion liters of corn ethanol produced in 2011, while Brazil produced 20.7 billion liters of sugarcane ethanol in the same year (Chum *et al.*, 2014a, 2014b). Together, Brazil and USA produced 87% of the total fuel bioethanol in the world (Chum *et al.*, 2014a). However, the production of most of this biofuel demands high energy input (from fertilization, for example) and waste at least 60% of the biomass (Jones, 2011; Dias *et al.*, 2013; Karlen *et al.*, 2014). Thus, there is a need for more competitive biofuel production in terms of energy efficiency, reduce costs to grow the crop, and more complete use of the plant material.

The so called "first generation" biofuel (G1) consists primarily of ethanol production from the fermentation of sugars and starch, mainly from sugarcane, corn and sorghum, although cassava (*Manihot esculenta*), *Agave*, beets, Napier grass and *Erianthus* are also used (Naylor *et al.*, 2007; Somerville, 2007; Davis *et al.*, 2011; Jones, 2011). Many problems are associated with this type of bioenergy production. Because of inefficiency of the G1 fuel production, more farmland is necessary, increasing competition with food production (Haberl *et al.*, 2011; Mohr & Raman, 2013; Manning *et al.*, 2014), and leading to deforestation, erosion, and loss of global biodiversity (Tilman *et al.*, 2006; Robertson & Walker, 2008). Clearing lands for biofuel production also deplete soil carbon stocks, releasing to the atmosphere more carbon than removed (Fargione *et al.*, 2008; Searchinger *et al.*, 2009). In accounting for all the energy input for the production of bioethanol, each liter of sugarcane ethanol releases to the atmosphere approximately 0.5 kg of $CO_2$, while each liter of corn-ethanol releases 1.4 kg of $CO_2$ (Table 1.2). Table 1.2 shows that each hectare of sugarcane can provide 64 Mg of harvestable biomass of which 3.4 Mg returns to the atmosphere as $CO_2$. One hectare of corn can produce 18 Mg of harvestable of biomass which 5.8 Mg returns to the atmosphere as $CO_2$. Most of the energy required for G1 ethanol production originates from tillage, planting, weed control, fertilization, and irrigation (Oliveira *et al.*, 2005; Shapouri *et al.*, 2008). Thus, it is important that the energy required for the production of biofuel be significantly lower than the energy released from it (Shapouri *et al.*, 2008; Yuan *et al.*, 2008; Sang & Zhu, 2011).

Second generation bioenergy (G2) utilizes new technologies to improve the conversion of biomass (cellulose, hemicellulose, and lignin) to liquid fuel. Ideally, G2 crops have high yields of biomass, low energy and fertilization input (high nitrogen use efficiency), a highly positive carbon balance (store more carbon in the soil than they remove), and is successful in marginal lands to

avoid competition with food crops (Fargione *et al.*, 2008; Jones, 2011; Sang & Zhu, 2011). G2 bioenergy production can achieve higher efficiency because it utilizes cellulose, hemicellulose and lignin in addition to less recalcitrant sugars and starch (Belmokhtar *et al.*, 2012; Irmak *et al.*, 2013; Costa *et al.*, 2014; Karlen *et al.*, 2014).

Local production of biomass can be an ideal energetic source for isolated areas as it reduces the cost of transportation, while in populated areas, it can reduce energy costs for large industrial markets. In Ontario, the greenhouse industry could use 800,000 Mg of lignocellulose biomass per year (Vyn *et al.*, 2012), and in 2011, Ontario Power Generation Inc. (http://www.opg.com/) estimated a demand of about 2 million tons of biomass for blending in coal-fired power plants. If bioenergy crops can be implemented on the marginal lands around the Great Lakes, the low cost of transportation on the lakes could provide a cheap and profitable solution to this demand.

This thesis addresses the development of perennial $C_4$ bioenergy crops adapted to marginal lands at higher latitudes. These crops should be able to efficiently recycle nutrients back to the dormant tissues by the end of the growing season, therefore reducing the need for fertilization and the potential level of pollutants released upon combustion (Sims *et al.*, 2006). They should be productive at relatively low growing season temperatures; be resistant to chilling temperatures in the spring, producing a canopy early in the spring; and have dormant parts resistant to sub-zero temperatures. *Miscanthus x giganteus* has most of these characteristics, achieving outstanding biomass yield production in temperate regions (Beale *et al.*, 1996; Kahle *et al.*, 2001; Heaton *et al.*, 2004a, 2010). However, studies determining critical climate are needed for Canadian climates. Sugarcane (*Saccharum spp.*) has already been used for bioenergy since the 1970's and recently this plant has attracted interest for temperate regions. Hybridization with *Miscanthus* has been

developed to merge the high productivity of sugarcane with cold tolerance of *Miscanthus* (Tai & Miller, 1996; Eggleston *et al.*, 2004; Belintani *et al.*, 2012; Lee *et al.*, 2014). Thus, there has been high interest in selecting lines adapted to high productivity under low temperature. Both *Miscanthus* and sugarcane, as well as most of the lignocellulosic bioenergy crops being studied, are in an initial phase of domestication and are considered to have much room for improvement (Jakob *et al.*, 2009; Moon *et al.*, 2010; Lee *et al.*, 2014).

This chapter provides a background on $C_4$ photosynthesis, explaining its productive potential and why it is important for bioenergy crops. I will also discuss low temperature tolerance in $C_4$ plants and will briefly introduce *Miscanthus* and sugarcane, two of the leading potential bioenergy crops being considered around the world.

## 1.2 - $C_4$ photosynthesis

In general, the $C_4$ photosynthetic pathway is more efficient and productive than $C_3$ photosynthesis, because the $C_4$ pathway is able to raise $CO_2$ levels around Rubisco (ribulose 1, 5 biphosphatase carboxylase/oxygenase) to operate near $CO_2$ saturation concentration, thus obtaining higher carboxylation activity rates than $C_3$ plants (Hatch, 1987; von Caemmerer & Quick, 2000). In $C_3$ plants, one of the main limitations on Rubisco is the capacity to react with oxygen in a wasteful reaction that produces compounds toxic to the plant (Forrester *et al.*, 1966; Moroney *et al.*, 2013; Lin *et al.*, 2014). Rubisco affinity for $O_2$ relative to $CO_2$ increases with temperature. Thus, photosynthetic efficiency in $C_3$ plants is reduced as temperature increases at current $CO_2$ and $O_2$ levels (Osmond, 1981; Sharkey, 1988). This is the case for the $C_3$ pathway, a pathway that lacks a $CO_2$ concentration mechanism around Rubisco.

*Rubisco, photorespiration and the evolution of $C_4$ photosynthesis*

The terms $C_3$ and $C_4$ refer to the number of carbons in the first stable product of the photosynthetic fixation of $CO_2$: phosphoglycerate (PGA) in the $C_3$ pathway and oxaloacetate and/or aspartate in the $C_4$ pathway (Furbank, 2011; Covshoff *et al.*, 2014; Wang *et al.*, 2014). In the $C_3$ pathway, $CO_2$ reacts with Ribulose 1, 5 biphosphate (RuBP) in a reaction catalyzed by Rubisco (Figure 1.1). The resulting product, PGA, is metabolized to triose phosphates (TP). About 5/6 of the TP produced are used to regenerate the RuBP (Figure 1.1). The remaining TP is converted into sugars. This cycle is known as the Calvin-Benson cycle or photosynthetic carbon reduction (PCR cycle) (Taiz & Zeiger, 2006).

Due to the electrostatic similarity between $CO_2$ and $O_2$, Rubisco fails to discriminate between oxygen and $CO_2$, thereby promoting the oxygenation of RuBP, which results in one molecule of PGA and one phosphoglycolate (PG). The carbon diverted to PG by the oxygenation of RuBP is recovered in a process called the photorespiratory oxidative cycle or simply photorespiration (PCO; Figure 1.1) (Tolbert, 1997). PG is a two-carbon molecule that is toxic for the plant at high concentration and inhibits the regeneration of RuBP and starch decomposition (Bauwe *et al.*, 2010). Thus PG is converted to glycine in the peroxisomes, and then glycine decarboxylation (GDC) converts the glycine to serine releasing $CO_2$ in the mitochondria. Recovery of carbon in the PG pool occurs at the expense of 2 ATP and 2 NADPH equivalents per $CO_2$ fixed (Tolbert, 1997).

When photosynthesis first evolved,over 2000 million years ago, Earth's atmosphere was composed of high $CO_2$ and low $O_2$ levels that made oxygenase rates insignificant to the photosynthetic process (Sage, 1999, 2004; Berner & Kothavala, 2001; Edwards *et al.*, 2010). It

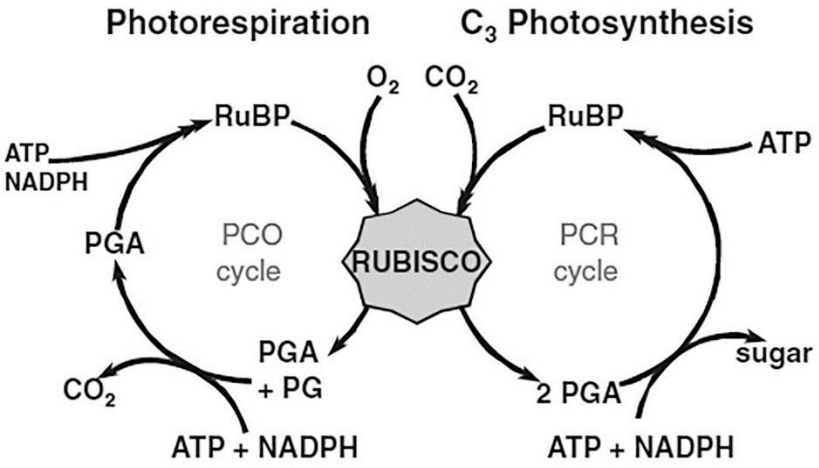



Figure 1.1 - Diagram of the $C_3$ pathway (photosynthetic carbon reduction = PCR cycle) and photorespiratoty carbon oxidative cycle (PCO). From Sage *et al.* (2014), modified from Tolbert (1997).

was estimated that in order for the oxygenation activity of Rubisco to become significant, $O_2$ concentration in the mesophyll (M) would have to be about 10 times higher than $CO_2$ concentration. That can only be achieved if $O_2$ concentration is 100 times greater than $CO_2$ concentration in the atmosphere due to their different solubility in the mesophyll (Jordan & Ogren, 1984; von Caemmerer & Quick, 2000). In the late Oligocene (around 25-30 million years ago), $CO_2$ levels decreased to 200-400 µmol $CO_2$ $mol^{-1}$. Temperature also increased, favoring Rubisco oxygenase activity, and thus photorespiration (Petit *et al.*, 1999; Zachos *et al.*, 2008; Gerhart & Ward, 2010). High temperature also favours $O_2$ solubility more than $CO_2$ solibility in the mesophyll, increasing firther the $O_2$ concentration around Rubisco (Jordan & Ogren, 1984). Although costly, the photorespiratory pathway is a solution to recover some carbon that would be lost by the oxygenation of RuBP (Moroney *et al.*, 2013).

*The $C_4$ cycle*

There are different variations of the $C_4$ cycle, mainly due to the form of decarboxylation that occurs in the bundle sheath (Furbank, 2011; Wang *et al.*, 2014). The classical NADP-ME sub-type, first described by Hatch & Slack (1966), is present in most of the plants with agronomical and bioenergetic value, such as maize, *Miscanthus*, sugarcane, sorghum, *Andropogon* and most of

the Panicaceae grasses. In this $C_4$ sub-type, the $CO_2$ diffuses from the air to the mesophyll and is converted to $HCO_3^-$ by carbonic anhydrase (CA) (Figure 1.2). Phosphoenol pyruvate carboxylase (PEPCase) will then combine $HCO_3^-$ and PEP to form oxaloacetate, which will enter the chloroplast located in the mesophyll. There the oxalacetate is converted to malate by the enzyme malate dehydrogenase with the expense of one NADPH. Plasmodesmata allow the rapid transport of malate from the mesophyll to the bundle sheath through the bundle sheath cell wall. Once malate enters the chloroplast in the bundle sheath it is decarboxylated by NADP-malic enzyme, releasing $CO_2$, NADPH and pyruvate. The $CO_2$ + NADPH released will be used in the $C_3$ cycle that occurs inside the bundle sheath. The pyruvate returns to the mesophyll and PPDK will convert it to PEP, thus closing the cycle (Figure 1.2) (Taiz & Zeiger, 2006).

The $C_4$ cycle occurs at the expense of two de-phosphorylations from one ATP (2 ATP equivalents). Thus, under conditions where photorespiration is minimal, $C_3$ photosynthesis is advantageous (Ehleringer & Pearcy, 1983). It was postulated that this is the reason why $C_3$ plants dominate the landscape in cold climate regions (Ehleringer, 1978). However, some $C_4$ plants such as *Miscanthus*, switchgrass, *Spartina*, and *Muhlenbergia* can be successful in cold environments (Beale *et al.*, 1996; Sage *et al.*, 1999; Sage & Sage, 2002; Lee *et al.*, 2014).

*$C_4$ advantage on marginal lands: WUE and NUE*

In dry environments most plants closely regulate stomatal aperture to reduce water loss, which also reduces $CO_2$ influx to the mesophyll (Franco & Lüttge, 2002; Peixoto, 2007; Franco *et al.*, 2014). The low $CO_2$ concentration around Rubisco in $C_3$ plants results in high rates of photorespiration. In $C_4$ plants, because of the high specificity of PEPCase for $HCO_3^-$ the $C_4$ cycle is able to maintain a high rate of $CO_2$ transport to the bundle sheath even under low $CO_2$

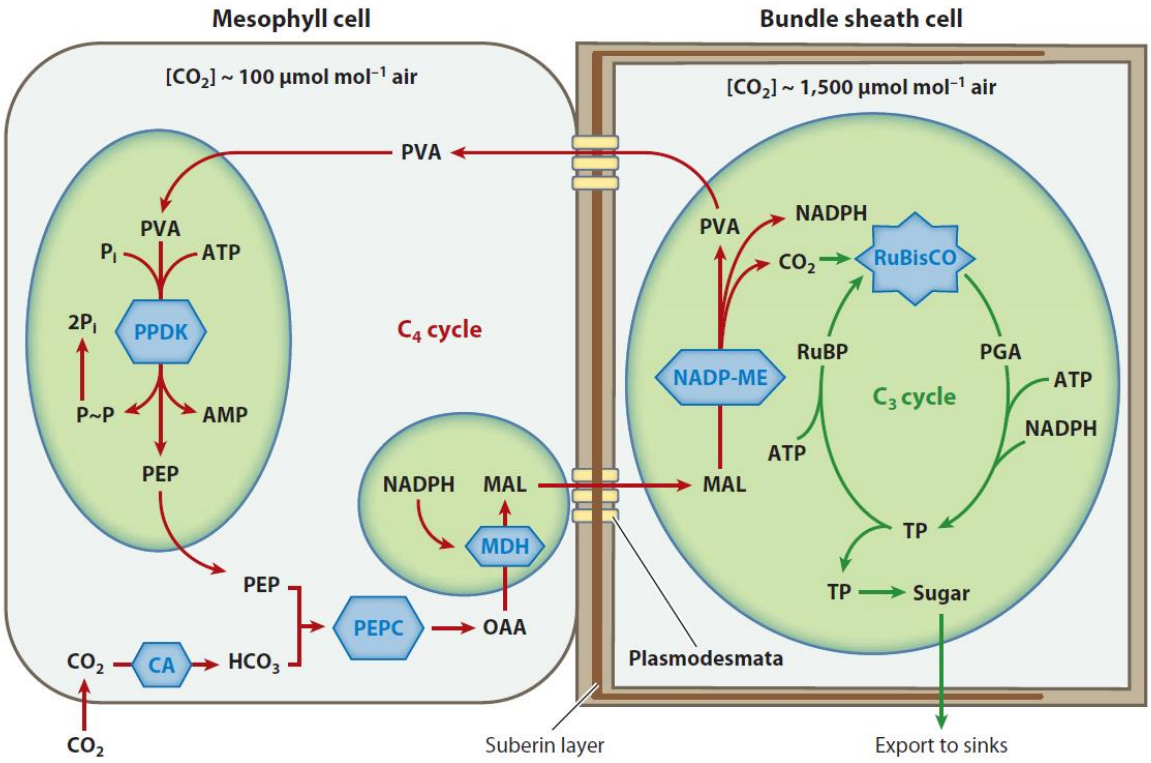



Figure 1.2 – NADP-ME $C_4$ photosynthetic pathway. Circles and oval shapes represent the chloroplasts. CA, carbonic anhydrase; PEP, phosphoenol pyruvate; PEPC, phosphoenol pyruvate carboxylase; OAA, oxaloacetate; MDH, malate dehydrogenase; MAL, malate; PVA, pyruvate; PPDK, pyruvate Pi-dikinase. Figure from Sage *et al.* (2012).

concentration in the mesophyll, reducing the sensitivity of $C_4$ photosynthesis to low stomatal conductance compared to $C_3$ plants (Sage *et al.*, 2012). Therefore, $C_4$ plants can fix more $CO_2$ per unit of water. This favors $C_4$ photosynthesis not only in dry but in saline environments, where water potential is low (Sage & Pearcy, 2000; Ripley *et al.*, 2007; Taylor *et al.*, 2014; Visser *et al.*, 2014).

$C_4$ plants also have an advantage in the use of N compared to $C_3$ plants. Because Rubisco operates under $CO_2$ saturation and with higher turnover rate, $C_4$ plants can maintain the same photosynthetic rate as $C_3$ plants with 70-80% less Rubisco than $C_3$ plants (Brown, 1978; Seemann *et al.*, 1984; Sage, 2002; Ghannoum *et al.*, 2005; Cousins *et al.*, 2010; Whitney *et al.*, 2011). Thus,

while in $C_3$ plants Rubisco accounts for 20% to 30% of the N invested in the leaf, in $C_4$ plants the investment in Rubisco is reduced to only 8% of leaf N (Wong *et al.*, 1985; Sage *et al.*, 1987; Evans, 1989; Makino *et al.*, 1992; Sage, 2002). The high N use efficiency reduces the need for fertilization and allows most $C_4$ plants to be present in poor and shallow soils (Sage *et al.*, 1999, 2014; Heaton *et al.*, 2009; Sage & Zhu, 2011; Cadoux *et al.*, 2012; Dohleman *et al.*, 2012).

### 1.3 - $C_4$ plants in a cold environment

Because $C_3$ plants dominate the landscape in cold climates, it was believed that $C_4$ photosynthesis is not compatible with cold temperatures (Long, 1983). Cold lability of the enzyme PPDK and the probability of photoinhibition (high light injury) at low temperature are some of the physiological processes that limit $C_4$ plants in cold environments (Sugiyama & Boku, 1976; Long, 1983, 1999; Du *et al.*, 1999a; Long & Spence, 2013). However, it has been recently proposed that $C_4$ plants are rare in cold regions because of phylogenetic constraints since the evolution of $C_4$ photosynthesis took place in warm climates (Edwards & Still, 2008; Edwards & Smith, 2010; Visser *et al.*, 2014).

Some $C_4$ plants can acclimate to low temperature growth or even to sudden chilling episodes and maintain a relatively high photosynthetic rate (*A*) at low temperature (Pearcy, 1977; Du *et al.*, 1999b; Purdy *et al.*, 2013; Friesen *et al.*, 2014). The ability to acclimate to sudden and prolonged drop in temperature was previously studied in upland Hawaiian sugarcane and some *Miscanthus* genotypes (Friesen *et al.*, 2014). After exposure for 24 hours at 12º/5ºC day/night temperature upland sugarcane grown at 25º/20ºC showed greater impact on the $CO_2$ assimilation rate (*A*) than *Miscanthus x giganteus* (Mxg) also grown at 25º/20ºC; after 7 days at this chilling temperature, when the temperature was returned to 25º/20ºC little recovery of *A* was observed in sugarcane compared to *M. x giganteus* (Friesen *et al.*, 2014). *Miscanthus x giganteus* grown at

14ºC or at 25ºC show similar photosynthetic response to temperature, showing the great acclimation capacity of this plant to low temperatures (Naidu *et al.*, 2003)

In most comparisons between $C_3$ and $C_4$ plants, $C_3$ plants have better photosynthetic performance at low temperature (generally below 20ºC) while $C_4$ plants have improved productivity at higher temperatures (Sage *et al.*, 2011). Yet, $C_4$ plants are present in cold climates. On a plant from alpine region grown at moderate temperatures (20ºC - 25ºC), the $C_4$ *Muhlenbergia richardsonis* had a better photosynthetic rate than the $C_3$ *Koeleria macrantha* and *Carex helleri* above 15ºC (Sage *et al.*, 2011). Under field conditions, the $C_3$ grass *Sesleria albicans* from the arctic-alpine region did not outperform the cold adapted $C_4$ *Spartina anglica*, or $C_4$ Mxg grown at 14ºC at any measured temperature (Long *et al.*, 1975; Long & Woolhouse, 1978; Naidu *et al.*, 2003). Mxg also had an advantage in light-saturated photosynthetic rate ($A_{sat}$) and in daily canopy $CO_2$ uptake ($A_c$) when compared to the arctic-alpine $C_3$ grass *Sesleria albicans* (Long, 1999). Osborne *et at.* (2008) also showed no advantage of the $C_3$ subtype of *Alloteropsis semialata* compared to the $C_4$ subtype of the same species, even at lower temperatures.

The physiological limitation of $C_4$ photosynthesis under low temperature results from the lability and activity of the enzymes PPDK and/or Rubisco. In desalted extracts from sugarcane leaves, PPDK activity was 35% less in measurements at 15ºC relative to measurements at 23ºC, and 70% less if the shift was to 5ºC (Du *et al.*, 1999a). In cold-adapted $C_4$ plants, PPDK is either in a cold-stable form, like in *Flaveria brownii* (Burnell, 1990), or increased in concentration as in Mxg (Naidu & Long, 2004; Wang *et al.*, 2008a). However, in the cold-adapted *Muhlenbergia montana* and *Bouteloua gracilis* the *in vitro* activity of fully activated Rubisco matches the *in vivo* gross photosynthesis at temperatures below 20ºC, indicating strong control of Rubisco at these temperatures (Pittermann & Sage, 2000, 2001; Kubien *et al.*, 2003). There is also evidence that

under low temperatures, $CO_2$ leaks from the bundle sheath, indicating that Rubisco capacity to consume $CO_2$ is lower than the $C_4$ cycle capacity to deliver $CO_2$ to the bundle sheath (Kubien *et al.*, 2003).

### 1.4 - Photoinhibition

In leaves, photons are absorbed by the photosynthetic pigments (mostly chlorophyll *a* and *b*), which are located in the thylakoid membrane of the chloroplast. The excitation energy absorbed from the photon is transferred to the photosystem II reaction center (PSII), which in turn, offers three different pathways for this available energy: photochemistry, dissipation as heat, or fluorescence (Krause & Weis, 1991). In the photochemical process, the excitation energy is transferred to electrons that are then transported through the electron transport chain. These electrons originate from the splitting of water, which releases $O_2$ and $H^+$ to the thylakoid lumen. The $H^+$ derived from water splitting and from co-transport via oxidation and reduction of plastoquinones increases the pH gradient across the thylakoid membrane, which then drive ATP synthesis. The products of the photosynthetic light reactions, ATP and NADPH, power the biochemical reactions of photosynthesis (Lambers *et al.*, 1998). If the absorbed energy cannot be used for photochemistry, it will be dissipated as heat or by emitting fluorescence. For simplification, this thermal dissipation is divided into non-photochemical quenching (NPQ) and constitutive quenching, and these are of vital importance to the protection of the plant.

Early development of leaves in the beginning of the spring gives plants a significant advantage in the formation of a canopy to capture maximum sunlight on warm days in late spring. However, under low temperature, the consumption of ATP and NADPH by the Calvin cycle is dramatically reduced, causing a feedback on photosynthetic electron transport (Long *et al.*, 1983;

Powles, 1984; Krause, 1988). The lack of consumption of the products from the light reactions are particularly aggravated in $C_4$ plants because of their restriction of photosynthesis at low temperature by Rubisco capacity which becomes a strong limitation (Sage, 2002; Kubien *et al.*, 2003; Sage & McKown, 2006; Sage *et al.*, 2011) and due to the lack of alternative sinks of dissipation for this energy (Long, 1983; Long *et al.*, 1983; Kubien & Sage, 2004a). If the excitation energy from the sunlight cannot be used in photochemistry, it will result in a buildup of energy (excitation pressure) which must be dissipated to prevent the formation of reactive oxygen species (ROS) (Ledford & Niyogi, 2005; Demmig-Adams & Adams-III, 2006; Murata *et al.*, 2012). ROS are formed when an excited electron is transferred to an $O_2$ molecule, generating super-oxide, which is an unstable and reactive molecule.

In general, non-photochemical quenching (NPQ) is responsible for the primary portion of photosynthetic photoprotection by dissipating excess energy as heat. NPQ can be regulated by the pH gradient and indirectly by light or sustained. Regulated NPQ is mediated by the xanthophyll cycle which is rapidly reversible (Demmig-Adams, 1990; Demmig-Adams & Adams, 1992, 1996; Ruban *et al.*, 2007). It can also be called flexible NPQ or dynamic photoinhibition, because it can easily be reversed, yet when activated it competes for the excitation energy with photochemistry and thus reduces the efficiency in the use of light energy. In the presence of light, the trans-thylakoid pH gradient is built up, and in association with the protein PsbS causes the fast conversion of violaxanthin (V) to antheraxanthin (A) and finally to zeaxanthin (Z) (Li *et al.*, 2002a, 2004; Niyogi *et al.*, 2005). At high light, the de-epoxidated xanthophylls antheraxanthin and zeaxanthin are responsible for the dissipation of the excess excitation as heat, and at low light they can be quickly re-converted to the inactive form (violaxanthin) (Demmig-Adams, 1990; Demmig-Adams & Adams, 1996).

The sustained non-photochemical quenching or chronic photoinhibition is a slowly reversible form of NPQ that may or may not be associated with light and pH gradient, but rather with constitutive changes to the PSII reaction center (Long *et al.*, 1994; Gilmore, 1997; Demmig-Adams *et al.*, 2006; Ensminger *et al.*, 2006). If the energy captured from sunlight cannot be used in photochemistry or dissipated by flexible NPQ, this excess excitation energy may cause configuration modifications or even damage to proteins of the PSII reaction centers (Long *et al.*, 1994; Melis, 1999; Allakhverdiev *et al.*, 2005; Murata *et al.*, 2007). These alterations to PSII cause it to lose its functional properties and represent a damaged-induced form of photoinhibition (Ensminger *et al.*, 2004; Ebbert *et al.*, 2005). To be reversed, the sustained photoinhibition requires the repair of PSII, which is slowed by low temperature (Demmig-Adams & Adams-III, 2006; Kato & Sakamoto, 2009).

Because cold-tolerant $C_4$ plants lack sufficient sinks for absorbed light energy they appear to produce large amounts of xanthophyll cycle pigments when grown under low temperatures, particularly in *Miscanthus* (Haldimann *et al.*, 1995; Leipner *et al.*, 1997; Kubien & Sage, 2004a; Farage *et al.*, 2006). Kubien & Sage (2004a) found that the cold-adapted $C_4$ *Muhlenbergia glomerata* grown at 14ºC produces twice the amount VAZ (xanthophylls) compared to the ones grown at 25ºC. In addition, *M. glomerata* grown at 14ºC produced 73% more of these carotenoids than the cold-adapted $C_3$ *Calamagrostis canadensis* grown under the same conditions (Kubien *et al.*, 2001; Kubien & Sage, 2004a). At 14ºC, *Miscanthus x giganteus* does not appear to significantly increase the amount of these pigments compared to plants grown at 25ºC; however, when grown at 10ºC *Miscanthus* plants nearly double the amount of VAZ (Farage *et al.*, 2006). This lack of response of VAZ production in *Miscanthus* grown at 14ºC might be an indication that at this temperature this plant can maintain high photosynthetic rates, which serves as a sink for the

absorbed light energy. Still, at chilling conditions (14ºC) *Miscanthus* leaves increased transcriptional responses of genes related to light harvesting complex, PsbS, D1 protein, chloroplasts, cytochrome f, among others related to the electron transport chain (Spence *et al.*, 2014). To complement these studies, detailed fluorescence analysis of *Miscanthus* responses to chilling under field conditions are needed. Such work would establish an understanding of how plants can cope with the excess energization on cold-sunny days, which is when photoinhibitory damage is most likely.

Investigation into the extent to which these plants are protected from sudden temperature changes in the field is still needed. This should establish a reliable threshold of the magnitude of excessive-energization stress which this plant can cope with and identify the environmental envelope in which this plant can be successful. In Chapter 4, I will focus on the partitioning of light absorbed by PSII in *Miscanthus*, which can be easily measured using chlorophyll fluorescence techniques.

### 1.5 - Low-temperature tolerance

In this thesis, I will examine two different types of low temperature tolerance: freezing or frost tolerance, which is when there is ice crystal formation under sub-zero temperatures; and chilling tolerance, which is the tolerance to temperatures between 0ºC and 15ºC. In general, there are two different strategies to overcome exposure to sub-zero temperatures: freezing avoidance and freezing tolerance (Levitt, 1980; Guy, 1990; Banuelos *et al.*, 2008). Freezing avoidance consists of avoiding ice formation in the tissue by increasing the solute concentration and producing supercooling compounds to prevent ice nucleation (Ruelland *et al.*, 2009; Wisniewski *et al.*, 2009). Freezing-avoiding species (i. e. with supercooling capacity), such as some boreal forest trees, can

maintain the cellular solution in the liquid phase even at -40°C when the plant is cold acclimated (Fujikawa & Kuroda, 2000; Kuroda *et al.*, 2003). However, in freezing-tolerant plants ice formation begins near 0°C in the extracellular spaces; as the temperature gradually drops the ice crystals grow removing water from the intracellular space and causing dehydration of the cell (Gusta & Wisniewski, 2013). Tolerance of ice crystal formation and cellular dehydration are the biggest assets for plants with this strategy (Levitt, 1980; Banuelos *et al.*, 2008; Gusta *et al.*, 2009). Steffen *et al.* (1989) found in leaves of wild potatoes that supercooling to -4.5°C caused minimal damage, while more damage was observed in plants cooled to the same temperature when ice formation occurred near 0°C. However, when ice formation was induced in supercooled leaves, the flash ice nucleation caused a great amount of damage. Although supercooling prevents injury better than the freeze-tolerating strategy, if the temperature drops below the supercooling threshold of that particular plant tissue, the consequences of the flash nucleation are disastrous.

The ability of plants to survive winter depends on their capacity to physiologically acclimate to low temperature. Cold acclimation has basically two phases: one occurs as soon as the temperature drops to near 0ºC, and the other occurs under sub-zero temperatures (Weiser, 1970; Gusta & Wisniewski, 2013; Chen *et al.*, 2014). Once the temperature approaches 0ºC, a series of molecular changes occur to promote the stabilization of the proteins and lipids of cell membranes, to increase the degree of dehydration of the cell and the detoxification of reactive oxygen species (ROS) (Steponkus, 1984; Gusta *et al.*, 2003; Verslues *et al.*, 2006; Chen *et al.*, 2014). There are two gene families responsible for cold acclimation including regulating and functional genes (Thomashow, 1999). The main regulatory genes are the C-repeat binding factor (CBF) family, cold regulated genes (COR) and late embryogenesis abundant (LEA) genes (Lucau-Danila *et al.*, 2012; Al-Issawi *et al.*, 2013). Upon the initial cold stress, CBF genes are expressed and their

transcripts activate the expression of the dehydration responsive element/C-repeat genes (DRE/CRT), which in turn stimulate the expression of COR and LEA genes (Gilmour *et al.*, 1998; Leubner-Metzger *et al.*, 1998; Fowler & Thomashow, 2002). This will activate the expression of the functional genes responsible for increasing the concentration of sugars and other solutes in the apoplastic space, improving antioxidative mechanisms, producing abscisic acid, accumulating dehydrins, and rearranging the lipids in the membranes (Stone *et al.*, 1993; Thomashow, 1999; Reyes *et al.*, 2008; Ozden *et al.*, 2009).

In orchard grass (*Dactylis glomerata* L.) the increase of lipid unsaturation and glycopeptide composition of the plasma membrane during the first phase of cold acclimation is associated with a shift in $LT_{50}$ (lethal temperature for 50% of the crop) from -8ºC in October to -18.0ºC in December (Yoshida & Uemura, 1984). Sugar accumulation enhances freezing tolerance in *Miscanthus* (Purdy *et al.*, 2013), *Arabidopsis* (Kaplan & Guy, 2004, 2005; Yano *et al.*, 2005), maize (Hodges *et al.*, 1997), sunflower (Paul *et al.*, 1991), and sugarcane (Du & Nose, 2002). Dionne *et al.* (2001) found an increase in sugar composition during the cold acclimation process of annual bluegrass (*Poa annua* L.); specifically, there was an increase in the concentration of sucrose and the number of fructans with high molecular weight.

Sub-zero acclimation is observed in alfalfa (Castonguay *et al.*, 1993, 1995; Monroy *et al.*, 1993), in cereals (Olien & Lester, 1985; Livingston III & Henson, 1998; Livingston III *et al.*, 2005; Herman *et al.*, 2006), and in *Arabidopsis* (Livingston III *et al.*, 2007; Le *et al.*, 2008), where plants are sub-zero acclimated at -2ºC or -3ºC. Upon long exposure to freezing temperature the activity of the ROS removal system is reduced (Chen *et al.*, 2014). However, some plants have the capacity to simultaneously convert starch into soluble sugars that act as osmotic regulators, thus stabilizing

the cell membrane stability that otherwise would be disrupted by ROS (Jonak *et al.*, 1996; Kang & Saltveit, 2002; Chen *et al.*, 2014). In alfalfa (*Medicago sativa*) that after two weeks that plants were transferred from 2ºC to -2ºC, the transcripts of *cas*15 (cold acclimation-specific) were increased when compared to plants maintained at 2ºC, indicating that a genetic component involved in freeze cold acclimation exists (Monroy *et al.*, 1993).

There is evidence that sub-zero acclimation is also related to the meristematic tissue. In silver birch (*Betula pendula* Roth), Li *et al.* (2002b) showed that buds and young leaves acquire freezing tolerance faster than the stem (which has fewer meristematic cells) and it becomes more cold tolerant than other parts of the plant. Wanner & Junttila (1999) found in *Arabidopsis* that the younger leaves (which have more meristematic cells) are more freezing tolerant than older leaves. Meristematic regions may have high accumulation of substances associated with freezing tolerance on the plant buds, including LEA-like proteins, carbohydrates and proline (Kaurin *et al.*, 1981; Salzman *et al.*, 1996; Wanner & Junttila, 1999; Purdy *et al.*, 2013).

Cold resistance of biofuel candidates has been studied for introduction on cold, unproductive lands. Willow (*Salix sp.*) has grown well in Scandinavia (Rosenqvist & Laakso, 1992), and its aerial parts can tolerate temperatures as low as -35ºC (Ogren, 1999). Hope & McElroy (1990) report switchgrass crowns surviving temperatures between -19ºC to -22ºC, although in their freezing experiments plants were sampled and thawed as soon as the temperature reached the nadir (lowest) temperature, allowing little time for adjustments at it. Lee *et al.* (2014) mention different varieties of Switchgrasses that are present in extremely cold areas in North Dakota, although soil temperature is not reported. In Europe, *Miscanthus* rhizomes tolerated temperatures as low as -3.4ºC to -6.5ºC depending on the genotype (Clifton-Brown &

Lewandowski, 2000a). The $C_4$ rhizomatous praire grasses *Spartina gracilis* and *Spartina pectinata* showed tolerance to -29ºC and -25ºC, respectively (Schwarz & Reaney, 1989; Friesen *et al.*, 2015). Cold-adapted strains of sugarcane and maize, which are primarily tropical, have been selected for study, but, the geographic ranges for these species are still limited by their intolerance to low temperature (Du *et al.*, 1999b; Du & Nose, 2002; Naidu *et al.*, 2003; Wang *et al.*, 2008b).

### 1.6 - Testing low temperature tolerance

Given the importance of low temperature tolerance for bioenergy crops that are being considered for cold climates, reliable methods are necessary to determine the threshold of crop survivability. Many low-temperature tolerance tests are reported in the literature, but there is little uniformity between them, and there is no clear explanation regarding the rationale for a certain procedure. Thus, in Chapter 2 I will describe the development of a methodology to properly test the low temperature tolerance of *Miscanthus* rhizomes. Here, I will review some of the important factors to be considered when promoting an artificial freezing test and the different methods to assess the injuries caused by low temperature.

*Artificial freezing experiment*

Artificial freezing experiments are intended to determine the cold tolerance of plants by reproducing natural-like temperature events. Water status, cooling and thawing rate, ice nucleation, and incubation time at the nadir exposure temperature are important factors that considered in the experimental design (Verslues *et al.*, 2006; Gusta & Wisniewski, 2013; Hincha & Zuther, 2014). Water status affects tissue solute concentration, which in turn influences cold acclimation (Steponkus, 1984; Gusta *et al.*, 2009). Small increments in solute concentration and/or

dehydration of plant tissues are part of the primary responses of the cold acclimation process (Hodges *et al.*, 1997; Kaplan & Guy, 2004, 2005; Verslues *et al.*, 2006). Low water concentration results in delayed ice nucleation which is the change in state of water from liquid to solid, that promotes ice crystal formation. Hence, impacts on the natural water status of plant tissue can affect experimental results. In the absence of ice nucleation, ice crystal growth in the apoplast is hindered (Fujikawa *et al.*, 2009; Gusta *et al.*, 2009; Wisniewski *et al.*, 2009). If nucleation is triggered in a supercooled tissue, flash ice formation can occur within cells, thus damaging the tissue (Steponkus, 1984; Wisniewski *et al.*, 2009).

A slow cooling rate coupled with early ice nucleation causes less damage to the plant tissue than either slow cooling with late ice nucleation or a fast cooling rate with either early or late ice nucleation (Steffen *et al.*, 1989; Verslues *et al.*, 2006; Gusta & Wisniewski, 2013). Regardless of whether cooling is slow or fast, late ice nucleation promotes flash ice formation without proper orientation of the ice crystals, and it can disrupt the membrane systems of cells (Steffen *et al.*, 1989; Ruelland *et al.*, 2009; Wisniewski *et al.*, 2009). Further, if a fast cooling rate with early ice nucleation is induced - typically by adding a nucleating agent such as ice or a salt crystal - the combination can cause more damage due to rapid dehydration of the cell (Steponkus, 1984; Verslues *et al.*, 2006). Dropping the temperature in small increments ensures that the sample temperature will be similar to the chamber temperature, and it may allow for acclimation; however, in some cases, natural freezing cycles are too fast to allow for acclimation. This is more of a problem for aboveground tissues that often experience rapid changes in air temperature (Ball *et al.*, 2006; Bykova & Sage, 2012). The thawing process can also injure the tissue. Rapid thawing promotes a high water flux across the cell membrane, which can cause lysis due to expansion (Gusta *et al.*, 2009). Rapid thawing may also cause the formation of bubbles by gas dissolved in

the solution, leading to cavitation in xylem conduits and cellular disruption (Steponkus, 1984; Ball *et al.*, 2006).

Different cooling rates have been used in freezing experiments to reproduce various environmental situations. When cooling leaflets of *Solanum commersonii* at rates of 1º and 6ºC $hr^{-1}$, slow cooling rates were found to produce less damage (Steffen *et al.*, 1989). Many authors use relatively fast cooling rates such as 4º to 20°C $hr^{-1}$ in their artificial freezing experiments (Webb *et al.*, 1994; Clifton-Brown & Lewandowski, 2000a; Márquez *et al.*, 2006; Bykova & Sage, 2012). A cooling rate of 10ºC $hr^{-1}$ was used to supercool leaf discs in mature stands of mixed chaparral plants, and 1ºC $hr^{-1}$ for treatment without supercooling (Boorse *et al.*, 1998). Ball *et al.* (2006) used 2ºC $hr^{-1}$, which is the natural field cooling rate for leaves of *Eucalyptus pauciflora*. Other experiments used 3ºC $hr^{-1}$ in *Miscanthus* leaves (Farrell *et al.*, 2006), 4.5ºC $hr^{-1}$ in leaves of *Bromus tectorum* and *Bromus rubens* (Bykova & Sage, 2012), 5ºC $hr^{-1}$ for shoots of *Picea rubens* (Murray *et al.*, 1989), and 12ºC $hr^{-1}$ for leaves of 9 species of grasses in the Venezuelan Andes (Márquez *et al.*, 2006). The use of fast cooling rates and short incubation time may be valid when simulating the coldest winter night in aerial plant parts, which are exposed to sudden atmospheric changes. When considering below ground parts, sudden changes are not likely to occur (Sharratt, 1993; Carey & Woo, 2005; Henry, 2007). Therefore, slower cooling rates and longer incubation time at the treatment temperature are more appropriate for roots and rhizomes, as they better reflect natural conditions.

Because *Miscanthus* overwinters as below-ground rhizomes, which are well-insulated by the soil, their cooling rate in the field is generally slow compared to above-ground tissues. Even an experimental cooling rate of 1°C $hr^{-1}$ may still be fast compared to normal conditions (Henry,

2007). Due to the soil and snow pack insulation, underground temperatures do not experience drastic changes at high latitudes with different winter air temperatures even at 5 cm deep in the soil (Henry, 2007). Clifton-Brown & Lewandowski (2000a) used 3ºC $hr^{-1}$ as a cooling rate for *Miscanthus* rhizomes. Also, Schimming & Messersmith (1988) used the rate of 1ºC $hr^{-1}$ to cool roots of Canada thistle and perennial sowthistle, roots and crowns of leafy spurge, and rhizomes of quackgrass, collected in frozen soil in North Dakota, USA.

Incubation time at the nadir temperature is also a key factor that must be addressed in a freezing experiment. Too short of an incubation time may not capture the harmful effects of the nadir temperature that plants experience in the field. The distribution of ice crystals in the tissue is not uniform, so not all cells come into contact with it (Ashworth, 1992). Thus, time is necessary for the cold to penetrate all the cells, and for water movement from the cell to allow apoplastic ice crystal formation. When no incubation time was allowed, *Brassica napus* plants could tolerate temperatures between -17ºC and -19ºC (Waalen *et al.*, 2011); however, most plants were killed if incubated at -8ºC for 12 days (Waalen *et al.*, 2011). In some cases long exposure to a sub-zero temperature can further increase cold acclimation (Monroy *et al.*, 1993; Livingston III *et al.*, 2007). In Chapter 3, I examine the effects of acclimation to subzero temperatures in four *Miscanthus* genotypes.

*Assessing freezing tolerance*

Several methods can be used to assess freezing injury in plants. The most common are (a) the triphenyltetrazolium chloride method, (b) leaf chlorophyll fluorescence, (c) quantification of reactive oxygen species, (d) electrolyte leakage, and (e) re-growth assays and visual scoring. I briefly review these methods below.

The triphenyltetrazolium chloride method consists of measuring the formation of red formazan by the reduction of tetrazolium salts in mitochondria (Iborra *et al.*, 1992; Fryer, 1998). Tissues that suffer greater damage due to freezing form less red formazan and stain less. The relative number of living cells after the treatment is estimated by the ratio of the absorbance reading of the treated sample compared to a non-treated control (Steponkus & Lanphear, 1967; Towill & Mazur, 1975; Iborra *et al.*, 1992). This is one of the best methods used for dormant tissues, although a major limitation is that photosynthetic pigments can interfere with absorbance readings, giving potentially inaccurate results.

Leaf chlorophyll fluorescence is a non-invasive assay to rapidly screen photosynthetic tissues for chilling and freezing injury in the lab or in the field (Fracheboud *et al.*, 1999; Rizza *et al.*, 2001; Bykova & Sage, 2012). Low temperatures can disrupt photosynthetic membranes that results in irreversible loss of variable fluorescence detected using any type of fluorescence protocol (Meyer *et al.*, 2001; Chaerle *et al.*, 2004). However, this method is only functional with photosynthetic tissues, and therefore it has little utility for screening below-ground winter dormant tissues in perennials.

Assays that measure the accumulation of reactive oxygen species (ROS) are complex and somewhat indirect. This approach estimates cell mortality by quantifying the production of ROS; high ROS concentration is likely to produce higher cell death (Jambunathan, 2010). Low temperature stress increases ROS production in plant cells (Suzuki & Mittler, 2006). ROS is generally quantified by staining the tissue, usually with 2'-7'-dichlorofluorescein, but, ROS are reactive and unstable so staining procedures can produce inconsistent results (Jambunathan, 2010). To reduce this variability, other approaches including measurement of electrolyte leakage,

chlorophyll fluorescence, and luminescence are also used to quantify ROS. The need for these additional assays – that can directly assess cold injury - reduces the value of the ROS method (Jambunathan, 2010).

The electrolyte leakage assay has been widely used to assess cold injury because it is simple, relatively fast, and useful for large sample sizes (Murray *et al.*, 1989; Gusta *et al.*, 2003, 2009; Verslues *et al.*, 2006). It works with the premise that cold-damaged tissues lose membrane integrity and will hence leak electrolytes. Following cold treatment, the sample is infused in deionized water to dissolve the electrolytes in the bathing solution. After 24 hours, the electrolyte conductivity is measured using a conductivity meter. Next, the bathing solution with the sample is boiled or frozen in liquid nitrogen to release all remaining electrolytes into the bathing solution, enabling a measure of final, or total, electrolyte concentration. Relative conductivity is calculated as the ratio of initial versus final electrolyte content, and provides an estimate of relative cell damage caused by the treatment.

Re-growth assays can be the most reliable way to assess freeze-induced survivability in plant tissues, but it can be problematic with large sample sizes (Steponkus & Lanphear, 1967; Márquez *et al.*, 2006; Gusta *et al.*, 2009; Skinner & Garland-Campbell, 2014). The assay requires planting the sample and then determining vegetative growth from rhizomes segments with buds or roots after the freezing treatment. Although this provides a direct measurement of plant survival after cold treatment, results may be influenced by the dormancy of vegetative parts, which may be broken by artifacts. For non-regenerative organs, visual scorings can be used such that the visual characteristics of dead and living sample are observed and given scores that reflect the severity of injury (Boorse *et al.*, 1998; Livingston III *et al.*, 2007; Waalen *et al.*, 2011; Jurczyk *et al.*, 2013).

This is a subjective method and validity depends on clarity of differences and consistency of the researcher's judgement.

### 1.7 - *Miscanthus*

The "Giant" *Miscanthus* is a promising potential bioenergy crop due to its high productivity, cold and chilling tolerance, water and nitrogen use efficiency (Beale & Long, 1995, 1997; Clifton-Brown & Lewandowski, 2000a; Naidu *et al.*, 2003; Farrell *et al.*, 2006; Heaton *et al.*, 2008a; Lee *et al.*, 2014). *Miscanthus* is a genus with 11-14 species originating from southeastern Asia, and the most productive and studied species is *Miscanthus x giganteus* (Mxg), an allotriploid hybrid that naturally occurs in Japan (Clifton-Brown *et al.*, 2008; Heaton *et al.*, 2010; Jones, 2011; Sacks *et al.*, 2013). This species is a sterile hybrid from the diploid *M. sinensis* and the allotetraploid *M. sacchariflorus* (Hodkinson *et al.*, 2002). Since it is sterile, Mxg presents little risk of becoming invasive (Lewandowski *et al.*, 2003). However, the lack of sexual propagation increases the cost of establishment of this crop and leads to a narrow genepool that results in plants prone to diseases, insect infestation and stress events (Clifton-Brown & Lewandowski, 2000b; Lee *et al.*, 2014).

Although Mxg is a $C_4$ species with high water use efficiency, water can be a limiting factor and Mxg responds well to irrigation in some regions. In the United Kingdom, final biomass yield increased by 20% if the crop was irrigated on the second and third year after establishment of the crop (Beale & Long, 1997). Also in the UK, Richter *et al.* (2008) found a correlation of 70% between the soil water capacity and the final biomass yield in Mxg crops. In New Jersey, USA, a dry and hot year caused a reduction of 50% in the biomass yield of Mxg grown in poor soil conditions (Maughan *et al.*, 2012). It was also observed in different regions that the soil moisture content of *Miscanthus* crops is lower than switchgrass and maize-soybean crops, showing

increased utilization of water in the soil by *Miscanthus* (Beale *et al.*, 1999; McIsaac *et al.*, 2010). Although water may limit biomass production, *Miscanthus* is relatively drought resistant, and even after a long dry season it is able to re-grow once water status is re-established (Lee *et al.*, 2014).

*Miscanthus* is able to recycle nitrogen and other mobile nutrients by relocating it to the dormant rhizomes during leaf senescence, which reduces the need for fertilizer (Beale & Long, 1997; Davis *et al.*, 2009; Heaton *et al.*, 2009, 2010; Propheter & Staggenborg, 2010; Dohleman *et al.*, 2012). In general, continuous harvesting of a crop depletes the N content of the soil. However, *Miscanthus* can mobilize a greater part of the N back to the rhizomes such that it has an average C/N ratio of 142.6 in senesced biomass while switchgrass has C/N ratio of 95.9, when harvested in December in Illinois (Heaton *et al.*, 2009). This requires, however, that cold-tolerant lines properly senesce rather than being killed by a hard freeze. Nitrogen-fixing microorganisms have also been observed in association with *Miscanthus* roots (Eckert *et al.*, 2001; Miyamoto *et al.*, 2004). Adding N to the soil by applying fertilizer did not significantly increase biomass yields of *Miscanthus* crops in Germany, England, and Ireland (Himken *et al.*, 1997; Clifton-Brown *et al.*, 2007; Christian *et al.*, 2008). Heaton *et al* (2004a) examined 97 peer-reviewed studies about the effects of N in Mxg and concluded that the data did not show a significant change in *Miscanthus* crops when treated with fertilization. Eight years later Cadoux *et al.* (2012) suggested that there are still not enough data to support claims about the effect of fertilization in Mxg. Recently, Arundale *et al.* (2014b) showed in a long term trial in Illinois, USA, that biomass yield of *Miscanthus* increases significantly with fertilization depending on the soil type. However, the authors conclude that the increase in biomass yield does not pay back the cost of fertilization. In any case, while supplemental fertilization may be occasionally needed to maintain yields, the overall cost is well below that of annual crops such as maize.

A characteristic of *Miscanthus* that makes this plant one of the most promising bioenergy crops is its performance under low temperature. The high photosynthetic rates at low temperatures (10ºC 20ºC) relative to most $C_4$ crops, allows *Miscanthus* to sprout new leaves when temperatures are below 10ºC, and to create a canopy early in the spring, increasing growing season limits (Beale & Long, 1995; Heaton *et al.*, 2004b, 2008b; Farrell *et al.*, 2006; Zhu *et al.*, 2008). This contributes to high biomass yield in cool climates (Beale & Long, 1995). Good photosynthetic rates at low temperature also serves as a sink for electrons, thus protecting the plant from photoinhibition. *Miscanthus* maintains high amounts of xanthophyll, especially as zeaxanthin that helps in the protection against photoinhibition (Farrell *et al.*, 2006). In *Miscanthus* plants grown at 25ºC/20ºC day/night temperature, the triploids Mxg "Illinois" and "Nagara" (M116) showed smaller reductions of photosynthetic rate, carboxylation efficiency, and maximum quantum yield of photochemistry (Fv/Fm) than diploid and tetraploid *Miscanthus* genotypes after 6 days of exposure to 12º/5ºC day/night temperature (Friesen *et al.*, 2014). Supporting these findings, Głowacka *et al.* (2014) found that among 51 different *Miscanthus* genotypes, chilling for 11 days at 10º/5ºC resulted in smaller reductions of photosynthetic rate and PSII operating efficiency (Fq'/Fm') for Mxg (3n) and *M. sacchariflorus* (4n) compared to *M. sinensis* (2n). It was also observed that after a mild chilling event (12ºC), the triploid Mxg had the smallest decrease in the photosynthetic rate and in leaf extension rate than *M. sinensis* and *M. sacchariflorus*, while these later species had a significant increase in the expression of genes related to cold stress (Purdy *et al.*, 2013).

Although Mxg shows good performance under chilling events, its rhizomes have poor tolerance to sub-zero temperature compared to *M. sinensis* genotypes. In *Miscanthus* genotypes grown in sites in Denmark, England, Germany and Sweden, Mxg and *M. sacchariflorus* rhizomes were killed at -3.4ºC, while rhizomes of *M. sinensis* could survive to temperatures as low as -6.5ºC

(Clifton-Brown & Lewandowski, 2000a). Purdy *et al.* (2013) studied the same *Miscanthus* genotypes and showed that *M. sinensis* had greater expression of genes related to cold stress after exposure to 12ºC, while Mxg maintained higher photosynthetic rates. Possibly, this is a result of two different strategies: while Mxg uses its improved chilling tolerance and photosynthetic rate to increase the growing season and produce more biomass, *M. sinensis* invests in winter cold hardiness, increasing the freezing tolerance of the rhizomes.

Most of these studies were performed in regions where cold stress is not as extreme as Canada. More investigation about the limits of survivability of the new *Miscanthus* lines in high latitudes is needed. Chapters 3 and 4 of this thesis will investigate the freezing tolerance of *Miscanthus* rhizomes, and the resistance to chilling events on *Miscanthus* leaves.

### 1.8 - Sugarcane

On a biomass basis, sugarcane (*Saccharum spp.*) is the most productive crop in the world, and shows a global yield of 1661 Mt year$^{-1}$ (the second most productive is maize with 819 Mt year$^{-1}$) (Moore *et al.*, 2014). This plant was reported to have endophytic diazotrophic bacteria associated with its roots, fixing atmospheric N and making it available to the plant, which maintains high yield with less need for fertilization (Andrews *et al.*, 2003; Samson *et al.*, 2005). Thus, the high productivity associated with low inputs result in an energy output/input ratio between 3.0 and 4.0 compared with corn which is between 1.5 and 3.0 (Mrini *et al.*, 2001; Shapouri *et al.*, 2008; Yuan *et al.*, 2008). In Brazil, Macedo *et al.* (2008) and Goldemberg (2008) describe an even more optimistic scenario: in 2005/2006 the energy output/input for sugarcane was 9.3 and it is predicted to increase to 11.6 by 2020, taking into consideration the use of the bagasse and the electricity surplus.

Some authors observed that sugarcane has a high photosynthetic rate in young leaves, but it declines as the leaf develops, having rates comparable to $C_3$ species (Bull, 1969; Bull & Glasziou, 1975; Irvine, 1983; Allison *et al.*, 2007). Similar phenomena were observed for the leaf N content, where young leaves had high N content but that decreased in adult leaves (Bull, 1969; Stevenson *et al.*, 1992; Vu *et al.*, 2006; Allison *et al.*, 2007). It was concluded that the decrease in photosynthetic rates in adult leaves is due to the dilution of N in the leaf as the plant grows. If the plant is loaded with N, it can achieve similar photosynthetic rates as maize (Sage *et al.*, 2014). Thus, the sugarcane strategy appears to involve increasing the N use efficiency, producing a greater leaf canopy, thus having greater total $CO_2$ assimilation by the plant (Sage *et al.*, 2014).

Sugarcane is a long duration crop (10 to 24 months) with slow early development, but after the third month it grows exponentially forming a competent canopy to intercept solar radiation all year (Inman-Bamber, 1994; Allison *et al.*, 2007). That makes this crop one of the most efficient in intercepting solar radiation on a yearly basis (Inman-Bamber, 2014). Also, sugarcane has the capacity to maintain active leaves with high water use efficiency longer than most crops, reducing the cost of leaf production (Allen *et al.*, 1998).

Brazil is the country with the highest production of sugarcane. In Brazil, the Southern Center region of the country has an average yield of 78-80 Mg $ha^{-1}$ while in São Paulo state it is 80-85 Mg $ha^{-1}$, and it should reach 110 Mg $ha^{-1}$ before 2020 (Macedo *et al.*, 2008; Dias *et al.*, 2013; Galdos *et al.*, 2013). In an experimental field in Louisiana, USA, it was reported that sugarcane yielded between 41 to 100 Mg $ha^{-1}$ when it was treated with 135 Kg $ha^{-1}$ of N (Lofton *et al.*, 2012). In Australia, the maximum yield recorded was 72 Mg $ha^{-1}$ (Muchow *et al.*, 1994; Jones, 2011).

Since the 1970's, the sugarcane industry in Brazil has developed complete infrastructure for producing bioethanol. This includes a system for cultivation, irrigation, pest, disease and weed control, transportation and the power plant structure (Costa & Sodré, 2010; Botha & Moore, 2014). The shift to G2 crops producing lignocellulosic biomass requires a big change in most of this structure. For example, new sugarcane lines should invest in the production of fibers rather than in sucrose, fibrous plants should require more robust machinery for harvesting and changes in the processing plant to implement the technology to convert lignocellulose into ethanol (Dias *et al.*, 2013). Thus, to reduce the impact of the shift from G1 to G2 production, gradual investments in new technologies are being made to initially integrate the traditional G1 ethanol production using sucrose with extension to lignocellulose utilization from the bagasse and trash leaf in the field (G2) (Nigam & Singh, 2011; Dias *et al.*, 2012).

Because of the success of sugarcane as a bioenergy crop and its high productivity, efforts have been made to extend the ranges of production of this crop. These includes hybridization with chilling-tolerant species or genetic engineering to increase the tolerance to low temperature (Tai & Miller, 1996; Eggleston *et al.*, 2004; Belintani *et al.*, 2012). Alternatively, one could reduce the growing season length to avoid the winter (Eggleston & Legendre, 2003). Recently, breeding programs aimed at hybridizing sugarcane and *Miscanthus* have been made to associate the high productivity of sugarcane with the cold performance of *Miscanthus* (Jones, 2011; Lee *et al.*, 2014). However, numerous upland lines, such as these from Hawaii, already appear to have greater chilling tolerance (Tai & Miller, 1986, 1996). In Chapter 5, I investigate the capacity of upland and lowland Hawaiian sugarcane to acclimate to different temperatures, an important feature to maintain the high productivity of this plant in colder climates.

## 1.9 - Thesis objectives

The objective of this thesis is to study the physiological traits that make a plant an ideal bioenergy crop in marginal lands at high latitudes. In this thesis, using the potential bioenergy species *Miscanthus* I evaluate the freezing tolerance that allows winter survival (Chapters 2 and 3), the chilling tolerance that will allow early canopy development in the spring (Chapter 4), and the ability of photosynthesis in sugarcane to acclimate in a warm and cool summer (Chapter 5).

Chapter 2 evaluates methods to test freezing tolerance. Many freezing tolerance tests are described in the literature, but there is no consistency between them. To overcome this limitation, I performed a series of experiments to develop a protocol to evaluate the freezing tolerance in *Miscanthus* rhizomes. This chapter will be submitted as Peixoto & Sage to *Global Change Biology Bioenergy*.

Chapter 3 address the freezing tolerance of *Miscanthus* rhizomes. Two freezing protocols were used to test freezing tolerance: one tests the intrinsic freezing tolerance of the rhizome, and the other allows for additional cold acclimation of the rhizomes at sub-zero temperatures. Freezing tolerance is compared in *Miscanthus* hybrids with different ploidy because it was previously reported that low temperature survivability is different depending on the ploidy of the *Miscanthus* hybrid (Clifton-Brown & Lewandowski, 2000a). This chapter has been submitted as Peixoto, Friesen & Sage to the *Journal of Experimental Botany*.

Chapter 4 evaluates the chilling tolerance of young *Miscanthus* leaves in the field. Episodic chilling events are common in the spring in Canada. Low temperatures and high light can result in irreversible damages due to an excess of sun energy in the leaf (photoinhibition). Thus, using chlorophyll fluorescence, the stress caused by low temperatures in the spring was studied in

*Miscanthus* leaves. Part of this chapter was published as Friesen, Peixoto, Busch, Johnson & Sage (2014) in *Journal of Experimental Botany*, and a more complete version will be submitted in 2015, possibly to *Global Change Biology Bioenergy*.

In Chapter 5, photosynthesis acclimation to mild and warm temperature was studied in lowland and upland sugarcane varieties. Sugarcane was chosen for this study because it is the main Brazilian bioenergy crop, thus it has ability for the Brazilian Government, that generously granted a scholarship for my PhD studies through the Coordination for the Improvement of Higher Education Personnel (CAPES), to exploit the results. If sugarcane can be grown in higher latitudes or elevations, acclimation of photosynthesis would be important to maintain high productivity. Also, in São Paulo, the state with the biggest sugarcane production in Brazil, the mean day temperature between May and August is below 23ºC. Thus it is important that plants quickly acclimate to sub-optimal temperatures to maintain high productivity in these months, and then acclimate to warm temperatures for the rest of the year, when day temperatures commonly reaches 35º-37ºC (http://www.inmet.gov.br/portal/index.php?r=clima/normaisClimatologicas, assessed in 20 November 2014). This work will be submitted for publication in *BioEnergy Research*.

Table 1.1 - Conversion efficiencies and estimation of land requirements (in mega hectares = Mha) to produce the 60 billion of liters of ethanol mandated by US Energy and Security Act (EISA). Superscript indicate the references for the values cited. G1, first generation bioenergy production; G2, second generation bioenergy production.

| Crop | Bioenergy Generation | Max biomass yield (Mg $ha^{-1}$) | Location | Conversion rate (L $Mg^{-1}$) | Land (Mha) |
|---|---|---|---|---|---|
| *Miscanthus x giganteus* "Illinois" (3n) | Lignocellulosic Biomass G2 | 23[1] | Illinois,USA | 380[2] | 7[1] |
| *Panicum virgatum* "Cave-in-Rock" | Lignocellulosic Biomass G2 | 10[1] | Illinois,USA | 380[2] | 16[1] |
| *Miscanthus x giganteus* "Nagara" (3n) | Lignocellulosic Biomass G2 | 23[3] | Ontario, Canada | 380[2] | 7 |
| *Panicum virgatum* "Cave-in-Rock" | Lignocellulosic Biomass G2 | 9[3] | Ontario, Canada | 380[2] | 18 |
| *Spartina pectinata* "Red River" | Lignocellulosic Biomass G2 | 6.5[3] | Ontario, Canada | 380[2] | 24 |
| Sugarcane | Stem sucrose G1 | 85[4,5,6] | São Paulo, Brazil | 87[5] | 8 |
| Sugarcane | Stem sucrose G1 + Lignocellulosic Biomass | 85[4,5,6] | São Paulo, Brazil | 110[5,7] | 6 |
| Corn | Grains G1 | 10[8, 9] | Midwest USA | 418[8] | 14 |
| Corn | Grains G1 + corn stover G2 integrated | 18[8] | Midwest USA | 402[8] | 8 |
| | | | Total cropland in Ontario – Canada[10] | | 3642 |
| | | | Total cropland in Illinois – USA[11] | | 11331 |

[1] Arundale *et al.* (2014a); [2] US Department of Energy (2006); [3] Deen *et al.* (2011); [4] Macedo *et al.* (2008); [5] Galdos *et al.* (2013); [6] wet weight, where 52% is dry weight, 15% is moisture, and 33% is trash, as suggested by Thompson (1978) (cited by Waclawovsky *et al.*, 2010); [7] Dias *et al.* (2012); [8]

Heaton *et al.*(2008b); [9] Gallagher & Baumes (2012); [10] http://www.statcan.gc.ca/ca-ra2006/analysis-analyses/ont-eng.html, assessed on 18 November 2014; [11] http://www.agr.state.il.us/about/agfacts.html, assessed on 18 November 2014.

Table 1.2 - Estimation of the $CO_2$ release from the production of bioethanol produced from corn grains and sugarcane sucrose. Numbers consider variuous sources of $CO_2$ inputs to the production of ethanol. Numbers do not take in account the soil carbon stock change.

| | Biomass yield (Mg $ha^{-1}$) | Conversion rate (L $Mg^{-1}$) | Ethanol yield (L $ha^{-1}$) | $CO_2$ released (Kg $L^{-1}$ ethanol)[1] | $CO_2$ released (Mg $ha^{-1}$) |
|---|---|---|---|---|---|
| Sugarcane (Brazil) | 64[2] | 87 | 7,395 | 0.461 | 3.4 |
| Corn (USA) | 18[3] | 418[2] | 4,180 | 1.392 | 5.8 |

[1] Oliveira *et al.* (2005); [2] dry weight + trash, estimated as suggested by Thompson (1978) (cited by Waclawovsky *et al.*, 2010); [3] grains + corn stover; estimated as suggested by Heaton et al. (Heaton *et al.*, 2009).

# Chapter 2 - Improved experimental protocols to evaluate cold tolerance thresholds in *Miscanthus* rhizomes

## 2.1 - Abstract

This study developed experimental protocols to evaluate the cold tolerance thresholds for *Miscanthus* rhizomes. Ice nucleation occurred near -1ºC in the rhizomes cooled at 1ºC $hr^{-1}$, but at variable temperatures (-1ºC to -11ºC) under cooling rates of 3º and 5ºC $hr^{-1}$. A cooling rate of 1ºC $hr^{-1}$ was demonstrated to be suitable, because rhizome temperature closely followed the chamber temperature allowing for proper ice nucleation and proper incubation time at the nadir temperature. Less than 4 hours of incubation at the treatment temperature did not allow for full injury, while there was no change in injury when samples were incubated between 4 and 20 hours. While continuous cooling allowed for an estimate of the cold tolerance of the rhizome at that moment, lowering the temperature in stages reduced the $LT_{50}$ of diploid *Miscanthus* rhizomes to -12ºC. Thawing diploid rhizomes from -10ºC to 4ºC and incubating them for 24 hours at 4ºC was not sufficient to deacclimate them, and no additional injury resulted when treated at -10ºC immediately after thawing. Logistic regressions are shown to be more accurate for the estimations of $LT_{50}$ and $LEL_{50}$, allowing for effective estimation of mortality using the electrolyte leakage method.

**Abbreviations:** EL = electrolyte leakage. RC = relative conductivity. $LT_{50}$ = temperature at which the sample has a 50% mortality. $LEL_{50}$ = percentage of electrolyte leakage at which the sample has a 50% mortality.

## 2.2 - Introduction

The development of novel bioenergy crops will require evaluation of environmental tolerances to best match prospective cultivars with suitable climate zones. To exploit higher latitude landscapes of Canada, the northern USA and Eurasia, environmental evaluations will need to specifically determine cold-tolerance thresholds. This is particularly necessary in the case of perennial $C_4$ grasses, which can show superior productivity in the long warm summers of high latitudes but they typically originate in warm-temperate latitudes (Jones, 2011). In *Miscanthus*, which is a leading contender for bioenergy production in cool climates, the source material can range from upland regions of southern China to the cold regions of northeastern Siberia, such as along the Amuri river basin at latitudes exceeding 45°N (Clifton-Brown *et al.*, 2008; Anzoua *et al.*, 2011; Liu *et al.*, 2014; Sage *et al.*, 2015). Screening new lines generated from this potentially large gene pool will require rapid, efficient and comprehensive methods for assessing cold tolerance. The objective of this study is to evaluate protocols to rapidly assess cold tolerance of overwintering rhizomes from *Miscanthus* lines bred for cool climates.

The ability to evaluate thermal tolerance in plants has improved in recent years due to the advent of relatively sophisticated computer-controlled, temperature-test chambers. These chambers reduce the risk of artifacts in freezing tolerance trials, as the solid-state computers provide excellent control over cooling and heating rates. In larger test chambers, more plant material can be accommodated in the experimental treatment, allowing for more comprehensive screens of a greater variety of plants. For remote locations, miniaturized, portable test chambers can be used, thus enabling field studies *in situ* away from established labs. In addition, improvements in analytical equipment have expanded options for designing high-throughput

methods. New, smaller conductivity meters, for example, allow for smaller sample volumes and faster assay turnaround, while reducing costs and space requirements (Bykova & Sage, 2012). Together, these developments justify a reassessment of cold tolerance protocols, particularly in the context of assessing novel bioenergy stocks, such as varieties of *Miscanthus* bred for high latitude climates.

New tools and methodologies also allow for a re-evaluation of the ideal indexes of cold tolerance. The most widely used index of cold tolerance has been $LT_{50}$ (lethal temperature for 50% mortality), which has often been estimated by visual extrapolation to the temperature axis from plots of mortality (or electrolyte leakage) versus treatment temperature (Burr *et al.*, 1990; Gusta & Wisniewski, 2013). Instead of simple extrapolation, $LT_{50}$ should be considered a statistical parameter that can be estimated using regression procedures (Coiner, 2012). $LT_{50}$ assessments can also be slow and cumbersome given the need to regrow treated plants to establish viability. Re-growth of exposed tissues is widely used for the determination of $LT_{50}$ in studies of cold tolerance, (Palta *et al.*, 1982; Gilmour *et al.*, 1988; McKay, 1992; Boorse *et al.*, 1998; Ryyppö *et al.*, 2008; Skinner & Garland-Campbell, 2014; Thalhammer *et al.*, 2014). If whole plants are used, large chamber volume may be required to accommodate planted pots, and substantial greenhouse space may be needed for regrowth of the treated plants, which will drive up costs and limit the range of genotypes or conditions examined. The use of whole plants also increases the chance of artificially damaging tissues that normally do not experience extreme cold; roots for example are typically buffered from thermal extremes by soil and snow insulation (Farrell *et al.*, 2006; Henry, 2007; Ryyppö *et al.*, 2008; Gusta *et al.*, 2009). To overcome these limitations, accurate physiological assessments that can use small tissue samples are desirable. Electrolyte leakage (EL) has been

recommended as a suitable procedure where it is impractical to use whole plants (Eldridge *et al.*, 1983); however, discrepancies in $LT_{50}$ estimates have been observed between the EL and regrowth methods (Zhang & Willison, 1987; Teutonico *et al.*, 1993; Maier *et al.*, 1994; Boorse *et al.*, 1998; Dunn *et al.*, 1999; Waalen *et al.*, 2011). It is possible these discrepancies reflect incorrect assumptions of the lethal threshold for electrolyte leakage. Many EL studies use 50% electrolyte leakage as the critical viability threshold, although many plants perish after suffering more than 30% EL (Palta *et al.*, 1982; Bykova & Sage, 2012; Coiner, 2012). Given their potential utility but potential for error if an improper mortality threshold is assumed, EL protocols should be calibrated by regrowth protocols when first examining a new group such as *Miscanthus*. This can establish the EL value that corresponds to the mortality limit, thus allowing for high through-put screening of *Miscanthus* lines using small tissue samples.

In this study, I use regrowth and electrolyte leakage to assess cold tolerance thresholds in three *Miscanthus* genotypes, as well as to evaluate different procedural options in cooling rate, ice nucleation, electrolyte leakage incubation, and acclimation time. Cold treatments were conducted using a series of computer-controlled, temperature-test chambers that allow for precise regulation of sub-zero temperature exposure down to -40ºC.

### 2.3 - Materials and methods

I divided this study into a series of five experiments. Experiment 1 evaluates the cooling rate and the point temperature of ice nucleation in cold acclimated and non-acclimated samples (Table 2.1). Experiment 2 examines incubation temperature for electrolyte leakage in addition to replicating the cooling rate assessment. Experiment 3 evaluates electrolyte leakage versus time in shaken

versus unshaken samples. Shaking of samples is often recommended in electrolyte leakage protocols, but can stress tissues and involves additional equipment and cost. Experiment 4 evaluates duration of exposure to the treatment temperature, termed here the "nadir" temperature in acclimated and non-acclimated samples. Experiment 5 uses the recommended procedures identified in the above experiments to evaluate cold tolerance thresholds in field grown-material. Samples in this experiment were chilled using two different protocols to evaluate acclimation potential to subzero temperatures.

*Plant material*

Four *Miscanthus* genotypes were obtained from New Energy Farms (http://newenergyfarms.com) in Leamington-ON, Canada. These were the hybrids "Nagara" (M116, 3n; Kludze *et al.*, 2013; Friesen *et al.*, 2014), "Illinois" (M161, 3n; Heaton *et al.*, 2008b; Arundale *et al.*, 2014a; Friesen *et al.*, 2014), "Amuri" (M115, 2n; Friesen *et al.*, 2014) and "Polish" (Deen *et al.*, 2011 unknown ploidy), from crosses between *Miscanthus sacchariflorus* and *Miscanthus sinensis*. Plants were grown at either the University of Toronto in pots in a greenhouse or outdoor platform (experiments 1-4), or in field plots in Leamington, Ontario managed by New Energy Farms (experiment 5).

Plants used in experiments 1-4 were grown in 20 L pots filled with growing medium (50% Pro-mix, 25% loam, and 25% sand), watered daily and fertilized weekly with 20-20-20 fertilizer (Miracle Gro™ http://www.scotts.com). A full strength Johnson-Hoagland's solution was applied biweekly to augment the commercial fertilizer preparation. Greenhouse day/night temperature was set to 26°/20°C with a photoperiod of 14 hours. Plants were transferred outdoors to the roof of the

Earth Sciences Building (University of Toronto) on 6 June 2008 and received the same water and fertilizer regime. Air temperature was monitored with an OWL2pe weather station (EME systems, Berkeley-CA, USA, http://www.emesystems.com/OWL2pepr.htm) installed on the building's roof. On 1 October 2008, when outdoor day/night temperature was near 13°/11°C, a subset of the plants were moved back to the greenhouse (non-acclimated plants). The remaining plants were kept outdoors to acclimate to declining autumn temperatures (cold acclimated plants). Average daily air temperature fell to 2°C by 2 November 2008, and it dropped below 0°C on 17 November 2008. Pots with the plants were wrapped with a heating tape connected to a thermostat on 13 December 2008, to maintain a minimal temperature of soil and rhizome near -3°C.

*General cooling procedure and tolerance assessment*

For each experiment, plants were randomly sampled by removing the rhizome masses from the pots or soil in the field and separating individual rhizomes from the rhizome mass. Five cm of the distal part of the rhizomes was sampled. Rhizomes were then placed on a series of trays that corresponded to the treatments for each of the experiments described below. Because it is important to maintain the water status of rhizomes during freezing experiments, a thin layer of moist media (50% Pro-mix, 25% loam, and 25% sand) was laid over the rhizomes on each tray. The tray was then enclosed with clear plastic wrap and stored at 0ºC in a Thermotron 2800 temperature test chamber (Thermotron Industries, Holland-MI, USA http://www.thermotron.com) until freezing experiments commenced.

All experimental freezing trials were performed using two Thermotron 8200 and one Thermotron 2800 programmable temperature test chambers. Tissue viability after freezing was

evaluated with electrolyte leakage (EL) and rhizome re-growth assays. The EL protocols were adapted from Zhang & Willison (1987), Murray *et al.* (1989), Steffen *et al.* (1989).

After the cold treatment, the rhizomes used for EL measurements were gently rinsed with distilled water to remove dust. Rinsing did not remove a noticeable amount of tissue electrolytes (data not shown). A one cm section of the sample was placed in 7 ml double-deionized water ($ddH_2O$) and incubated for 24 hours at room temperature (21ºC) to allow electrolytes to diffuse out of the tissue. The electrolyte conductivity of the treated sample ($EL_{treat}$) was then measured by transferring 4 ml of the bathing solution into the assay well of a calibrated Ultrameter 4P conductivity meter (Myron L Company, Carlsbad-CA, USA, http://www.myronl.ca/ultrameter_II.htm) at room temperature. The solution was returned to the vial containing the sample and boiled for 1 hour to kill all cells causing complete leakage of electrolytes. When the temperature of the resultant solution reached around 21ºC, the total electrolyte content was measured ($EL_{total}$). Relative conductivity (RC) was calculated as:

$$RC\% = \left( \frac{EL_{treat}}{EL_{total}} \right) x100\% \qquad \text{Equation 1}$$

To measure re-growth, treated rhizomes were planted in soil (described above) and kept in a greenhouse at a day/night temperatures near 24ºC/18ºC with daily watering. If a rhizome sprouted new leaves or formed new roots within six weeks, it was considered to have survived the cold treatment. The background sprouting rate of rhizomes from each genotype was previously determined using dormant rhizomes in the greenhouse and observed to be above 90%, and thus there was no need to artificially induce buds to break dormancy.

*Experiment 1 and 2: Assessment of cooling rate and ice nucleation temperature*

For both experiments, rhizomes from *Miscanthus* genotypes Nagara, Illinois, and Polish were studied (Table 2.1). The purpose of these experiments was to evaluate the effects of cooling rate on cold tolerance of *Miscanthus* rhizomes and how ice nucleation occurs in the rhizome. In Experiment 1, the response of cold acclimated and non-acclimated rhizomes to the cooling rate was evaluated, and on Experiment 2, the post-treatment temperature for diffusion of electrolytes was analysed. Therefore, it was used a split-plot design with 4 plants per genotype (replicate).

The temperature of sampled rhizome was lowered to -15ºC. The chamber temperature was maintained for 4 hours, and then raised back to 15ºC. For Experiment 1, the cooling and thawing rates were 1º, 3ºC and 5ºC $hr^{-1}$.The same procedure was used in Experiment 2, but the rates tested were 1º and 3ºC $hr^{-1}$ plus the control (rhizomes were kept at 4ºC).

During the cold-trial in Experiment 1, the temperature of individual rhizomes was monitored by type T (copper/constantan) thermocouples using the Thermotron 8200 datalogger or a Veriteq Spectrum 1700 thermocouple data logger (Veriteq Instruments Inc., Richmond- BC, Canada, http://www.vaisala.com).

*Experiment 2 and 3: Post-treatment electrolyte diffusion in the bathing solution: temperature, time, and shaking*

Published protocols for EL incubate samples in bathing solution at room temperature (21ºC) or at 4ºC for the diffusion of the electrolytes (Knowles & Knowles, 1989; Murray *et al.*, 1989; Campos *et al.*, 2003; Ebeling *et al.*, 2008). Pairs of samples used in Experiment 2, collected

from the same plant and taped together were used to evaluate the effects of incubation temperature on post-treated rhizomes. After the cold treatment, one rhizome of each pair was placed in a vial filled with 7 ml of $ddH_2O$ and incubated for 24 hours at 4°C, and the other was incubated for 24 hours at 21°C prior to the electrolyte leakage measurement.

In Experiment 3, the effects of agitating the vials or not with bathing solution for diffusion of electrolytes after the treatment are compared. Because the measurement of the EL of samples was repeated at various times, this experiment was designed as repeated measures with 8 replicates. Rhizomes of the *Miscanthus* hybrids Amuri M115 and Illinois were frozen using a continuous cooling rate at 5ºC $hr^{-1}$ to -20ºC and incubated for 10 hours at this temperature, and then thawed at 5ºC $hr^{-1}$. This procedure was performed to promote complete leakage of electrolytes. After the cold treatment, 16 rhizomes of each genotype were placed in vials with $ddH_2O$ for electrolyte diffusion. From these vials, 8 per genotype were randomly chosen for incubation on a G10 Gyratory Shaker (New Brunswick Scientific Co. Inc. New Brunswick-NJ, USA, http://newbrunswick.eppendorf.com/) and shaken at 150 rpm, while the other 8 samples of each genotype were left undisturbed. Electrolyte conductivity was measured after 2, 4, 10, 20, 24, 28, and 32 hours of incubation of the samples in vials with 7 ml of $ddH_2O$. Then, all samples were boiled for 1 hour. After another 24 hours at room temperature, the total electrolyte conductivity was measured. The order of each measurement was randomized.

*Experiment 4: Relationship between incubation time and nadir temperature*

Treatment time can potentially affect EL given that samples are detached from the parent plant. To minimize experimental time without creating artifacts from too rapid treatment time, the

effect of time spent at the nadir temperature was evaluated. Acclimated and non-acclimated plants from genotypes Nagara, Illinois, and Polish, were used (Table 2.1) with 5 replicates for each treatment temperature at each sampling time. This experiment was designed as a repeated measurement experiment.

Non-acclimated rhizomes were cooled to -2º, -8º, or -12ºC. Based on preliminary observations, non-acclimated rhizomes had nearly complete leakage when treated at -10ºC (data not shown). Cold-acclimated rhizomes were frozen to -2º, -8º, -12º, -14º, -18º, and -22ºC. In all treatments, rhizomes were continuously cooled to the nadir temperature at a rate of 1ºC $hr^{-1}$. Once the nadir temperature was reached, rhizomes were sampled after 4, 8, 12, 16, and 20 hours of incubation. After sampling they were placed in a second programmable freezer, set at the respective nadir temperature, and thawed to 21ºC at 1ºC $hr^{-1}$ after which EL was assessed.

*Experiment 5: Evaluation of staged cooling rate, acclimation potential, and de-acclimation*

This experiment was designed to test whether an artificial cooling protocol can induce greater cold tolerance. In addition, temperature was raised to 4ºC after treatment at -10ºC to simulate a winter warming event. Because a plant was considered the experimental unit, and survivability and RC were measured at various temperatures, this experiment was designed as repeated measures with 6 replicates.

Entire underground rhizome masses of six *Miscanthus* plants from each of the two genotypes (M115 and Illinois) were collected from field plots at New Energy Farms (Leamington-ON) on 8 February 2010, when the mean air temperature was around -4ºC (Environment Canada; http://climate.weatheroffice.gc.ca/climateData/dailydata_e.html?timeframe=2&Prov=ONT&Stat

ionID=4647&dlyRange=1960-10-01|2013-05-14&Year=2010&Month=2&Day=01, accessed in 22 April 2013). In the laboratory, a pair of rhizomes of each genotype was immediately sampled for EL and re-growth assays (control). Rhizomes were separated from the root mass and placed on trays with a thin layer of soil before being allocated in one Thermotron 8200 test chamber. The temperature was then lowered in stages to -1º, -2.5º, -5º, -7º, -9º, and -10ºC at a cooling rate of 1°C $hr^{-1}$. At each nadir temperature, samples were incubated for 16 hours and, except at -1ºC and -2.5ºC, a pair of rhizomes from each plant was sampled.

Episodic warming to above freezing temperatures can occur at mid-to-boreal latitudes. To test if warming events affect *Miscanthus* rhizomes, samples treated at -10ºC were warmed to 4ºC at the rate of 1ºC $hr^{-1}$. Samples were kept at 4ºC for 24 hr and then cooled to -10ºC in stages as described above, allowing 16 hours of incubation at each stage. Cooling then continued to the treatment temperatures of -12º, -14º, -18º, and -22ºC, in this order, with 16 hr incubation at each nadir temperature. Rhizomes were sampled at each treatment temperature (-10º, -12º, -14º, -18º and -22ºC). Sampled rhizomes were placed in another Thermotron 8200 pre-cooled to the treatment temperature, and then thawed at 1°C $hr^{-1}$ to 21°C. Electrolyte leakage was evaluated in one rhizome of the pair while the other was planted to determine mortality using re-growth assays.

*Determination of $LT_{50}$ and $LEL_{50}$*

Data from Experiment 5 were used to determine $LT_{50}$ and $LEL_{50}$. The survivability of samples was analyzed as a function of the treatment temperature or relative conductivity (RC), using a logistic regression given by a binary generalized linear mixed-effects model (GLMM) as will be described in the next section. After determining the optimal model, the predicted values

for rhizome survival were determined using the "predict" function using R statistics (R-Core-Team, 2013) for each (a) temperature or (b) RC, to estimate the temperature ($LT_{50}$) or RC ($LEL_{50}$) at which rhizomes have a 50% chance of mortality.

*Statistical analysis*

All experiments were analyzed using a Generalized Linear Mixed Model (GLMM) on the beta or binomial family. In Experiments 1, 2, 3, and 4 relative conductivity was analyzed as the response variable. Because relative conductivity is constrained between 0 and 1, these experiments were analyzed using a beta regression using the package "gamlss" (Rigby & Stasinopoulos, 2005). In Experiment 5, (a) survivability as a response to the treatment temperature, (b) survivability as a response to the relative conductivity (RC), and (c) RC as a response to the treatment temperature were analyzed. Thus, logistic regressions (logit binomial family) were performed for each model because survivability is either 0 or 1, and the RC responses are constrained between 0 and 1. On all the statistical analysis performed here, plant was considered as the experimental unit, so the difference between the rhizomes tested from the same plant was assumed to be null. Also, the plant was considered as a random effect. The binomial GLMM was performed using the "lme4" package (Bates *et al.*, 2011). Because the binomial GLMM in the package "lme4" is made by Laplace approximation, only p-values $<0.002$ were considered significant, as suggested by Zuur *et al.* (2009). All statistical procedures were conducted using R Statistical software (R-Core-Team, 2013).

## 2.4 - Results

*Nucleation temperature and cooling rate (Experiments 1, 2)*

Cooling rate had a large effect on the time required to complete an experiment with a nadir temperature of -15ºC (Figure 2.1). A freezing experiment using a cooling rate of 1ºC $hr^{-1}$ required 34 hours, at 3ºC $hr^{-1}$ it lasted 14 hours, and at 5ºC $hr^{-1}$ about 10 hours. At 1ºC $hr^{-1}$, *Miscanthus* rhizomes exhibited exotherms near -1ºC (Figure 2.1c). For the cooling rates of 3º and 5ºC $hr^{-1}$, exotherms occurred at variable temperatures ranging from -1ºC to -11ºC (Figure 2.1a, b). Exotherms indicate when ice formation occurs (Akyurt *et al.*, 2002; Gusta *et al.*, 2009). Therefore, it is an indication that in *Miscanthus* rhizomes cooled at 3ºC $hr^{-1}$ and faster, water may supercool before ice crystals are formed. Rhizome temperature lagged behind chamber temperature by 1 to 2 hours at the faster cooling rates (Figure 2.1a, b) but it was close to chamber temperature at 1ºC $hr^{-1}$ (Figure 2.1c). This result shows that at the faster cooling rates, rhizomes are not being incubated at the nadir temperature for the full 4 hours.

In Experiments 1 and 2, cooling at 1ºC $hr^{-1}$ resulted in greater relative conductivity (RC) than observed with faster cooling rates ($p < 0.05$; Figure 2.2 and Figure 2.3). No significant differences in RC among the three *Miscanthus* genotypes (Nagara, Illinois, and Polish) were detected ($p > 0.05$). Experiment 1 tested cold-acclimated versus non-acclimated rhizomes, and showed that cold acclimated rhizomes had lower RC than the non-acclimated ones (Figure 2.2).

*Post-treatment electrolyte diffusion: temperature, time and agitating the bathing solution (Experiment 1 and 3)*

In Experiment 1, the two temperatures used for the incubation of treated rhizomes for electrolyte diffusion in dd$H_2O$ produced similar results ($p > 0.05$; Figure 2.3). The main difference between electrolyte diffusion at 4ºC versus 21ºC was that at 21ºC the bathing solution temperature was equivalent to the conductivity meter and the air temperature in the room, which stabilizes the conductivity reading.

There was no significant difference in electrolyte diffusion in the bathing solution between samples shaken at 150 rpm and samples without agitation at any incubation time up to 32 hours at room temperature ($p > 0.05$; Figure 2.4). There was also no difference between genotypes. Relative conductivity reached its maximum at 24 hours. A strong smell was noticed in the samples after 32 hours, indicating they were beginning to rot so they were immediately boiled.

*Relationship between incubation time and nadir temperature (Experiment 4)*

The time that samples were incubated at various treatment temperatures had no significant effect on RC ($p > 0.05$; Figure 2.5). In this experiment, differences among genotypes were also not significant and thus the genotypes were pooled ($p > 0.05$). However, differences between cold-acclimated and non-acclimated rhizomes were significant, as was the interaction between temperature and acclimation status. For instance, cold-acclimated rhizomes treated at a nadir temperature of -8ºC had an RC near 30%, while non-acclimated rhizomes at the same temperature had more than double the RC (about 70%). At -12°C, non-acclimated rhizomes had almost complete leakage of electrolytes and cold-acclimated rhizomes exhibited an RC around 50%.

*Evaluation of staged cooling rate and potential to acclimate (Experiment 5)*

Because cooling occurred in stages, this experiment required around 22 days for the -22°C treatment be completed. The results of Experiment 5 were evaluated in three different ways. First, the survivability of rhizomes at each temperature tested was evaluated. One out of six Illinois rhizomes was alive after 16 hours at the nadir temperature of -10ºC, and that was the lowest temperature a rhizome from this genotype could survive (Figure 2.6a). At -10ºC, M115 had survivability of 83% before and after the freeze-thaw cycle. Even at -12ºC and -14ºC, M115 had survivability of 66% and 33%, respectively. The estimated $LT_{50}$ was -12.8ºC for M115 and -6.3ºC for Illinois.

When analyzing the RC that causes death to the rhizomes, M115 rhizomes had significantly higher survivability at a common RC than Illinois (Figure 2.6b). The $LEL_{50}$ was 28.6% for M115, and 18.6% of RC for Illinois. Finally, injury as indicated by RC value was analyzed for each genotype as a function of temperature (Figure 2.6c). *Miscanthus* Illinois was more sensitive to cold than M115. At -7ºC and below, Illinois had consistently higher RC than M115. For Illinois, RC rose sharply above 20% RC, which was reached at temperatures below -10ºC; in M115 the rise in RC was more gradual at lowered temperature.

M115 had similar survivability and RC at -10ºC before and after (star symbols on Figure 2.6) the freeze-thaw cycle. Illinois suffered a significant increase in RC after the freeze thaw cycle, and survivability at -10º was minimal before and null after the freeze-thaw cycle.

Table 2.1 - List of experiments performed to identify the acclimation condition of the rhizomes, the test applied in the experiment, and the genotypes (with ploidy) used. The superscript numbers on each genotype indicate references where these genotypes were also studied.

| Experiment number | Rhizome acclimation | Test | Genotypes |
|---|---|---|---|
| 1 | Acclimated<br>Non-acclimated | Cooling rate;<br>Nucleation temperature. | Illinois (3n)[1,2]<br>Nagara (3n)[1.3] |
| 2 | Non-acclimated | Cooling rate;<br>Post-treatment incubation temperature. | Polish (?)[4] |
| 3 | Non-acclimated | Post-treatment diffusion agitation. | Amuri M115 (2n)[1,4]<br>Illinois (3n)[1.2] |
| 4 | Acclimated<br>Non Acclimated | Incubation time at the treatment temperature. | Illinois (3n)[1,2]<br>Nagara (3n)[1.3]<br>Polish (?)[4] |
| 5 | Winter field acclimated | Sub-zero acclimation;<br>Winter warming event de-acclimation. | Amuri M115 (2n)[1,4]<br>Illinois (3n)[1.2] |

Figure 2.1 - Chamber and rhizome temperature during freezing trials using a continuous cooling rate of (a) 5ºC $hr^{-1}$, (b) 3ºC $hr^{-1}$, and (c) 1ºC $hr^{-1}$ (Experiment 1). Solid line represents the chamber temperature and the dotted lines represent each of the four rhizomes temperatures. Arrows indicate the freezing exotherm.

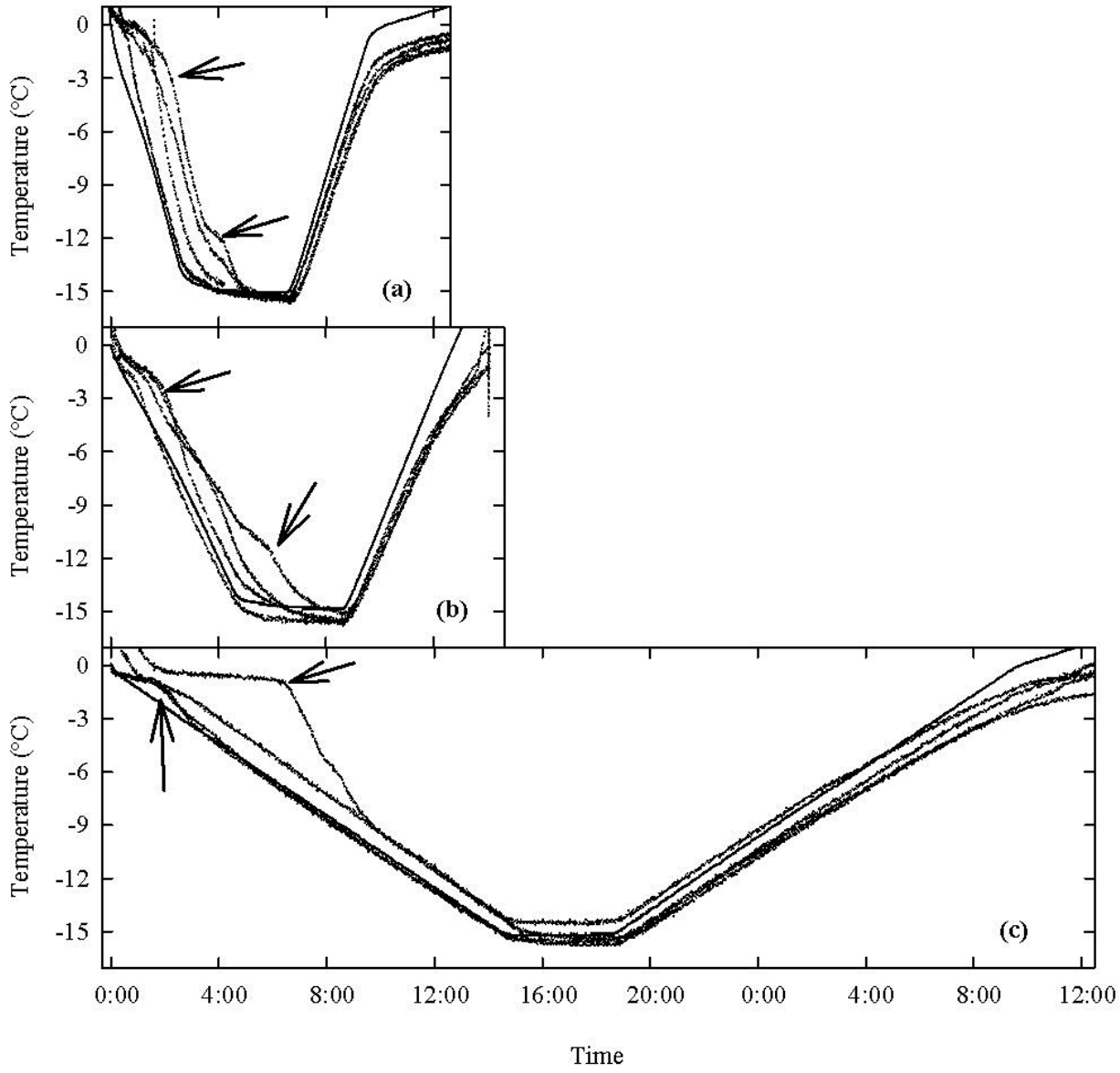


Figure 2.2 - Effects of cooling rate on the relative conductivity (RC) of cold acclimated and non-acclimated rhizomes from three *Miscanthus* genotypes. Samples were chilled to -15°C at cooling rates of 1º, 3 º or 5°C $hr^{-1}$ and incubated at this temperature for 4 hours before thawing. Cooling at 1ºC $hr^{-1}$ resulted in higher RC than any of the faster cooling rates. No difference was observed between the 3ºC $hr^{-1}$ and 5ºC $hr^{-1}$ trials. Differences between cold-acclimated (open symbols) and non-acclimated (filled symbols) rhizomes were significant. No significant difference between the *Miscanthus* genotypes was detected. Circles for Illinois (M161), squares for Nagara (M116) and triangles for Polish. Mean ± SE, (n = 4).

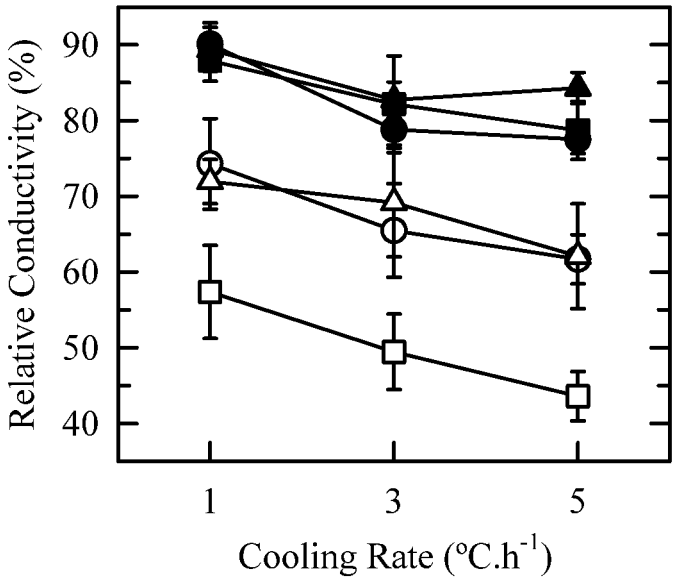


Figure 2.3 - Effects of cooling rate and post-treatment incubation temperature for electrolyte diffusion on relative conductivity (RC) of rhizomes from three different of *Miscanthus* genotypes. The control was kept at 4ºC while other samples were chilled to -15°C at 1º and 3°C $hr^{-1}$ and maintained at -15ºC for 4 hours. A pair of rhizomes was collected from each plant: one was incubated at 21ºC (white boxes) and the other at 4°C (grey boxes) following cold treatment. According to the post-hoc Tukey test at $p < 0.05$, the cooling rate of 1ºC $hr^{-1}$ resulted in higher RC than 3ºC $hr^{-1}$ and the control. Differences between *Miscanthus* genotypes or electrolyte diffusion temperatures were not significant (n = 4).

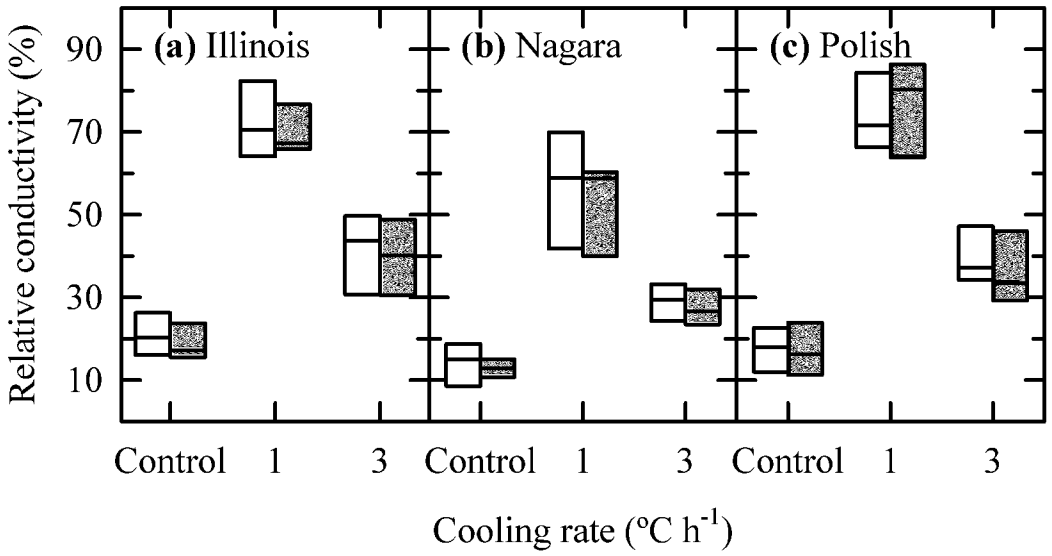


Figure 2.4 - Diffusion of electrolytes in the bathing solution after cold treatment. Samples were placed in vials with 7 ml of double deionized water and electrolyte conductivity was measured after 2, 4, 10, 20, 24, 28, and 32 hours. No difference between the genotypes or the methods (shaking or not) were found ($p > 0.05$). The relative conductivity was relatively stable between 24 hours to 32 hours ($p > 0.05$). Circles for *Miscanthus* genotype Illinois, triangles for *Miscanthus* genotype Amuri M115, open symbols for shaken samples, filled symbols for non-shaken samples. (N = 8).

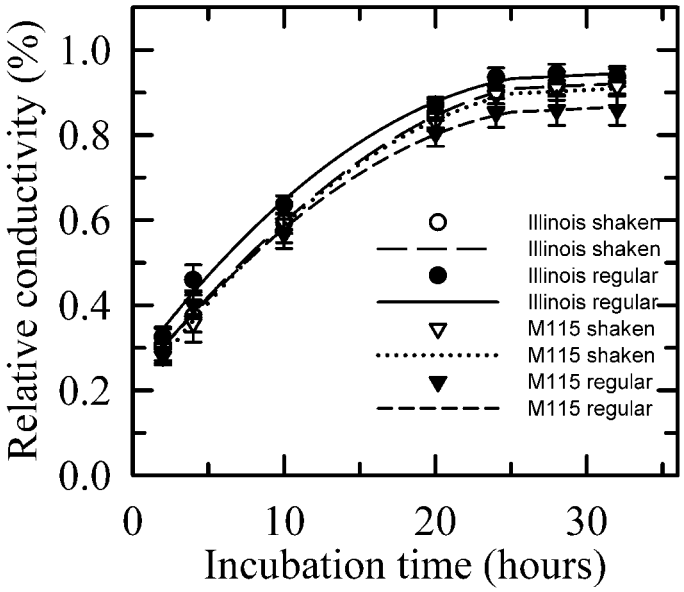
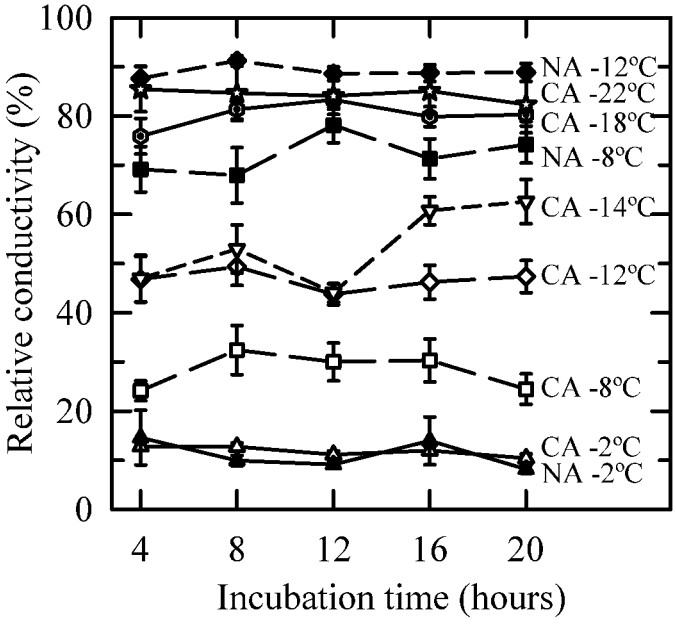


Figure 2.5 - Effects of incubation time at various temperatures on relative conductivity (RC) of cold acclimated (CA, open symbols) and non-acclimated (NA, filled symbols) rhizomes of *Miscanthus*. Based on preliminary observations, non-acclimated rhizomes had nearly complete leakage when treated at -10ºC (data not shown). Genotypes were pooled together because there was no difference between them. Once rhizomes reached the nadir temperature, they were sampled every 4 hours up to 20 hours. No difference between incubation time lengths were observed. Differences between acclimation state, treatment temperature, and the interaction were significant. Up triangles for treatment at -2ºC, squares for -8ºC, diamonds for -12ºC, down triangles for -14ºC, hexagon for -18ºC, and star for -22ºC. Mean ± SE (n = 12).



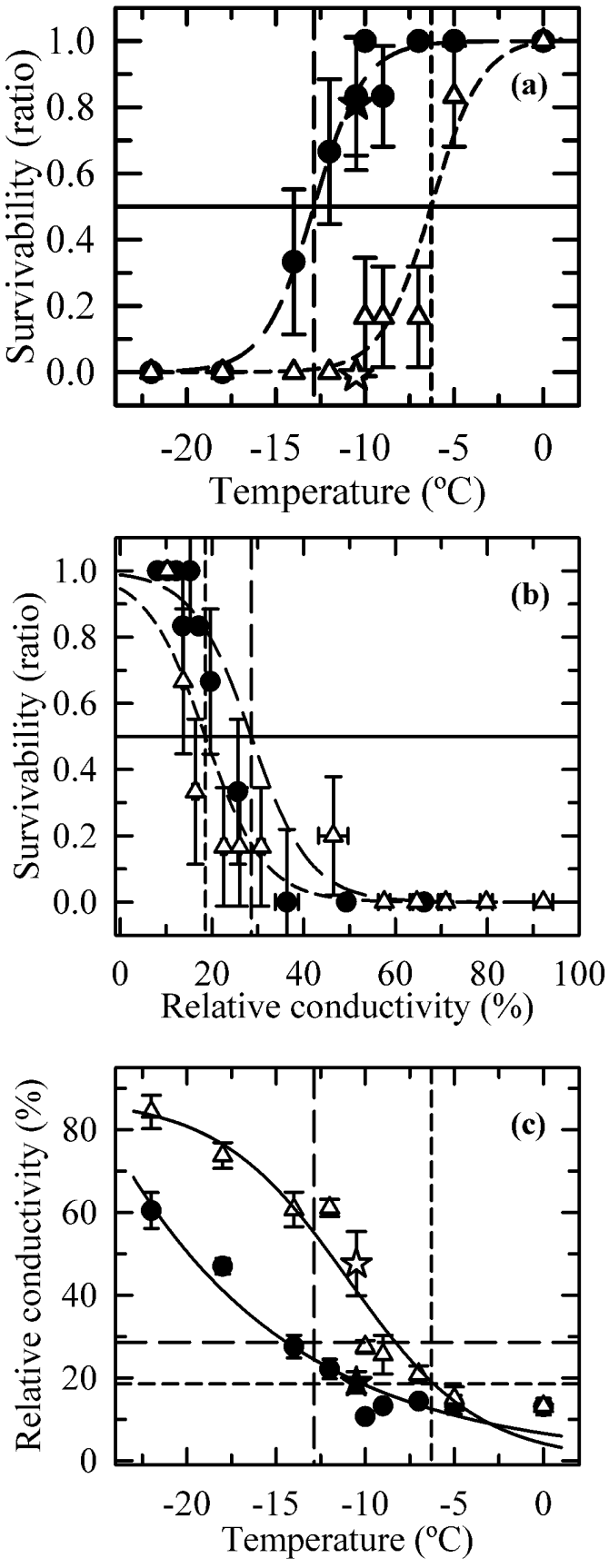



Figure 2.6 - Staged cooling rate in *Miscanthus* genotypes M115 (filled circles, long dashes lines) and Illinois (open triangles, short dashes lines). (a) Survivability of *Miscanthus* rhizomes as a function of temperature. Vertical lines represent the temperature at which the sample has a 50% chance of mortality (LT50). (b) Survivability of *Miscanthus* rhizomes as a function of relative conductivity (RC). Vertical lines represent the RC at which the sample has a 50% chance of mortality (LEL50). Sigmoid lines are the fitted values indicating the probability for a rhizome to survive at a given temperature (panel a) or at a given RC (panel b). (c) RC as a function of temperature. Vertical lines represent LT50 and horizontal lines represent LEL50. Sigmoid lines are the logistic regression for each genotype. In panel (a) and (c), the stars (filled symbol for M115 and open symbol for Illinois) indicate results for the incubation at -10 ºC after the freeze-thaw cycle. Panel (a): differences in survivability as a function of temperature (including LT50) between genotypes are significant. In panel (b): differences in survivability between the two genotypes as a function of RC (including LEL50) are significant. In panel (c), difference between genotypes are significant. LEL50 and LT50 values were calculated based on the equation given by the logistic regression. For M115 the LT50 = -12.8ºC and LEL50 = 28.6% of RC. For Illinois the LT50 = -6.3ºC and LEL50 = 18.6% of RC. (n = 6). Significant p-values are < 0.002 because the statistical model used Laplace approximation.

## 2.5 - Discussion

In the present study, a series of trials were performed to identify robust methods for assessing cold tolerance in *Miscanthus*, a $C_4$ grass that may serve as novel bioenergy feedstock in higher latitudes. In *Miscanthus* rhizomes covered with a thin layer of soil and cooled at 1ºC $hr^{-1}$, ice nucleation occurred near -1°C, indicating no need to artificially induce ice nucleation. At cooling rates of 3ºC $hr^{-1}$ and 5ºC $hr^{-1}$, samples temperature did not follow the chamber temperature, therefore reducing incubation time at the nadir temperature and causing ice crystal formation to occur at variable temperatures. Also, a cooling rate of 1°C $hr^{-1}$ avoided a drastic post-nucleation drop in the temperature of the rhizome, and maintained the rhizome temperature near the chamber temperature. Thus, 1ºC $hr^{-1}$ is a suitable cooling rate for cold tolerance trials in *Miscanthus* rhizomes. Samples incubated for less than 4 hours at the nadir temperature had less electrolyte leakage (EL) than those cooled for 4 hours or more. There was no difference in EL for the post-treatment incubation between chilling (4°C) and room temperature (21ºC). In addition, there was no effect of shaking on samples incubated for 24 hours during the leaching phase. Rhizomes of *Miscanthus* M115 (2n) showed improved cold tolerance when incubated to sub-zero temperatures, and it did not de-acclimate if exposed to a 24 hour warming event that raised the temperature to 4ºC. Finally, an effective estimation of $LT_{50}$ was obtained based on statistical protocols. This allows for a reliable assessment of survivability using the electrolyte leakage method.

*Artificial freezing trial*

Ice crystal formation is intimately related to the cooling rate in artificial freezing experiments, and it affects survivability in natural systems. In *Solanum acaule* leaves, the

temperature at which ice nucleation occurs influences the $LT_{50}$: if nucleation occurred at -1ºC, the $LT_{50}$ was -7ºC; if nucleation occurred at -2ºC, the $LT_{50}$ was -3ºC (Rajashekar *et al.*, 1983). Early ice nucleation causes less injury to plants tissues than late ice nucleation because it allows for proper organization of ice crystals in the apoplast, preventing the cell membrane from being ruptured (Boorse *et al.*, 1998; Griffith & Yaish, 2004; Ruelland *et al.*, 2009). In Experiments 1 and 2, some rhizomes cooled at 3º and 5ºC $hr^{-1}$ showed ice crystal formation at a lower temperature than the ones cooled at 1ºC $hr^{-1}$. Although injury level should be higher on the rhizomes cooled at the faster cooling rates, the experiments using the cooling rate of 1°C $hr^{-1}$ led to considerably more damage (based on RC values) than faster cooling rates (3ºC $hr^{-1}$ and/or 5°C $hr^{-1}$).

The unexpected results obtained for different cooling rates could be because cooling at 3º and 5ºC $hr^{-1}$ is too fast to allow rhizomes temperature to equilibrate to the chamber temperature, so rhizomes would be incubated at the nadir temperature for less than the designed incubation time. Incubation time is very important in consider in a freezing experiment because the equilibrium between the cell membrane and the extracellular solution may take hours or even days to be reached (Gusta & Wisniewski, 2013). Waalen *et al.* (2011) show in different cultivars of *Brassica rapa* and *B. napus* that if no incubation time is allowed, plants have an $LT_{50}$ below -17ºC, but when incubated at -8ºC most of the cultivars have less than 50% survivability after 4 days. Also, in this experiment, short term survivability had no correspondence with long term survivability. The same was found for wheat. Spring wheat showed an $LT_{50}$ of -9ºC if no incubation time was allowed, but $LT_{50}$ was -3ºC if incubated for 48 hours (Gusta *et al.*, 1997).

Based on these results two questions arose. Can the increased injury caused by cooling rate of 1ºC $hr^{-1}$ also be due to the longer time necessary to complete the experiment (34 hours for 1ºC

$hr^{-1}$ vs 14 hours for 3ºC $hr^{-1}$ and 10 hours for 5ºC $hr^{-1}$)? Is 4 hours of incubation time reproducing the effects of the treatment temperature or RC can increase with longer incubation time? Previous studies have used an incubation time at the nadir temperature between 5 to 10 minutes (Ball *et al.*, 2006; Márquez *et al.*, 2006) and 1-4 hours (Murray *et al.*, 1989; Steffen *et al.*, 1989; Farrell *et al.*, 2006; Bykova & Sage, 2012) for leaves and shoots. Hope & McElroy (1990) used no incubation time for switchgrass (*Panicum virgatum*) crowns that were thawed as soon as minimal temperature was reached. Experiment 4 was designed to test if an incubation longer than 4 hours and the total experiment time increased injury in the samples. When rhizomes were cooled to different nadir temperatures and sampled from the programmable freezer every 4 hours up to 20 hours, the incubation time and the length of the experiment resulted in no significant change in RC at any temperature tested, either in acclimated or non-acclimated rhizomes. Therefore, in *Miscanthus* rhizomes, long experiments (e. g. 19 days incubationa in Experiment 5 at 14ºC showed viable rhizomes) and more than 4 hours of exposure to the treatment temperature did not increase RC on the rhizomes.

*Electrolyte diffusion*

Also analyzed was the temperature at which post-treated tissue is incubated to assess diffusion of the electrolytes from damaged tissues in the bathing solution. Prior studies have used 4ºC (Murray et al., 1989; Sheppard et al., 1993; Ebeling et al., 2008) or room temperature (21ºC) (Knowles & Knowles, 1989; Fan & Blake, 1994; Tarhanen, 1997; Campos et al., 2003), but no clear explanation for this choice was provided. It may be that 4°C for the electrolyte extraction in de-ionized water is a strategy to avoid thawing injury. However, the results of Experiment 2 showed that Miscanthus rhizomes experienced no significant difference in RC whether electrolyte

extraction occurs at 4°C or 21ºC. In addition, it was observed that if the bathing solution is in thermal equilibrium with the measuring device, the electrolyte conductivity readings are more stable, as the conductivity meter is temperature sensitive. Thus, incubating treated Miscanthus rhizomes in de-ionized water at room temperature is a suitable option.

Another concern about the incubation for determining electrolyte diffusion in the bathing solution is the incubation time and whether samples need to be agitated or not during this incubation period. Many experiments report the use of a shaker for leaching of electrolytes. Thalhammer *et al.* (2014) suggest to incubate leaves of *Arabidopsis thaliana* on a shaker at 150 rpm for 24 hours. Płażek *et al.* (2012) used a shaker at 100 rpm for 2 hours on *Miscanthus* stolon disks. Other experiments report the use of a shaker for short incubation periods (Kaplan & Guy, 2005). Agitating the samples for leaching of electrolytes might be used where there is a need to shorten the incubation time. However, care should be taken not to add further disturbance to cell membranes that might already be sensitive due to the stress caused by the freeze-thaw procedure. Because of that, samples were treated to extreme conditions to kill almost all cell, to avoid creating a confounding factor if shaking adds more injuries to the sample. *Miscanthus* rhizomes showed no difference between shake and non-shake treatments for electrolyte diffusion in the bathing solution, demonstrating no need for agitation for leaching of electrolytes. In addition, incubation of the samples in the bathing solution for less than 24 hours was not enough time for complete diffusion of the electrolytes that leaked due to the treatment.

*Sub-zero acclimation and de-acclimation of* Miscanthus *rhizomes*

Experiments 1 and 4 showed that cold-acclimated rhizomes are more cold tolerant compared to non-acclimated rhizomes (Figure 2.2 and Figure 2.5). In Experiment 5, it was tested whether cold tolerance could be increased if samples were incubated at sub-zero temperatures. When samples were cooled in stages and incubated for 24 hours at each stage until reaching the nadir temperature, rhizomes could tolerate lower temperatures than when cooled using a continuous cooling rate (data not shown). M115 (2n) showed greater acclimation of rhizomes than Illinois genotype (2n), and had $LT_{50}$ values of -12.8ºC for M115 and -6.3ºC for Illinois. The $LT_{50}$ value for M115 is much lower than the ones previously reported for *Miscanthus* hybrids (-3.4ºC to -6.5ºC) which were not tested on a staged cooling rate (Clifton-Brown & Lewandowski, 2000a). It has already been reported that in some species acclimation can increase with exposure to sub-zero temperatures (Weiser, 1970; Livingston III & Henson, 1998; Chen *et al.*, 2014). Here, I show that depending on the genotype, *Miscanthus* can also increase acclimation when exposed to sub-zero temperature. Further investigation of the thresholds of the cold tolerance of the rhizomes of the new *Miscanthus* genotypes is still needed.

Because acclimation is first induced late in the growing season, as evidenced by expression of cold-acclimation genes and relocation of nutrients (Zhu *et al.*, 2008; Gusta & Wisniewski, 2013; Purdy *et al.*, 2013), it was hypothesized that sub-zero acclimated rhizomes could enhance acclimation by gene expression if temperature rose just above the freezing point. However, promoting a freeze-thaw cycle where the samples were thawed from -10ºC to 4ºC and maintained at this temperature for 24 hours before being cooled again to -10ºC caused no change in survivability or RC of the diploid M115. In the triploid Illinois, this freeze-thaw cycle caused a

sharp increase in the RC. This result indicates that even though acclimation is not enhanced by a freeze-thaw cycle, de-acclimation does not occur in M115 *Miscanthus* rhizomes if a short warming event occurs in the winter. The capacity to not de-acclimate can be determinant in the survivability of plants in regions where warm episodes in the winter or cold episodes in the beginning of the spring are common (Kalberer *et al.*, 2006; Bykova & Sage, 2012). Data from 1950-1998 shows that winter warming episodes and spring cold episodes are particularly frequent in Canada (Shabbar & Bonsal, 2003).

*The use of $LT_{50}$ and $LEL_{50}$*

$LT_{50}$ is defined as the lethal temperature that kills 50% of the samples and it is commonly used as an index for the threshold between survival and death of the sample (Murray *et al.*, 1989; Huner *et al.*, 1998; Verslues *et al.*, 2006; Gusta & Wisniewski, 2013). Estimating $LT_{50}$ is usually made by visual observations rather than using a statistical protocol that generates confidence intervals. Boorse *et al.* (1998) estimated the $LT_{50}$ of chaparral shrubs by doing a linear regression of viability as a function of temperature. However, the viability response as a function of temperature is not linear (Figure 2.6b). Lim *et al.* (1998) suggested the $LT_{50}$ is the temperature where the plant had 50% of injury estimated by a Gompertz regression. Studying St Augustinegrass, Maier *et al.* (1994) used a sigmoidal regression for temperature vs EL and considered the inflection of the regression as the lethal point. In the present study, logistic regressions were used to estimate $LT_{50}$ (and $LEL_{50}$), which appears more appropriate for this type of data.

Many studies use the EL method to estimate $LT_{50}$, but a small number of them have estimated the amount of EL that results in sample death for that species (Palta *et al.*, 1982; Coiner, 2012; Chapter 2). Such studies estimate that no plant could survive if it had suffered more than 30% RC. In the present study, the RC that causes death of *Miscanthus* rhizomes is even lower: between 15% and 28% (Figure 2.6b). Some authors used the temperature at which 50% of EL occurs as an index for cold tolerance (Webb *et al.*, 1994, for oat and rye; Hannah *et al.*, 2006, for *Arabidopsis*; Bykova & Sage, 2012, for *Bromus spp*). That leads to an overestimation of cold tolerance because no sample has been reported to recover from this level of injury (Zhang & Willison, 1987; Teutonico *et al.*, 1993; Maier *et al.*, 1994; Boorse *et al.*, 1998; Dunn *et al.*, 1999; Waalen *et al.*, 2011).

Different genotypes may show different values of $LEL_{50}$, reinforcing the importance to evaluate $LEL_{50}$ for each strain prior to experimental trials. Waalen *et al.* (2011) reported that one cultivar of *Brassica napus* had low EL but high mortality in the field. Teutonico *et al* (1993) found that the cell membrane of *Brassica rapa* is more permeable to ion leakage than *B. napus*. It was suggested that this higher resistance of *B. napus* is due to a thicker wax layer in the leaf compared to *B. rapa* (Earnshaw *et al.*, 1990). Here I show that different *Miscanthus* genotypes are killed after suffering 15% (Illinois) and 28% (M115) injury. It is not necessary to compare EL with re-growth assays for all the samples. Once the $LEL_{50}$ for a given genotype is statistically determined, the $LT_{50}$ will be the temperature where the sample reachs the amount of EL determined by $LEL_{50}$.

The estimation of $LT_{50}$ and $LEL_{50}$ is important in cold tolerance experiments, because they provide the thresholds for survivability of the sample based on the temperature or on the amount of injury ti which the sample can recove. In general, the focus of low temperature tolerance studies

is to evaluate the lethal temperature for plants tissues, i. e. $LT_{50}$. $LEL_{50}$ is valuable when using the faster EL method, and it allows for the estimation of $LT_{50}$ if the relationship between RC and temperature is known.

*Conclusion*

Energy crops have appeared as an important alternative for a sustainable source of fuel. However, it is imperative that these crops are grown on marginal lands to avoid competition with food crops or deforestation to create more farmland (Tilman *et al.*, 2006; Fargione *et al.*, 2008; Searchinger *et al.*, 2008). At high latitudes such as Canada, marginal lands are abundant due to the low temperature. Thus, it is necessary to find a novel crop resistant to extreme cold temperatures. Before investing in such crops, thresholds of survivability to low temperature must be securely determined. New crops being considered for bioenergy, such as *Miscanthus*, are still not well domesticated and it is fundamental to promote fast and realistic techniques to screen for the cold tolerance of the new genotypes. A method such as electrolyte leakage allows for fast screening of large sample sizes if well calibrated by determining the EL corresponding to plant death as shown here. It is important to consider the cooling rate, ice nucleation temperature, and incubation time of the treatment temperature prior to the experiment for a realistic assessment of the effects of low temperature. Also, this study brings new perspectives on cold tolerance of *Miscanthus* by showing that in some genotypes the rhizomes can improve acclimation to low temperature upon incubation to sub-zero temperatures, and this acclimation is not lost if a warming even occurs in the winter. Yet, more studies about the thresholds of survivability of the existing genotypes are still needed.

# Chapter 3 - Winter cold tolerance thresholds in field grown *Miscanthus* hybrids rhizomes

## 3.1 - Abstract

The cold tolerance of winter-dormant rhizomes was evaluated on diploid, allotriploid, and allotetraploid hybrids of *Miscanthus sinensis* and *Miscanthus sacchariflorus* grown in a field setting. Two artificial freezing protocols were tested: one lowered the temperature continuously to the treatment temperature (nadir) and another lowered the temperature in stages until reaching the treatment temperature. Electrolyte leakage and rhizome sprouting assays were conducted after cold treatment to assess plant and tissue viability. Results from the continuous-cooling trial showed *Miscanthus* can tolerate temperatures as low as -6.5ºC. However, if *Miscanthus* rhizomes were given 24 hours to acclimate to sub-zero temperatures, rhizomes from diploid lines could survive and re-sprout at temperatures as low as -14ºC but not lower. Allopolyploid genotypes were still killed by exposure to temperatures below -7ºC. The results demonstrate diploid *Miscanthus* lines have superior cold tolerance that can be exploited to improve cold tolerance in new cultivated varieties. With expected levels of soil insulation, winter cold should not be lethal to tolerant genotypes of *Miscanthus* in temperate to sub-boreal climates (up to 60ºN); however extreme winter cold will likely harm plantations of existing high yielding varieties of *Miscanthus*.

**Abbreviations:** EL = electrolyte leakage. RC = relative conductivity. $LT_{50}$ = temperature at which the sample has a 50% chance of mortality. $LEL_{50}$ = percentage of electrolyte leakage at which the sample has a 50% of mortality.

## 3.2 - Introduction

*Miscanthus x giganteus* (*Miscanthus*) is a $C_4$ perennial grass that is a leading second generation crop for bioenergy production in temperate latitudes (Beale *et al.*, 1996; Naidu *et al.*, 2003; Heaton *et al.*, 2010). *Miscanthus* is chilling tolerant and capable of rapidly develops a large canopy, allowing it to intercept a substantial fraction of sunlight early in the growing season (Zhu *et al.*, 2008; Dohleman & Long, 2009). In temperate latitudes, peak annual biomass production of *Miscanthus* is at least twice that of switchgrass (*Panicum virgatum*), the other leading $C_4$ bioenergy crop being developed for temperate climates, and is two to three times that of promising $C_3$ biofuel crops (Heaton *et al.*, 2004a, 2008b; Somerville, 2007; Zhu *et al.*, 2008). While *Miscanthus* is suitable for mid-latitudes of mild-to-moderate winters, a major question for potential growers in the expansive landscapes of the northern USA and Canada and northern Eurasia is whether it can tolerate the more severe winters present in these locations. There also remains uncertainty as to which index best predicts suitability for a particular climate region. Because it is a new crop, there has not been time to generate sufficient data from field trials to allow for safe predictions regarding a region's suitability. Survival of most winters may provide false confidence that a perennial crop is suitable for a region, because perennial stands may be destroyed by isolated extreme cold events that are not reflected in regional climate summaries. Moreover, tolerance predictions for a given region could be misleading as climate data may not reflect temperatures actually experienced by overwintering tissues. In the case of perennial grasses, overwintering tissues are typically below ground rhizomes that are insulated by snow, soil and litter, and thus would experience a different thermal regime than reported by climate stations. To overcome these challenges, a physiological-

based assessment that identifies thermal tolerance limits of overwintering tissues could provide valuable predictive data of the cold tolerance of *Miscanthus* genotypes.

Previous studies of cold tolerance in *Miscanthus* indicate various genotypes are tolerant of only mildly sub-zero temperatures. In winter dormant rhizomes of *Miscanthus x gigantheus* harvested in January from a field site in Germany, the $LT_{50}$ (the lethal temperature at which 50% of the rhizomes died) was -3.4°C, while the $LT_{50}$ of diploid *Miscanthus sinensis* plants was -6.5ºC (Clifton-Brown & Lewandowski, 2000a). Heaton *et al.* (2010) noted Illinois-grown rhizomes can survive to soil temperatures as low as -6°C. In Elora, Ontario, Canada, the *Miscanthus x giganteus* genotype "Illinois" also could survive to -6ºC but had only 10% of survivability at -8ºC (Friesen *et al.*, 2015). These lethal temperatures are relatively warm in terms of low temperature tolerance of cold-climate plants, where cold tolerance of dormant tissues can be substantial. For example, crowns of Canadian $C_4$ grasses such as *Andropogon scoparius*, *Spartina gracilis*, and *Distichlis stricta* can survive to temperatures of -27º, -29º, and -35ºC respectively (Schwarz & Reaney, 1989). Dunn *et al.* (Dunn *et al.*, 1999) showed that rhizomes of numerous varieties of *Zoysia* spp. could sprout new shoots after 2 hours of incubation at -10ºC, and some varieties could sprout new shoots even after treatment of -18ºC. Hope and McElroy (1990) found $LT_{50}$ for switchgrass (*Panicum virgatum*) of -22ºC, however no incubation time at the target temperature was allowed, so the finding is tentative. *Spartina pectinata* has an $LT_{50}$ below -25ºC in southern Ontario, Canada (Friesen *et al.*, 2015). These differences in cold tolerance between *Miscanthus* and northern-latitude $C_4$ grasses indicates *Miscanthus* may be poorly prepared for winter conditions in Canada and northern Eurasia. This is somewhat surprising, since wild species of *Miscanthus* survive winter at higher elevations extending into Siberia, northern Japan, and China (Stewart *et al.*, 2009;

Shumny *et al.*, 2011; Yan *et al.*, 2014; Sage *et al.*, 2015). Trial plots of *Miscanthus sinensis* also show good winter survival in colder soils of Sweden, in contrast to hybrid *Miscanthus* lines which have poor survival in this northern location (Lewandowski *et al.*, 2000; Farrell *et al.*, 2006). These results indicate there may be much greater genetic potential for winter cold tolerance in the *Miscanthus* genus, and it is possible that new lines of hybrid *Miscanthus* have exploited this potential (Deuter, 2000; Jakob *et al.*, 2009; Lee *et al.*, 2014). Here, it is evaluated the cold tolerance thresholds of overwintering rhizomes of seven hybrid *Miscanthus* genotypes that have been selected for cold tolerance and potential production in higher latitude landscapes.

To evaluate cold tolerance in the *Miscanthus* hybrids, electrolyte leakage and re-growth of rhizomes were assessed using two different cold treatment procedures. In the first, which follows typical cold treatment protocols, the rhizomes were continuously cooled to the nadir temperature at the rate of 1°C $hr^{-1}$ (Chapter 2). In the second, the rhizomes were cooled gradually at the rate of 0.5°C $hr^{-1}$, and allowed for a 24 hour resting stage at 2.5°C to 4°C intervals. The first procedure was intended to determine the cold tolerance of material in the field at the time of harvest. The second procedure evaluated the absolute cold limit after tissues were allowed to acclimate to their greatest degree of cold hardiness (Chapter 2). Using these protocols it was determined the lowest temperature tolerated by winter dormant rhizomes using plants grown in a field plantation near Leamington-Ontario, in southern Canada.

## 3.3 - Materials and methods

*Plant material, temperature and weather information*

Rhizomes of seven hybrid genotypes from crosses of *Miscanthus sacchariflorus* and *M. sinensis* were harvested from field plantations maintained by New Energy Farms (http://newenergyfarms.com) in Leamington-Ontario (42º8'21"N, 82º38'35"W). The varieties are currently being considered for introduction into Canada because of high yield potential in cool climates (Dean Tiessen - late of New Energy Farms - and Dr. Bill Deen – University of Guelph, personal communication). For the continuous-cooling experiment, it was used *Miscanthus* Amuri genotypes M115 and M147 (diploids, 2n = 2x), M116 (Nagara) and M161 (*Miscanthus x giganteus* Illinois) (allotriploids, 2n = 3x), and Msa/Msi M118 (allotetraploid, 2n = 4x). For the staged-cooling experiment the diploids Amuri M115 and M147 were also used, however, due to a shortage of the hybrids Nagara, Illinois and M118 plants, the allotriploid genotype M1, and the allotetraploid Msa/Msi M119 were examined instead. For a comprehensive explanation for *Miscanthus* nomenclature and chromosomic description, see Hodkinson *et al.* (2002).

In order to monitor rhizome conditions prior to the experiment, soil temperature at the field site was monitored using thermistors attached to Hobo U23-003 two-channel dataloggers (Onset Hobo Data Loggers, Bourne-MA, USA, http://www.onsetcomp.com). In the winter of 2009-2010, six dataloggers were spaced at least 30 meters apart with one thermistor placed 1 cm deep and the other 7 cm deep in the soil at the site of a rhizome mass for the genotypes in the study. These depths corresponded to where most of the rhizomes occurred. A weather station (Hobo Micro Station data logger H21-002) in the center of the field site recorded air temperature, solar radiation

and wind speed. During the winter of 2010-2011, eight dataloggers were spaced 20 meters apart and an OWL2pe weather station (EME systems, Berkeley-CA, USA, http://www.emesystems.com) was used to measure air temperature. In addition, meteorological data was collected from Environment Canada, Kingsville station (Environment Canada, 2010), 11 km distant from the field site.

Soil temperature at 5 cm depth, and corresponding air temperature and snow depth were obtained for 84 weather stations in 73 locations across Canada from the Ontario Climate Centre (for soil temperature data) and Environment Canada (http://climate.weather.gc.ca/). The data were screened for the lowest soil temperature between 1984 and 2006, and is reported along with the associated air temperature and snow pack in Table 3.1 for all 73 locations (stations had between 3 to 22 years of recorded data).

Rhizomes were harvested from 3 to 4 year-old plants growing on a sandy-loam soil. Entire plants were randomly chosen and harvested by digging below the rhizome cluster and shaking the dirt from the cluster. In the winter, pick axes were used to trench around the plants in order to lift them out of the frozen soil. Plants were labelled, bagged, and transported to the laboratory at the University of Toronto. Each plant provided a pair of rhizomes for each treatment. From the pair, one rhizome was used for the electrolyte leakage assay, and the other for the re-growth assay. Before being placed in the programmable freezer, rhizome pairs from the plants from all genotypes were placed in trays (one per treatment), covered with moist soil and the tray was enclosed with plastic wrap. In the winter, the rhizomes' temperature during transport to the lab was near -1ºC. In August 2010, rhizomes were transported after harvest at ambient temperature (around 27ºC). For the continuous-cooling rate experiment, treatments were randomly assigned to each of the three

programmable freezers. Rhizomes were stored at -1ºC (or 21ºC in August 2010) until their treatment started. No rhizome was stored for longer than 5 days. For the staged-cooling rate experiment, all rhizomes were immediately placed in the programmable freezers and the experiment started. Collections were made in November 2009, January 2010 and August 2010 for the continuous-cooling trial, and in January 2011 and February 2011 for the staged-cooling experiment. All artificial freezing experiments were conducted using Thermotron programmable freezers (models 8200 and 2800; Thermotron, Holland-MI, USA - http://www.thermotron.com).

During the artificial freezing experiments, rhizome temperature was monitored using 36 gauge copper-constantan thermocouples that were inserted 4 mm deep into seven rhizomes (at least one rhizome of each genotype). Thermocouples were measured with either the Thermotron 8200 data logger or a Veriteq Spectrum 1700 thermocouple data logger (Veriteq Instruments Inc., Richmond-BC, Canada, http://www.vaisala.com). Rhizome temperature profiles during a freezing experiment are shown in Chapter 2.

All freezing protocols were performed as suggested in Chapter 2.

*Continuous-cooling experiment*

Rhizomes temperature was reduced from the storage temperature (-1ºC for winter collection, and 21ºC for August 2010 collection) at a constant rate of 1ºC $hr^{-1}$ to the treatment (nadir) temperature, and they were exposed to this temperature for 16 hours. The nadir temperatures used for the November 2009 and January 2010 harvests were -1°C, -5°, -10°, -15°, -20°, -25°, and -30°C, and 9ºC, 4°, -5°, -10°, -15°C for the August 2010 samples. Samples were thawed to 21ºC at the rate of 1°C hr-1. After thawing, one set of rhizomes was used for the

electrolyte leakage measurement and another for re-growth assays. The order of treatments, and the Thermotron used for each treatment, were randomly established. Seven days were required to complete all treatments within a trial.

*Staged-cooling trials*

For the staged-cooling trials, all rhizomes were placed inside a Thermotron 8200 freezer at -1ºC. The temperature of the chambers was lowered to -2.5ºC at the rate of 1ºC $hr^{-1}$ and then held at this temperature for 24 hours. Temperature was once again lowered at the same cooling rate to -5ºC. After 24 hours at this temperature, rhizome pairs were sampled and the samples were transferred to the Thermotron 2800 set at the treatment temperature and then thawed at 1ºC $hr^{-1}$ to 21ºC. The temperature of the Thermotron 8200 was sequentially lowered to -7ºC, -10º, -12º, -14º, -18º, and -22ºC at 1°C $hr^{-1}$ and the cycle (incubation at the nadir temperature followed by sampling) repeated at each of these treatment temperature (-22ºC treatment was only tested in the February 2011 experiment). After thawing, one rhizome of the pair was used for the electrolyte leakage measurement and the other for re-growth assays. The complete trial lasted 14 days.

*Electrolyte leakage and re-growth assays*

The electrolyte assay consisted of incubating a rhizome sample in a vial containing 7 ml of de-ionized water for 24 hours. After this period, an Ultrameter 4P conductivity meter (Myron L Company, Carlsbad, CA, USA) was used to determine the relative conductivity of the incubated solution ($EL_{sample}$). Tissues damaged by cold released greater levels of electrolytes, thus raising the solution conductivity (Murray *et al.*, 1989). The incubation solution was then returned to the vial (containing the sample) and boiled for 1 hour to release all the electrolytes to the bathing solution.

Once the temperature of the bathing solution returned to room temperature, the electrolyte conductivity was again measured ($EL_{total}$). The relative conductivity is given by the formula 100 x $EL_{sample}/EL_{total}$.

Re-growth assays were conducted in a warm greenhouse at about 26º/20ºC day/night temperature, by planting the second rhizome from a sample pair in soil (40% Pro-mix, 30% loam, and 30% sand) and observing whether the rhizome grew new roots and/or shoots in the following 6 weeks. A rhizome that produced new tissue was scored as alive; one that failed to produce new tissues after 6 weeks was scored as dead.

*Experimental design and statistical analysis*

In the continuous-cooling rate experiment, the experimental design consisted of replicated trials on three sampling dates (November 2009, January 2010, and August 2010). For the staged-cooling experiment, the experimental design was the same, however with just two sampling dates (January 2011 and February 2011). For each trial, rhizome clusters from six different plants per genotype were harvested randomly in the respective field plots. Each plant provided rhizomes for all temperature treatments from that trial, such that the plant was considered the unit of replication in the statistical analysis.

Results of both the re-growth and electrolyte leakage assays were analyzed using a logistic regression given by a generalized linear mixed-effects model (GLMM) on the binary distribution because survivability and relative conductivity are constrained between 0 and 1. These models were fitted using the package "lme4" (Bates *et al.*, 2011) on R statistical software (R-Core-Team,

2013). Post-hoc means comparisons were conducted using the Tukey's test in the package "multcomp" (Hothorn *et al.*, 2008).

After determining the optimal model, it was used the predicted values for rhizome survival at each temperature and relative conductivity to estimate the temperature ($LT_{50}$) or relative conductivity ($LEL_{50}$) where rhizomes have 50% chance of mortality.

## 3.4 - Results

*Soil temperature data*

The air temperature at the Leamington-ON field site declined to -14°C on 17 December 2009 and to -20°C on 10 January 2010 (Figure 3.1A). The soil temperature fell to the seasonal minima on 3 January 2010: at 1 cm below ground, soil temperature was -4°C, and at 7 cm depth, it was -1°C. These soil temperatures followed a warm spell in the previous week that melted the snow cover and in doing so reduced its insulation. Shortly after the observed low, new snow accumulated and the soil temperature returned near -1°C for the remainder of the winter. In the 2010-2011 dormant season, the coldest soil temperature recorded was -4ºC on 24 January 2011 at 1 cm depth, when the minimal air temperature reached -15ºC and only traces of snow were present on the ground (Environment Canada website; Figure 3.1B).

Soil temperature values at 5 cm depth have been recorded by Environment Canada Stations across Canada. In Ontario, southern British Columbia, southern Quebec, and southeast Labrador, most of the weather stations did not measure soil temperature below -6.5ºC at 5 cm depth (Table 3.1, Figure 3.2). During the same period at most stations in southwest Quebec, Nova Scotia,

Alberta, the southern Yukon and southern Northwest Territories, the recorded minimum soil temperature at 5 cm below ground was between -6.5ºC and -14ºC. However, in Saskatchewan, southern Manitoba, New Brunswick, northern and eastern Quebec, western Labrador and Nunavut, the stations recorded soil temperature below -14ºC at 5 cm depth.

*Continuous-cooling experiment*

In the continuous-cooling rate study, survivability of the rhizomes from 5 genotypes was similar when sampled in November 2009 and January 2010 (Figure 3.3). $LT_{50}$ values from the 5 genotypes ranged between -4.4ºC to -6.7ºC. Variety Illinois (the triploid used in Illinois trials by Heaton *et al.* 2008*a* and Dohleman & Long 2009) was the least cold tolerant, with 50% of the rhizome death at -5ºC, and an estimated $LT_{50}$ of -4.4ºC in January 2010. The diploid variety M115 was the most cold tolerant with no mortality at -5ºC for the November 2009 and January 2010 sampling, and corresponding $LT_{50}$ estimates of -6.3ºC and -6.7ºC. Rhizomes sampled in the summer (August 2010) were as cold tolerant as winter sampled rhizomes in the M115 and M147 lines, but less cold tolerant by approximately 2º to 3ºC in the triploids (Nagara and Illinois) and tetraploid (M118) lines. For example, Illinois genotype, the estimated $LT_{50}$ in the rhizomes for the August 2010 sampling rose to -1.5ºC, approximately 3ºC warmer than for rhizomes sampled in the previous winter. In all cases, no *Miscanthus* rhizome re-sprouted after exposed to ≤ -10ºC.

The $LEL_{50}$ was equivalent for all genotypes at all collection times and varied between 15% and 22% (Figure 3.4). In treatments at -5ºC, the rhizomes of all genotypes exhibited lethal relative conductivity near the predicted $LT_{50}$ values (Figure 3.5). Treatment at -10ºC significantly increased the RC to above 40% in all rhizomes, none of which were viable after the treatment.

*Staged-cooling experiment*

When rhizomes were frozen gradually in a staged manner, rhizome survivability did not differ between the January 2011 and February 2011 collections, but did differ between genotypes (Figure 3.6). M115 and M147 had more negative $LT_{50}$ values (-14.4ºC and -12.6ºC, respectively), with no rhizome surviving below -15ºC (Figure 3.6A, B). The allotriploid (M1) and the allotetraploid (M119) had similar survival patterns as the allotriplois and allotetraploid genotypes tested in the continuous-cooling experiment, and were considerably less cold tolerant than the diploids. Their $LT_{50}$ was -6.6ºC and -6.3ºC respectively (Figure 3.6C, D). At -10ºC, no rhizome of M1 and M119 genotypes survived in January 2011, and just one out of 6 rhizomes survived in the February 2011 sampling. Below -10ºC, no rhizome from M1 and M119 survived.

Diploid genotypes also exhibited higher $LEL_{50}$ (30% for M115 and 27% for M147; Figure 3.7A, B) in comparison to the continuous-cooling rate experiment, and to M1 and M119 (18% and 17%, respectively; Figure 3.7C, D). For the staged-cooling experiment, the $LEL_{50}$ in the diploid genotypes was reached at lower temperature than in the continuous-cooling rate, and complete leakage was not reached even after treatment at -22ºC (Figure 3.8A, B). For the allopolyploids, there was little difference in the relationship between temperature treatment and RC, and at -22ºC the rhizomes had nearly complete leakage (Figure 3.8C, D).

Table 3.1 - Soil temperature at 5 cm depth for 85 weather stations. Loc is for location of the station; Station ID and Stn name are the station identifications used by Environment Canada; Prov is for province; min soil temp at 5 cm deep is the minimal soil temperature recorded during years indicated. Min air temp is the minimal air temperature on the day the minimal soil temperature was registered (min soil temp reg date). 3-day mean temp and snow cover are the mean temperature and mean snow cover (respectively) on the three days before the minimal soil temperature was registered. Data provided by Environment Canada (http://weather.gc.ca/) and Ontario Climate Centre (http://climate.weather.gc.ca/). See Figure 3.2 for the location of the weather stations.

| Loc | Station ID | Stn Name | Prov | Latitude (degrees) | Longitude (degrees) | Min soil temp at 5cm (ºC) | Min air temp(ºC) | 3-day mean temp (ºC) | Snow cover (cm) | Min soil temp reg date | Record range | # of years |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1016940 | SAANICHTON CDA | BC | 48.62 | -123.42 | 0 | -3 | 0.833 | 0 | 1/6/1995 | 1984-1996 | 13 |
| 2 | 1100119 | AGASSIZ RCS | BC | 49.24 | -121.76 | -3.3 | -10.1 | -6.2 | NA | 1/7/1993 | 1991-1996 | 6 |
| 3 | 1108487 | VANCOUVER UBC | BC | 49.25 | -123.25 | 0 | -8.3 | -5.2 | 10 | 1/1/1985 | 1984-1990 | 7 |
| 4 | 1127800 - 112G8L1 | SUMMERLAND CS | BC | 49.56 | -119.65 | -9 | -15.3 | -11.33 | NA | 1/10/1993 | 1984-1996 | 13 |
| 5 | 3033890 | LETHBRIDGE CDA | ALTA | 49.7 | -112.77 | -14 | -31 | -25.2 | 3 | 2/3/1985 | 1984-1990 | 7 |
| 6 | 3036681 | VAUXHALL CDA | ALTA | 50.05 | -112.13 | -11.5 | -33.5 | -27.6 | 3 | 2/3/1985 | 1984-1992 | 9 |
| 7 | 3036652 | UNIVERSITY OF CALGARY | ALTA | 51.08 | -114.13 | -12.5 | -26.5 | -20.7 | 3 | 2/3/1985 | 1984-1990 | 7 |
| 8 | 3023720 | LACOMBE CDA | ALTA | 52.47 | -113.75 | -15 | -33 | -23.43 | 2 | 2/5/1988 | 1984-1995 | 12 |
| 9 | 3012295 | ELLERSLIE | ALTA | 53.42 | -113.55 | -8.9 | -35.5 | -14 | 21 | 2/3/1985 | 1984-1986 | 3 |
| 10 | 3016761 | VEGREVILLE CDA | ALTA | 53.48 | -112.03 | -13.9 | -40.5 | -31.43 | NA | 1/8/1991 | 1984-1994 | 11 |
| 11 | 3062244 | EDSON A | ALTA | 53.58 | -116.47 | -7 | -23.6 | -25.1 | 8 | 2/20/1986 | 1984-1993 | 10 |
| 12 | 3070560 | BEAVERLODGE CDA | ALTA | 55.2 | -119.4 | -12.8 | -31.5 | -25 | 6 | 3/2/1991 | 1984-1996 | 13 |

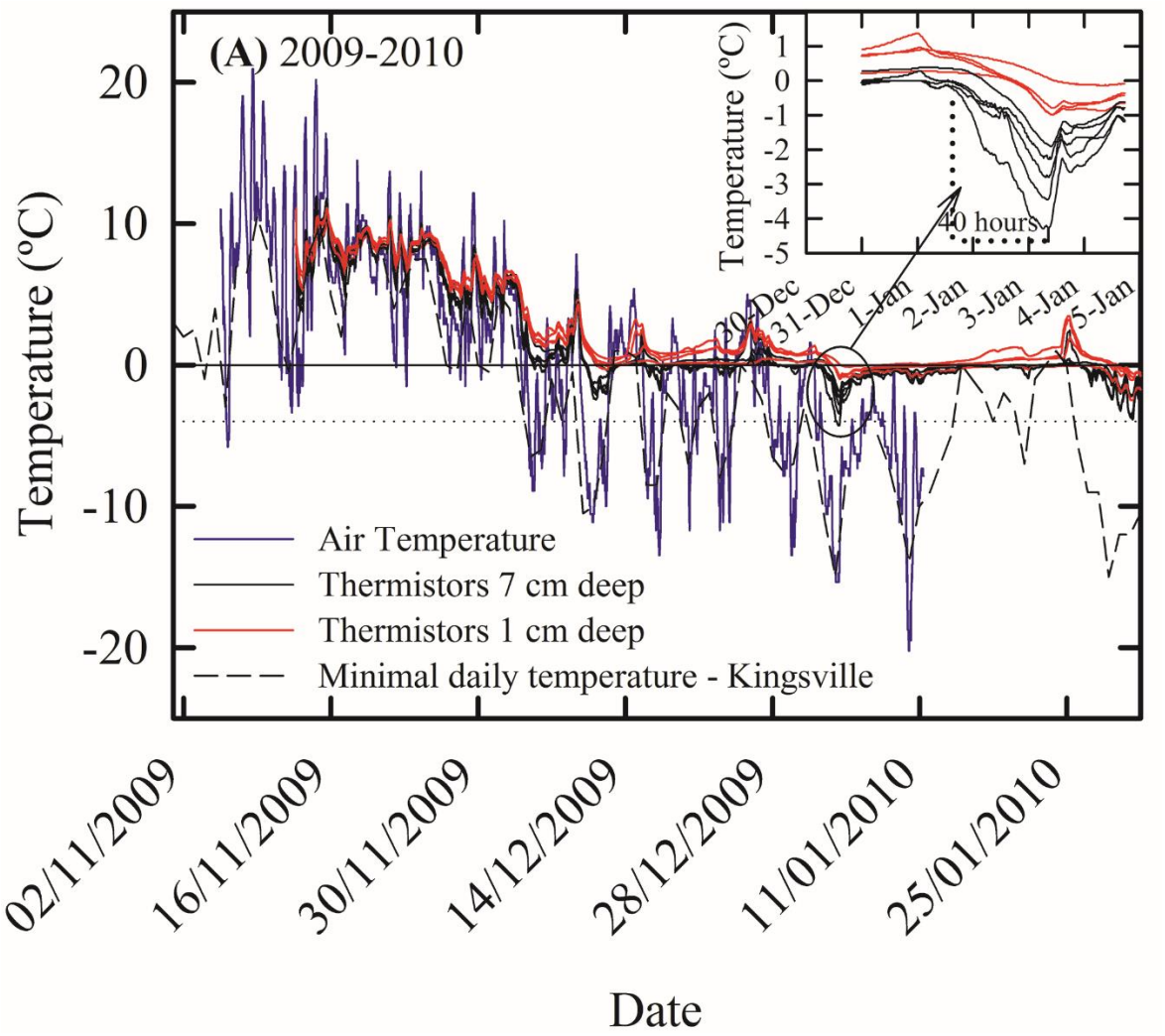
| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 13 | 3066001 | SLAVE LAKE A | ALTA | 55.3 | -114.78 | -13.5 | -34.2 | -28.35 | 20 | 12/24/1984 | 1984-1993 | 10 |
| 14 | 3075040 | PEACE RIVER A | ALTA | 56.23 | -117.45 | -8.5 | -22.5 | -17.5 | 4 | 1/9/1984 | 1984-1988 | 5 |
| 15 | 4012400 | ESTEVAN A | SASK | 49.22 | -102.97 | -16 | -30.8 | -20.85 | 4 | 1/18/1992 | 1984-2008 | 25 |
| 16 | 4028040 - 4028060 | SWIFT CURRENT | SASK | 50.27 | -107.73 | -24 | -34.5 | -29.43 | 8 | 2/3/1985 | 1984-2008 | 25 |
| 17 | 4010879 | BROADVIEW | SASK | 50.37 | -102.57 | -20 | -39.6 | -30.23 | 6 | 1/9/1991 | 1985-1994 | 10 |
| 18 | 4016640 | REGINA CDA | SASK | 50.4 | -104.57 | -21 | -38.5 | -30.6 | 7.33 | 2/4/1985 | 1984-1993 | 10 |
| 19 | 4013490 | INDIAN HEAD PFRA | SASK | 50.5 | -103.68 | -15.5 | -22 | -16.86 | 2 | 12/10/1988 | 1984-1995 | 12 |
| 20 | 4019080 | YORKTON A | SASK | 51.27 | -102.47 | -19 | -39.2 | -34.43 | 22 | 2/3/1989 | 1984-1991 | 8 |
| 21 | 404037Q | BAD LAKE IHD 102 | SASK | 51.32 | -108.42 | -14.5 | -35 | -26.5 | 3 | 12/2/1985 | 1984-1986 | 3 |
| 22 | 4055736 | OUTLOOK PFRA | SASK | 51.48 | -107.05 | -16.5 | -33 | -28.1 | 1 | 11/27/1985 | 1984-1986 | 3 |
| 23 | 4043900 | KINDERSLEY A | SASK | 51.52 | -109.18 | -25 | -35.2 | -30.03 | 7 | 2/5/1988 | 1987-2006 | 20 |
| 24 | 4019035 | WYNYARD | SASK | 51.77 | -104.2 | -22 | -33 | -27.53 | 1 | 1/5/2004 | 1984-2005 | 22 |
| 25 | 4057180 | SASKATOON SRC | SASK | 52.15 | -106.6 | -18.5 | -37 | -32.03 | 10 | 1/9/1991 | 1984-1997 | 14 |
| 26 | 4083321 | HUDSON BAY A | SASK | 52.82 | -102.32 | -9 | -37.8 | -35.33 | 15 | 12/21/1989 | 1984-1993 | 10 |
| 27 | 4075518 | NIPAWIN A | SASK | 53.33 | -104 | -21 | -33.6 | -31.43 | 10 | 2/3/1989 | 1984-1997 | 14 |
| 28 | 4064150 | LA RONGE A | SASK | 55.15 | -105.27 | -11 | -37.6 | -30.4 | 0.5 | 11/28/1985 | 1984-1995 | 12 |
| 29 | 4061861 | CREE LAKE | SASK | 57.35 | -107.13 | -15 | -41 | -29.4 | 12 | 1/19/1984 | 1984-1993 | 10 |
| 30 | 5021848 | MORDEN CDA | MAN | 49.18 | -98.08 | -21 | -30 | -24.4 | 0.5 | 1/20/1984 | 1984-1998 | 15 |
| 31 | 5021054 | GLENLEA | MAN | 49.65 | -97.12 | -19 | -33.5 | -27.65 | 23 | 1/7/1991 | 1984-2002 | 19 |
| 32 | 5023222 - 5023224 | WINNIPEG | MAN | 49.92 | -97.23 | -13 | -35.7 | -29.46 | 42 | 1/10/1989 | 1984-1997 | 14 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 33 | 5031038 | GIMLI | MAN | 50.63 | -97.02 | -12 | -32.4 | -26.6 | 6 | 12/26/1984 | 1984-1991 | 8 |
| 34 | 5043158 | MCCREARY | MAN | 50.71 | -99.53 | -12 | -24 | -18.83 | NA | 2/17/1993 | 1991-1997 | 7 |
| 35 | 5052060 | PASQUIA PROJECT | MAN | 53.72 | -101.53 | -17 | -40.5 | -32.5 | 13 | 1/9/1991 | 1984-2005 | 22 |
| 36 | 5062922 - 5062926 | THOMPSON | MAN | 55.8 | -97.86 | -13.5 | -34 | -30.27 | 29 | 1/3/1986 | 1984-2007 | 24 |
| 37 | 6133360 | HARROW CDA | ONT | 42.03 | -82.9 | -4.8 | -17.5 | -12.86 | 2 | 1/10/1988 | 1984-1989 | 6 |
| 38 | 6137730 | SIMCOE | ONT | 42.85 | -80.27 | -3 | -22 | -16.75 | 14.5 | 1/21/1985 | 1984-1986 | 3 |
| 39 | 6139145 | VINELAND STATION | ONT | 43.18 | -79.4 | -4 | -14.6 | -9 | 0.33 | 3/7/1989 | 1984-1989 | 6 |
| 40 | 6142285-614B2H4 | ELORA AUTOMATIC CLIMATE STATION | ONT | 43.65 | -80.42 | -7.8 | -24 | -14.75 | NA | 1/14/1988 | 1984-1995 | 12 |
| 41 | 6158740 | TORONTO MET RES STN | ONT | 43.8 | -79.55 | -4 | -16.5 | -11.6 | 4.33 | 2/27/1986 | 1984-1988 | 5 |
| 42 | 611KBE0 | EGBERT CARE | ONT | 44.23 | -79.78 | -8 | -23.5 | -16.75 | 7 | 2/7/1993 | 1988-1995 | 8 |
| 43 | 6104025 | KEMPTVILLE | ONT | 45 | -75.63 | -11 | -24 | -17.53 | 0 | 3/8/1989 | 1988-1989 | 2 |
| 44 | 6105976 | OTTAWA CDA | ONT | 45.38 | -75.72 | -9 | -21.7 | -15.56 | 3 | 2/18/1989 | 1984-1998 | 15 |
| 45 | 6020379 | ATIKOKAN | ONT | 48.75 | -91.62 | -2 | -37.6 | -26.43 | 27.33 | 2/13/1988 | 1984-1988 | 5 |
| 46 | 6073960 | KAPUSKASING CDA | ONT | 49.4 | -82.43 | -4.9 | -30 | -18.36 | 7.33 | 12/21/1984 | 1984-2000 | 17 |
| 47 | 6016525 | PICKLE LAKE (AUT) | ONT | 51.45 | -90.22 | -3 | -28.1 | -24.3 | 67 | 3/5/1989 | 1984-1990 | 7 |
| 48 | 7024280 | LENNOXVILLE | QUE | 45.37 | -71.82 | -4 | -22 | -14.87 | 5.66 | 3/7/1990 | 1984-1995 | 12 |
| 49 | 7026839 | STE ANNE DE BELLEVUE | QUE | 45.43 | -73.93 | -11.5 | -25 | -17.13 | 18 | 3/8/1989 | 1984-1992 | 9 |
| 50 | 7025250 - 7035290 | MONTREAL/ | QUE | 45.67 | -74.03 | -18.5 | -30.4 | -26.05 | 11 | 1/17/1992 | 1984-1999 | 16 |
| 51 | 7014160 | L'ASSOMPTION | QUE | 45.81 | -73.43 | -12.5 | -26 | -19.96 | 8 | 1/27/1992 | 1984-1995 | 12 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 52 | 7016900 | ST AUGUSTIN | QUE | 46.73 | -71.5 | -5.4 | -21 | -17.4 | 38.33 | 1/18/1987 | 1984-1987 | 4 |
| 53 | 7042388 | FORET MONTMORENCY | QUE | 47.32 | -71.15 | -1.5 | -30.5 | -15 | 39 | 2/1/1995 | 1984-1997 | 14 |
| 54 | 7054095 | LA POCATIERE CDA | QUE | 47.35 | -70.03 | -17.5 | -30 | -23.9 | 16 | 2/7/1993 | 1984-1996 | 13 |
| 55 | 7098600 | VAL-D'OR A | QUE | 48.06 | -77.79 | -9 | -31.1 | -24.07 | 43.67 | 2/8/1995 | 1984-1995 | 12 |
| 56 | 7065640 | NORMANDIN CDA | QUE | 48.85 | -72.53 | -12 | -35.5 | -26.26 | 18 | 2/6/1985 | 1984-1992 | 9 |
| 57 | 7040440 | BAIE-COMEAU A | QUE | 49.13 | -68.2 | -19.5 | -30.2 | -28 | 18.33 | 12/29/1993 | 1984-1999 | 16 |
| 58 | 7113534 - 7.113E+37 | KUUJJUAQ UA | QUE | 58.12 | -68.42 | -31.5 | -42.4 | -38.95 | 33 | 1/25/1994 | 1984-1995 | 12 |
| 59 | 8102234 | HOYT BLISSVILLE | NB | 45.6 | -66.57 | -16 | -21.5 | -15 | 0 | 1/27/1992 | 1984-1999 | 16 |
| 60 | 8101600 | FREDERICTON CDA | NB | 45.92 | -66.62 | -17 | -25.5 | -17.26 | 0 | 2/2/1994 | 1984-1999 | 16 |
| 61 | 8100592 - 8100593 | BUCTOUCHE CDA | NB | 46.43 | -64.77 | -8.5 | -20.5 | -16.83 | 13.33 | 2/21/1990 | 1984-1998 | 15 |
| 62 | 8202800 - 8202810 | KENTVILLE CDA | NS | 45.07 | -64.48 | -8 | -19 | -8.9 | 4.5 | 3/4/1995 | 1984-1998 | 15 |
| 63 | 8205990 | TRURO | NS | 45.37 | -63.27 | -8 | -18 | 11.25 | 0 | 2/9/1997 | 1984-1999 | 16 |
| 64 | 8300400 - 8300401 | CHARLOTTETOWN CDA CS | PEI | 46.25 | -63.13 | -7.5 | -19.2 | -14.9 | NA | 1/28/1992 | 1984-1998 | 15 |
| 65 | 8403600 - 8403605 | ST JOHN'S WEST CDA CS | NFLD | 47.52 | -52.78 | -8.4 | -13.7 | -10.3 | 0.5 | 2/9/1998 | 1984-1998 | 15 |
| 66 | 8504217 | WEST ST MODESTE | NFLD | 51.58 | -56.72 | -3 | -16 | -12.67 | NA | 1/8/1985 | 1984-1987 | 4 |
| 67 | 8503018 | PORT HOPE SIMPSON | NFLD | 52.53 | -56.3 | -2.5 | -20 | -13.5 | 12 | 12/8/1985 | 1984-1988 | 5 |
| 68 | 8501900 | GOOSE A | NFLD | 53.32 | -60.42 | -21 | -28.7 | -22.76 | 1 | 2/5/1996 | 1984-1999 | 16 |
| 69 | 8502800 | NAIN A | NFLD | 56.55 | -61.68 | -22.5 | -29.5 | -25.86 | 172 | 2/10/1988 | 1984-1991 | 8 |
| 70 | 2101200 | WATSON LAKE A | YT | 60.12 | -128.82 | -11 | -24.7 | -24 | 19 | 1/1/1984 | 1984-1988 | 5 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 71 | 2202208 | FORT SMITH UA | NWT | 60.03 | -111.93 | -11.4 | -42.2 | -34.13 | 18 | 1/11/1999 | 1984-2001 | 18 |
| 72 | 2400800 | CLYDE A | NU | 70.49 | -68.52 | -39.5 | -46.2 | 42.43 | 35 | 2/12/1987 | 1984-2000 | 17 |
| 73 | 2403500 | RESOLUTE CARS | NU | 74.72 | -94.97 | -25.5 | -42.2 | -41.86 | 25 | 3/4/1984 | 1984-1999 | 16 |

Figure 3.1 - Air and soil temperature recorded for (A) November 2009 to January 2010 and (B) November 2010 to March 2011, at or near the field site in Leamington, Ontario. Thermistors recorded soil temperature at 1 cm deep (black lines) and 7 cm deep (red lines). The blue line shows the air temperature at the field site until January 12th 2010 (A), and November 2010 to January 2011 (B; except for 15 November 2010 to 15 January 2011, lost data). The dashed line represents the daily minimum temperature registered by Kingsville-ON weather station, located 11 km from the field site (data from The Weather Office, Environment Canada http://www.weatheroffice.gc.ca). The horizontal dotted line marks the minimal temperature reached in the soil at 1 cm depth (-4° C for both years). The insert graph is a magnification of the area inside the circle.



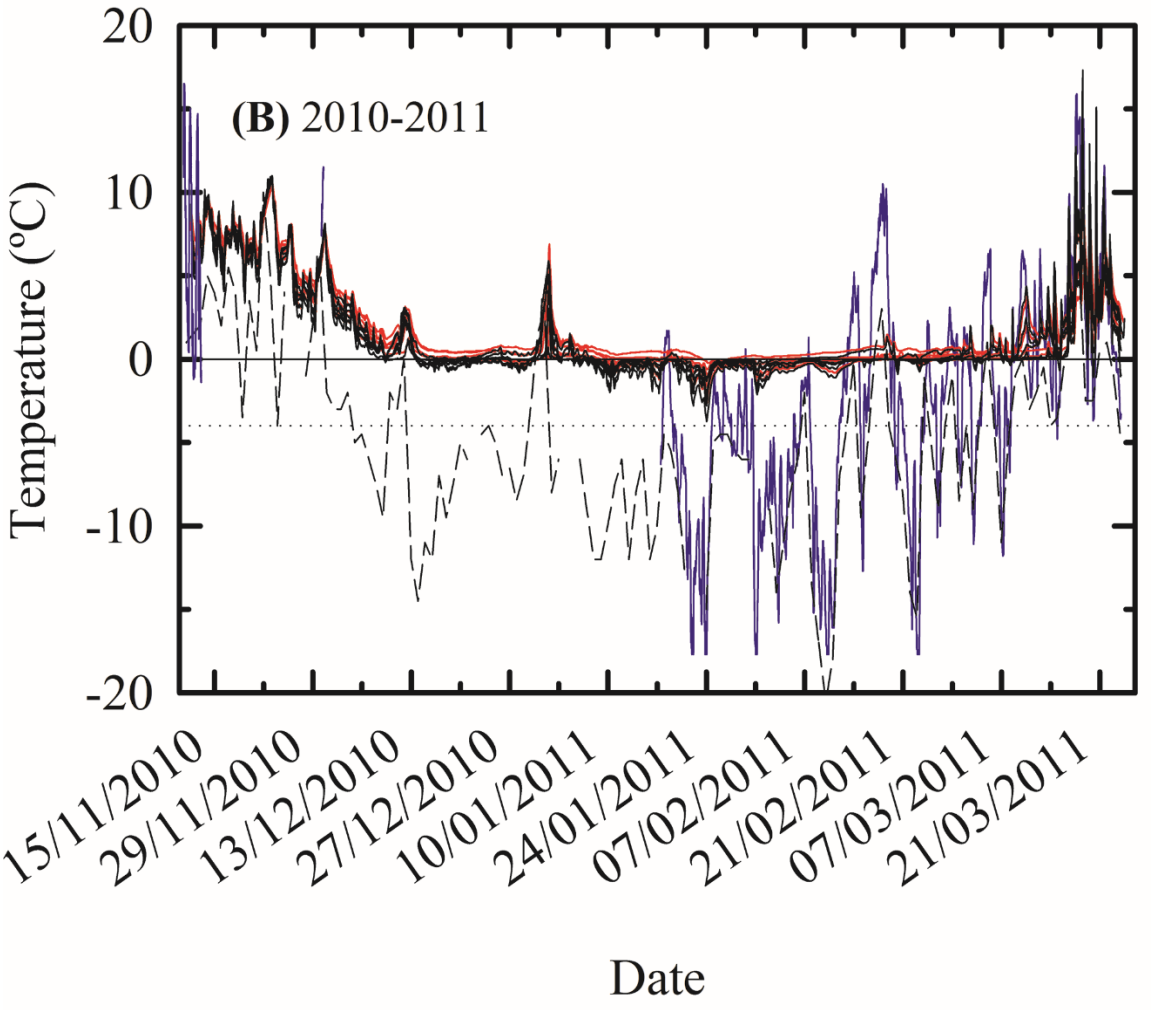

(B) 2010-2011
Temperature (ºC)
20
10
0
-10
-20
15/11/2010
29/11/2010
13/12/2010
27/12/2010
10/01/2011
24/01/2011
07/02/2011
21/02/2011
07/03/2011
21/03/2011
Date

Figure 3.2 - Map indicating the location of the weather stations where soil temperature at 5 cm was recorded. Green circles indicate weather stations with the minimal temperature registered above -6.4º C, yellow circles indicate weather stations with the minimal temperature registered between -6.4ºC and -14.1ºC, and red circles indicate weather stations with the minimal temperature registered below -14.1ºC during the measurement intervals for each station by Environment Canada (http://weather.gc.ca/) between 1984 and 2006. Weather stations with 5 years or less of recorded data are marked with a "*". Refer to Table 3.1 for more information about the weather station and other climatic conditions

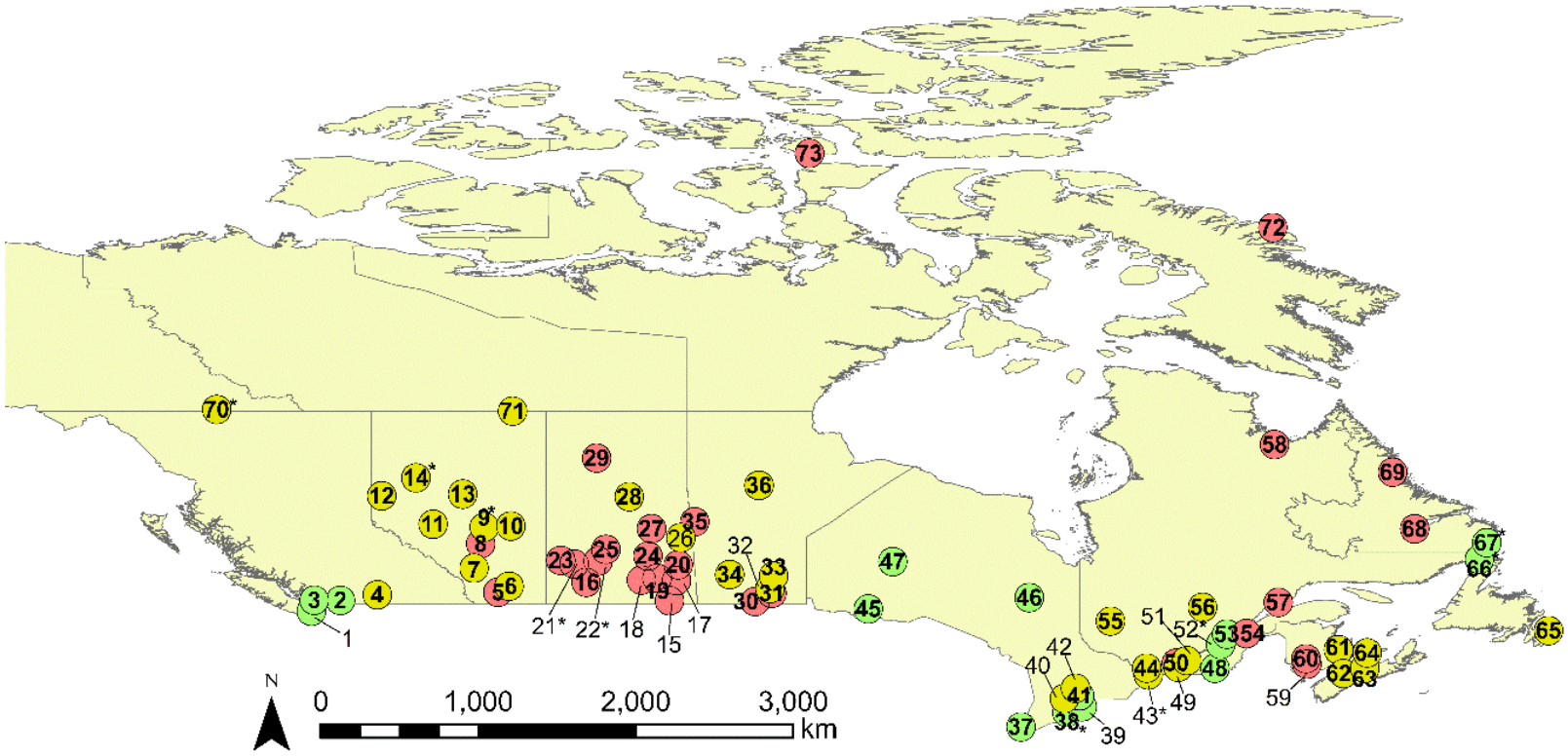


Figure 3.3 - Survivability for *Miscanthus* rhizomes as a function of temperature in the continuous-cooling experiment. Symbols are observed values (mean ± SE, n = 6), lines are predicted responses using a logistic regression. Filled diamonds and solid lines for November 2009, open circles and dotted lines for January 2010, filled stars and dashed lines for August 2010. Treatment temperature significantly affected rhizomes survivability ($p < 0.001$), while difference between the *Miscanthus* varieties is not significant ($p = 0.53$). The interaction between genotypes and the temperature effect on survivability is significant ($p = 0.001$), as is the interaction between genotype and sampling date ($p = 0.0014$). Differences in sampling date are not significant ($p = 0.08$).

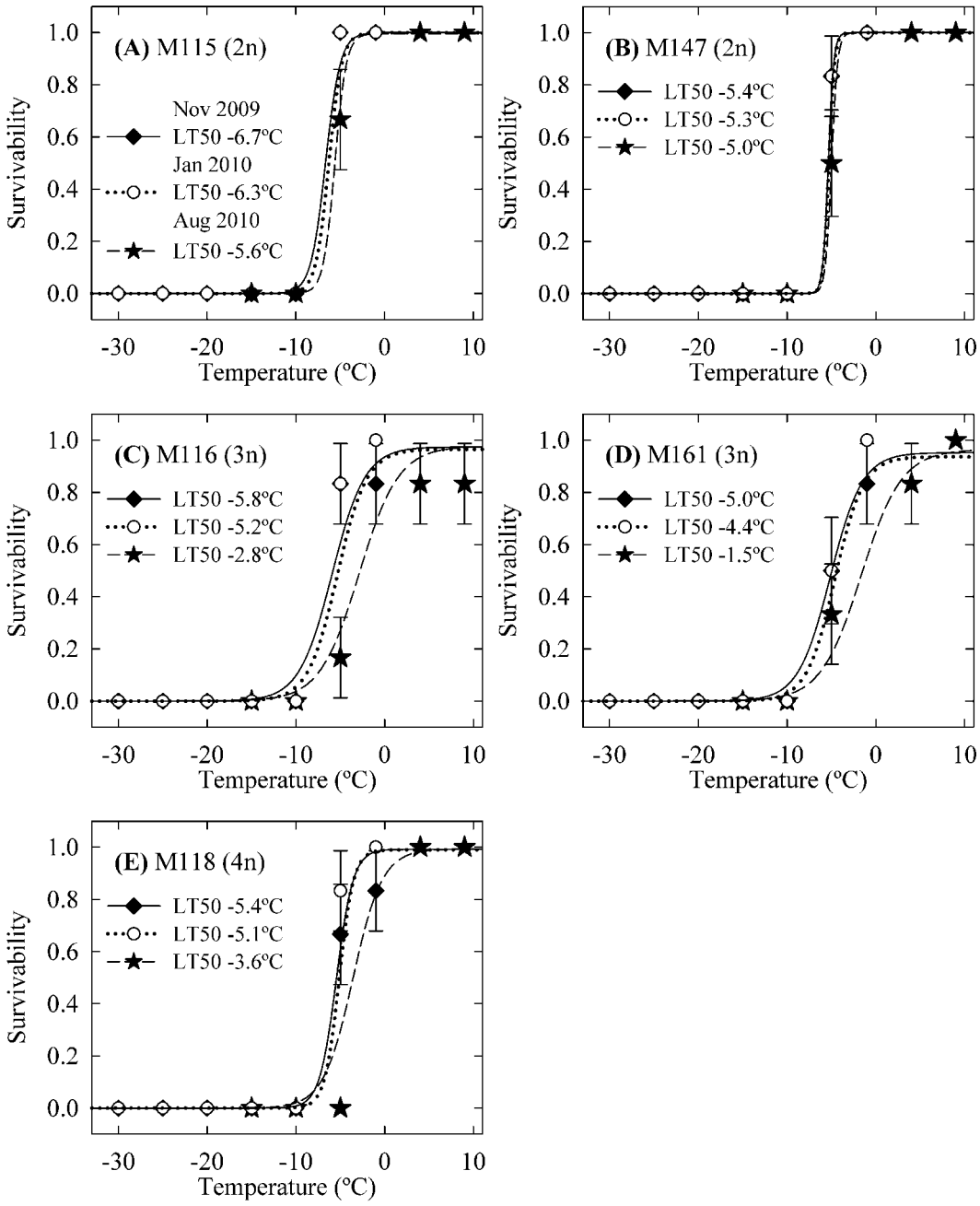


Figure 3.4 - Survivability for *Miscanthus* rhizomes as a function of relative conductivity (RC) in the continuous-cooling experiment. Symbols are observed values (mean ± SE, n = 6), lines show predicted responses using logistic regression. Filled diamonds and solid lines for November 2009, open circles and dotted lines for January 2010, Filled stars and dashed lines for August 2010. Collection time (p = 0.052) and genotype (p = 0.051) differences were not significant.

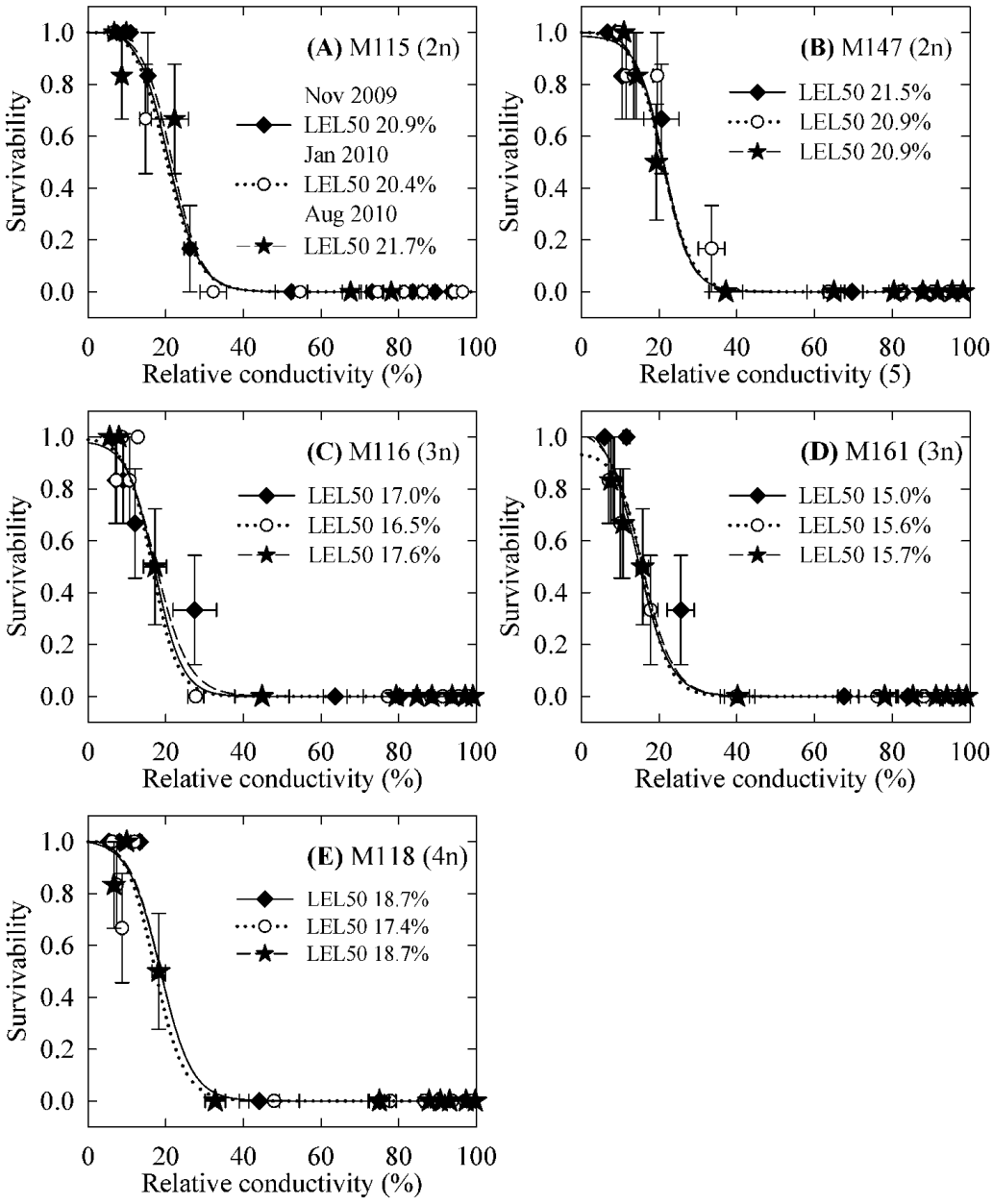


Figure 3.5 - Relative conductivity as a function of the lowest temperature at which *Miscanthus* rhizomes were tested in in the continuous-cooling rate experiment. Points show the mean ± SE (n = 12) of the pooled data from all collection times because there was no effect of time (P = 0.13). Genotypes also were not significantly different (P = 0.52). Vertical short dashed line is the predicted $LT_{50}$, and horizontal long dashed line is the predicted $LEL_{50}$. The filled line is a logistic regression. Error bars represent the standard error. N = 6

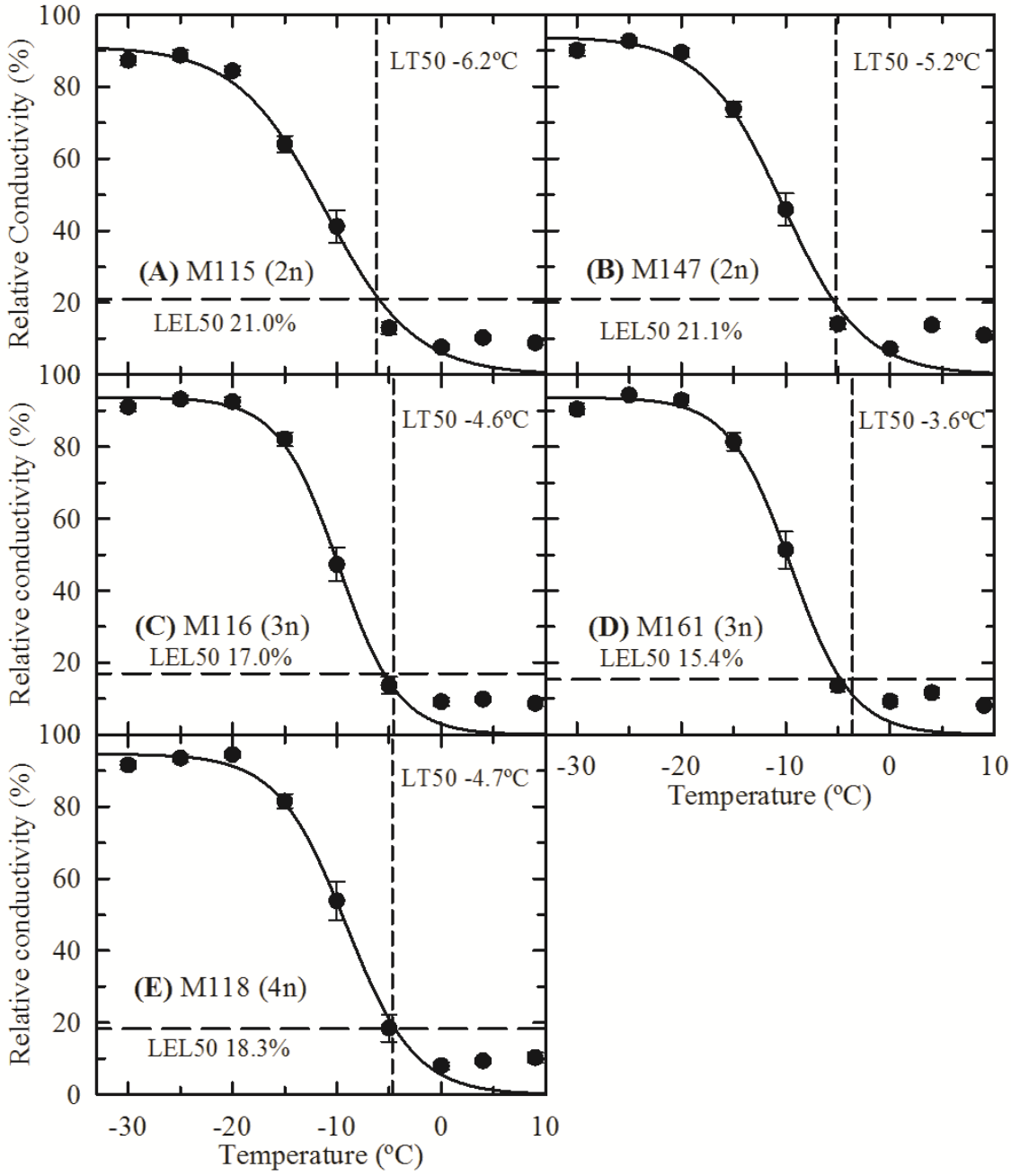


Figure 3.6 - Survivability for *Miscanthus* rhizomes as a function of temperature in the staged-cooling rate experiment. $LT_{50}$ is the temperature on which a rhizome has 50% of chances of being killed. Symbols are means ± SE (n = 6) and lines are the predicted response using a logistic regression fitted to the data. Filled diamonds for January 2011, open circles for February 2011. One regression line was drawn for both collection times because there is no difference between them (p = 0.479). The difference in survivability between the *Miscanthus* genotypes was significant (p < 0.001) and post-hock Tukey test differentiate the diploids from M1 and M119 (Pz < 0.001). No difference between diploids (Pz = 0.219) or between M1 and M119 (Pz = 0.993) was significant.

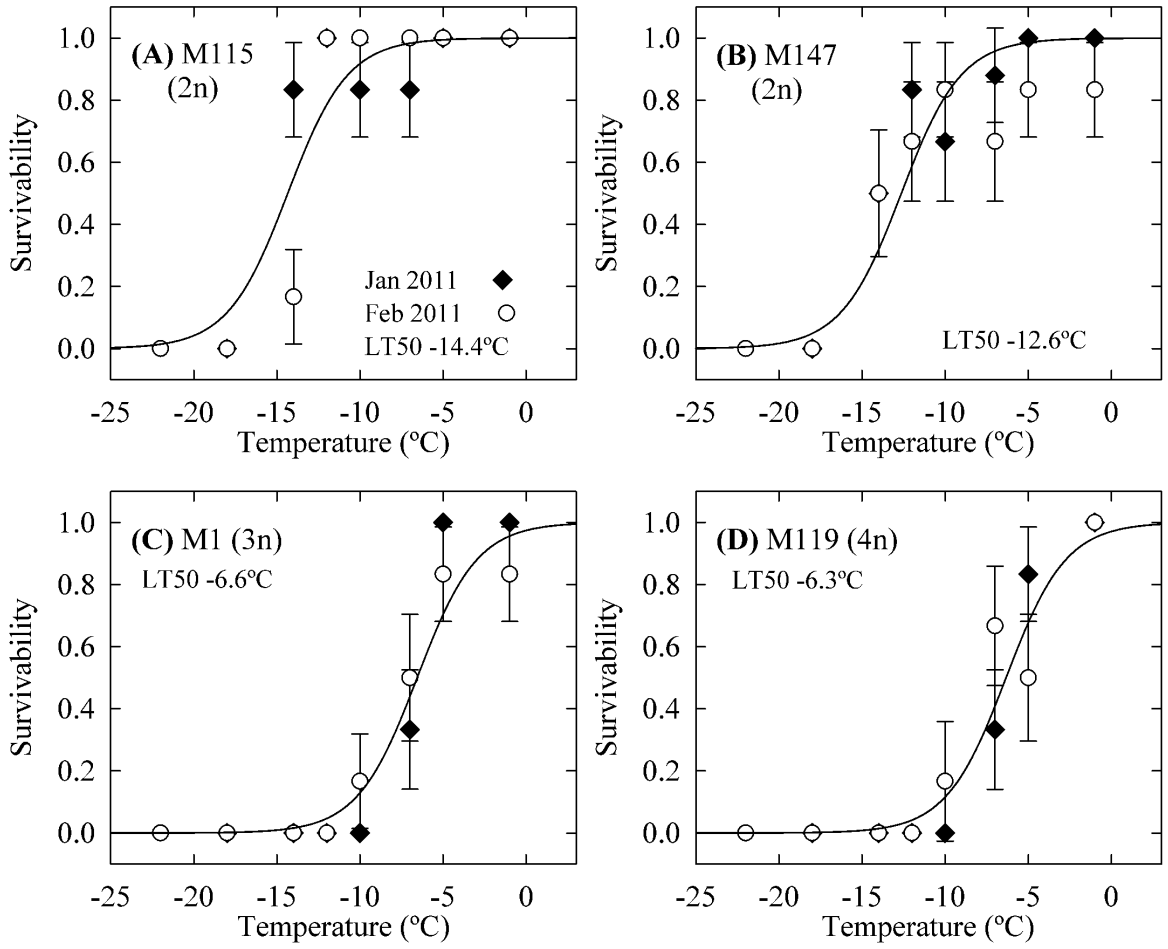


Figure 3.7 - The survivability of rhizomes as a function of relative conductivity in the staged-cooling experiment. Symbols are the observed mean ± SE (n = 6), and lines are the predicted values fitted using a logistic regression. $LEL_{50}$ is the relative conductivity at which a rhizome has 50% chance of being killed. Differences in collection time is not significant (p = 0.99); genotypes show different $LEL_{50}$ ($p < 0.001$). The post-hoc Tukey test indicates the diploid genotypes have similar $LEL_{50}$ (Pz = 0.82), as well as M1 and M119 (Pz = 0.99). However the $LEL_{50}$ for M115 and M147 are different from M1 and M119 ($Pz < 0.001$) for M115 and M1 or M119; Pz = 0.002 for M147 and M1 or M119).

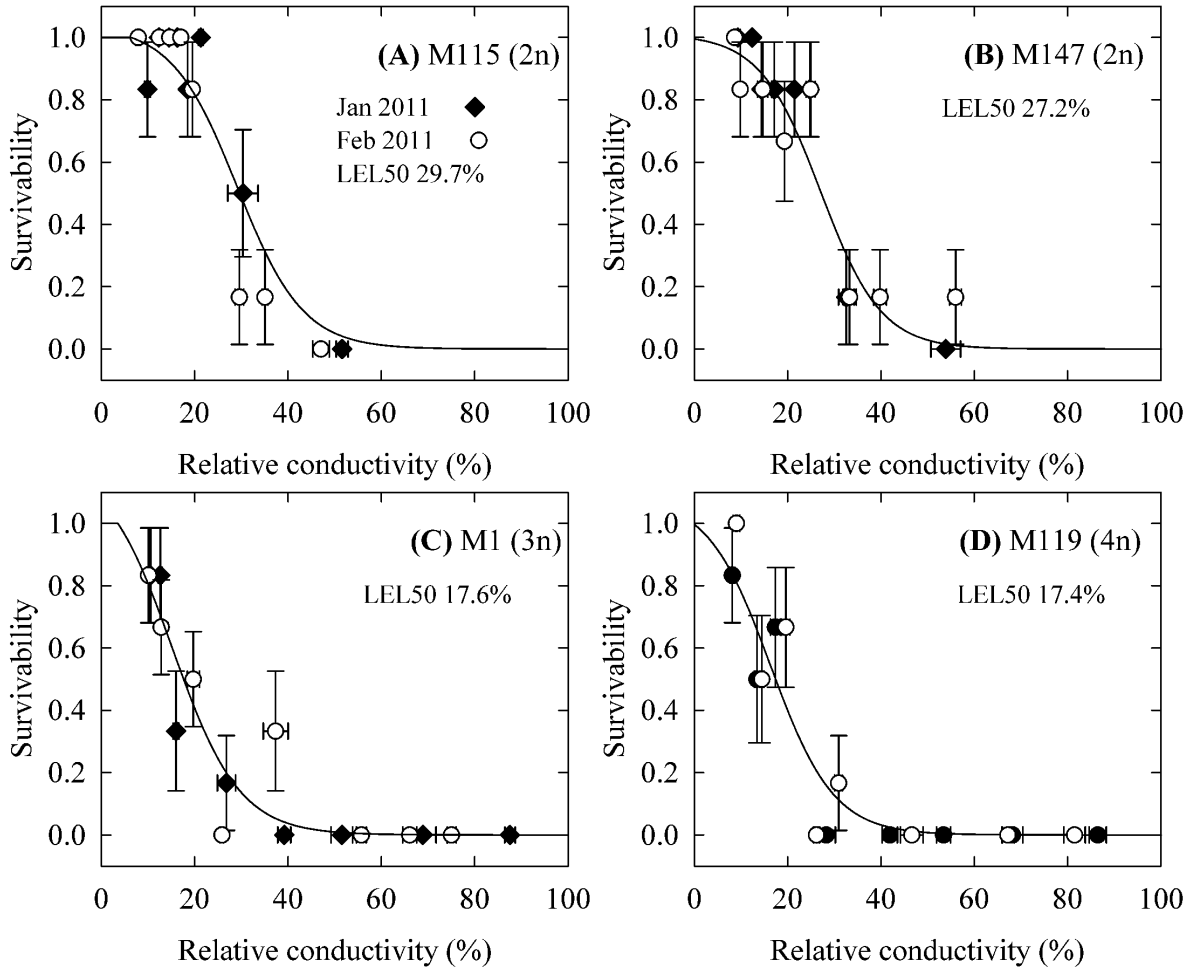
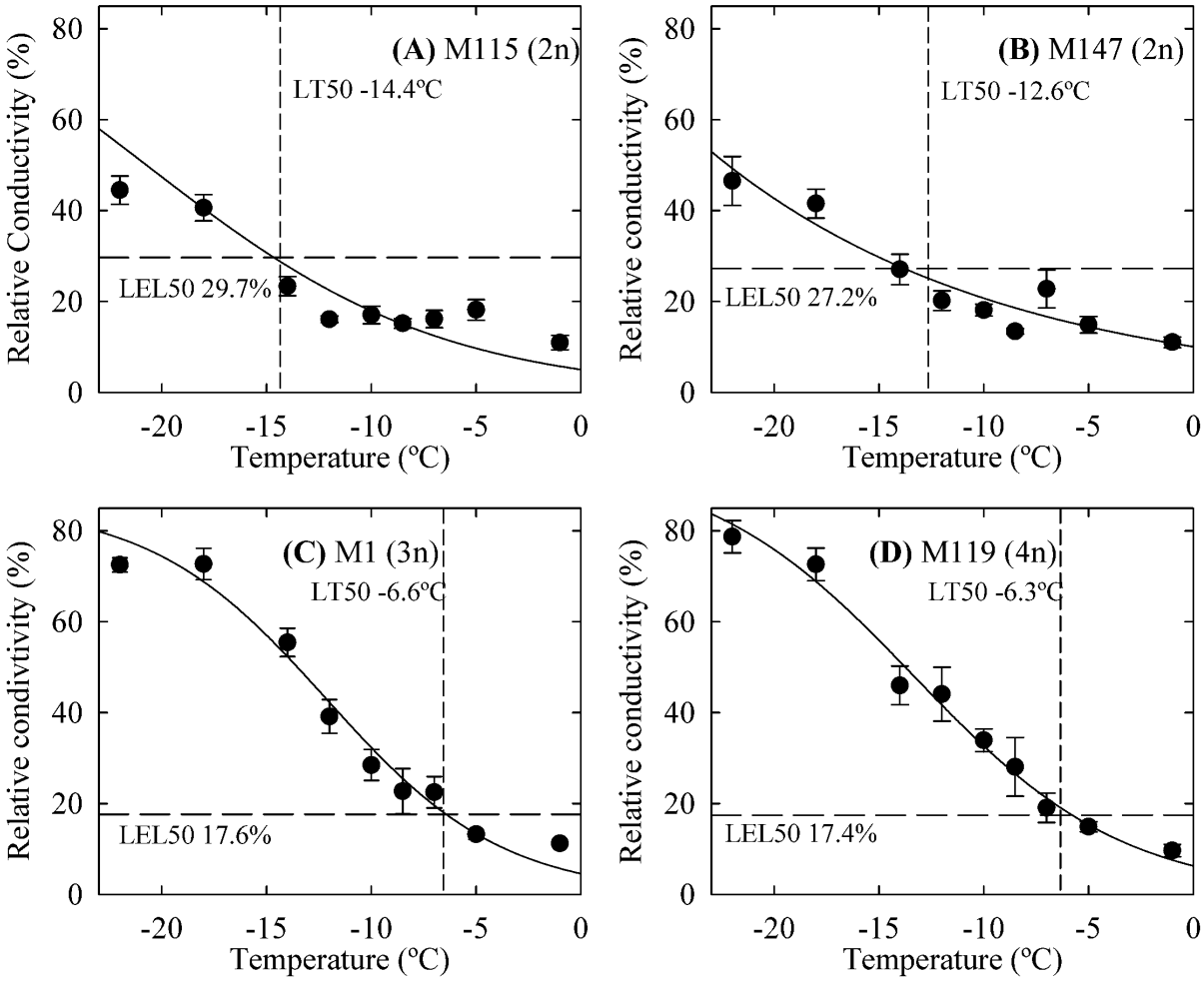


Figure 3.8 - Relative conductivity as a function of the nadir temperature in *Miscanthus* rhizomes from the staged-cooling rate experiment. Symbols are the mean ± SE (n = 12) of the pooled data from all collection times because there is no difference between collection times (P > 0.05). Difference between genotypes is significant (P = 0.001). Vertical short dashed line is the LT50, and horizontal long dashed line is the LEL50. Solid line is a logistic regression. Error bars represent the standard error. N = 6.



## 3.5 - Discussion

Prior research shows that *Miscanthus* rhizomes can survive down to -3.4ºC (allopolyploids) to -6.5ºC (diploids) in winter-hardened conditions (Clifton-Brown & Lewandowski, 2000a). These numbers are similar to the ones observed when *Miscanthus* rhizomes were continuously cooled in winter at 1ºC $h^{-1}$ (-4.4ºC to -6.7ºC). However, when temperature was lowered in a more gradual, staged manner, rhizomes of diploid hybrids of *Miscanthus* acclimated such that the lethal temperature was not observed until near -14ºC. Allopolyploid hybrids did not show such a shift, as they were still killed by exposure near -6.5ºC. Staged-cooled rhizomes of diploid *Miscanthus* re-sprouted even though they had higher EL than allopolyploids and continuous-cooled rhizomes, indicating greater tolerance to moderate tissue injury. These results show rhizomes from diploid *Miscanthus* lines have a substantial ability to acclimate to slowly increasing winter cold, in contrast to rhizomes of polyploid hybrids. Ploidy thus appears to affect potential cold hardness with the polyploids losing some of the genetic potential for cold tolerance that is present in the diploid lines. If widespread in the hybrid lines of *Miscanthus*, this absence of tolerance to -14ºC could represent a significant limitation on attempts to cultivate *Miscanthus* at latitudes above the warm temperate zone. The presence of greater cold tolerance in the diploid rhizome, demonstrates that there is genetic potential for survival to below -10ºC in the *Miscanthus* genepool, and it could be exploited through targeted breeding efforts that employ physiological surveys to detect greater cold tolerance in the hybrid offspring.

Cold acclimation begins before the end of the growing season, when photosynthetic rates are still high, producing sugars that will be important for sub-zero temperature tolerance (Gusta & Wisniewski, 2013). In *Miscanthus*, N mobilization to the rhizomes begins in August and might be

an indication of the beginning of cold acclimation (Dohleman *et al.*, 2009, 2012). This first stage of acclimation is marked by accumulation of sugars, cryoprotective compounds, anti-freeze proteins, amino and organic acids, and modification in the membrane lipid composition. Sugar accumulation enhances cold tolerance in *Miscanthus* (Purdy *et al.*, 2013) as well as in other species including *Arabidopsis* (Kaplan & Guy, 2004, 2005; Yano *et al.*, 2005), maize (Hodges *et al.*, 1997), sunflower (Paul *et al.*, 1991), and sugarcane (Du & Nose, 2002). At sub-zero temperatures, sugars will modulate tissue osmolarity, thus helping to maintain the membrane stability (Jonak *et al.*, 1996; Chen *et al.*, 2014). In the continuous-cooling rate, in August 2010, the diploids already had similar $LT_{50}$ to the ones measured in the winter, while this value for the triploids and tetraploid was significantly warmer than the winter values. It is possible that the allopolyploid lines of *Miscanthus* mobilize sugars toward the production of high yield of biomass, while diploid *Miscanthus* invest in cold tolerance as early as the end of summer.

At sub-zero temperatures the second stage of cold acclimation occurs and it is often called sub-zero acclimation, what involves a series of cellular adjustments to deal with the effects of low temperature (Weiser, 1970; Livingston III *et al.*, 2007). The capacity to survive sub-zero temperatures is mainly acquired by dealing with dehydration of the cells (Levitt, 1980; Verslues *et al.*, 2006; Gusta & Wisniewski, 2013). At moderate sub-zero temperatures, the cellular water content does not freeze due to high solute concentration and presence of antifreeze agents that occurred in the first stage of acclimation (Levitt, 1980; Steponkus, 1984; Gusta *et al.*, 2009). The prevention of early ice crystal formation in the cell, but not in the apoplast, causes water to exit the cell further decreasing the osmotic potential (Ruelland *et al.*, 2009).

*Miscanthus* rhizomes occur 5 – 9 cm deep in the soil, and will therefore be protected from intense winter cold by soil and snow insulation. To understand cold tolerance of winter conditions, it is important to consider the cold penetration to the depth where *Miscanthus* rhizomes occur. At the Leamington field site, soil temperature between 2009 and 2011 never fell below -4ºC at 1 cm below ground; this -4ºC extreme corresponded to a day when mean air temperature was -15ºC and snow cover was absent. To get a better idea of where *Miscanthus* could be grown in a northern country such as Canada, it was examined records available for the minimum soil temperature recorded by Environment Canada for sites across Canada. Minimum soil temperatures at 5 cm depth did not reach lethal temperatures for *Miscanthus* allopolyploids in southern British Columbia, Ontario, a few sites in southern Quebec and southeastern Labrador (Figure 3.2). If soil cooling allows for deep cold acclimation, diploids can also be grown up to in southern Yukon and Northwest Territories as well as in most of Alberta. However, in Saskatchewan, New Brunswick, north and eastern Quebec and west Labrador, temperature at 5 cm depth would exceed the lethal thresholds for all the *Miscanthus* lines tested, indicating these lines could not be safely cultivated there. Rosser (2012) analyzed the survival rate of *Miscanthus* in the second year after establishment (in 2009) in Leamington, Guelph, and Kemptville, in Ontario. In Leamington and Guelph, diploids M115 and M147, allotriploids Nagara and Illinois, and allotetraploid M118, had winter survival above 99%. In Elora, Ontario, Friesen *et al.* (2015) observed 20% of losses in first year *Miscanthus x giganteus* genotype "Illinois" in an unusual winter where soil temperature at 2 cm depth reached -6ºC and at 8 cm depth it reached -3.5ºC. In Kemptville, second year stands of diploids M115 and M147 had survivability of 49% and 88% respectively, while polyploids Nagara had 40% survivability, Illinois had 2.8%, and M118 had no survivability in 2009 (Rosser, 2012).

Because the allotriploid lines represent some of the major strains being considered for northern regions of the USA and Canada, caution should be used before investing in large plantations in northern locations. Given the rigor of the winter in high latitudes, the best strategy is to identify allotriploid lines which have inherited the acclimation ability of the diploid parents. This may be aided by genomic studies which can identify the genetic mechanism for greater cold tolerance at the diploids. In any case, further studies are needed to clarify the ability of *Miscanthus* lines to tolerate severe winter cold.

The limits for growing *Miscanthus* can be extended if soil insulation is increased. Rhizomes are safer deep in the soil and where snow cover is persistent. In addition to air temperature, the incident solar radiation, soil cover in general (snow, vegetation, organic matter), soil moisture content, depth, and type of soil also influence soil temperature (Paul *et al.*, 2004). For example, although the distance between Forêt Montmorency (site 53) and La Pocatiere (site 54;Table 3.1; Figure 3.2) is relatively small (around 85 km), minimal soil temperature registered is quite large: -17.5ºC in La Pocatiere and -1.5ºC in Forêt Montmorency. Differences in the snow cover (16 cm vs 39 cm), and mean air temperature (-23.5ºC vs -15ºC, respectively) from the 3 days prior to the day when the minimal soil temperature was measured can explain the difference in soil temperature. Soil structure, density, and consistency can affect soil temperature as well. Clay/silt and moist soil have higher thermal conductivity than sandy and dry soil (AgriInfo.in., 2011). Practices can be implemented to control soil temperature, such as regulating soil moisture and the proper soil management practices to have good drainage (AgriInfo.in., 2011). Use of mulching and addition of organic matter are also effective in moderating extreme soil temperatures (AgriInfo.in., 2011). However, in regions where soil temperatures potentially fall to lethal levels,

genetic improvement to increase cold tolerance, will be needed. This can involve increased biochemical mechanisms at the cellular level, or changes in the morphology of the rhizome bundles. For example, moving rhizome deeper in the soil, or keeping them together in a tight well-insulated cluster could prevent lethal surface cold from penetrating to rhizome depth.

*Conclusion*

*Miscanthus* has been identified as a potentially valuable biofuel feedstock for higher latitudes because it can maintain high productivity even at cool temperatures (Beale *et al.*, 1996; Heaton *et al.*, 2008a; Zhu *et al.*, 2008). The present study shows that if sub-zero acclimated, diploid *Miscanthus* rhizomes can survive temperatures as low as -14ºC, being the most cold tolerant *Miscanthus* hybrid. However, higher biomass yield is present in allotriploid hybrids (Lewandowski *et al.*, 2003; Clifton-Brown *et al.*, 2004; Vyn *et al.*, 2012). Because soil temperature is well buffered, not only by the soil itself but also by snow cover, there is significant potential for *Miscanthus* to be successfully grown in Canada up to 60ºN latitude. However, to safely avoid plantation loss due to episodic cold at rhizome depth, it will be necessary to move the superior cold tolerance of the diploid lines into the more productive allotriploids, and improve insulation properties of the soil. In areas where cold penetration is substantial, it may be unwise to grow *Miscanthus*, and hence other species like the $C_4$ *Spartina pectinata* or upland switchgrass, should be considered (Lee *et al.*, 2014; Friesen *et al.*, 2015). The findings described here indicate that greater potential for freezing tolerance exists within the *Miscanthus* gene pool. Future improvement efforts should be made to exploit this potential using a larger number of germplasm.

# Chapter 4 - Electron transport in photosystem II, photoprotection and photoinactivation in Miscanthus: the effects of episodic chilling events

## 4.1 - Abstract

*Miscanthus x giganteus* has been considered an important bioenergy crop for cold latitudes due to its good performance under low temperature. Previous studies have determined the capacity to acclimate and tolerate chilling events in growth chamber, where the light levels are less than half of the solar irradiation. The study of the chilling performance of plants is important because episodic frost and snow events in the spring and early summer are common in cool-temperate climates. The loss of a canopy in the spring or summer negatively impacts the productivity because it reduces the capture of solar radiation when it is abundant and when temperatures are higher. Here, I analyzed the performance of 9 *Miscanthus* hybrids, 3 *Miscanthus sinensis* and 2 switchgrass (*Panicum virgatum*) varieties in the field during the spring of 2010. In the second measurement time, on 10 May, a spring frost with predawn temperature reaching -2ºC and day temperatures below 12ºC caused death of all *Miscanthus* hybrids and Switchgrasses when exposed to solar radiation at 1300 PPFD. The exception were the *M. sinensis* that maintained living leaves, positive photosynthetic rate, and post irradiance Fv/Fm almost twice of the *Miscanthus* hybrids and Switchgrasses. Two weeks after the chilling event, all *Miscanthus* hybrids had built a competent photosynthetic canopy with Fv/Fm and photosynthetic values comparable to summer values, while *M. sinensis* had a healthy but poor canopy. At the warmer temperatures, the triploid *M. x giganteus* "Illinois" had the greatest quantum yield and photosynthetic rates while some diploid hybrids and the Switchgrasses had the worse. Although *M. sinensis* show improved

resistance to high light and chilling temperature, *M. x giganteus* have the greatest photosynthetic rate at cool to warm temperatures and soon built a vast canopy. This shows the capacity to increase chilling tolerance in the *Miscanthus* genepool.

## 4.2 - Introduction

*Miscanthus x giganteus* (Mxg) is a $C_4$ perennial grass well known for its ability to tolerate low temperatures. It can maintain high photosynthetic rates at chilling temperatures allowing it to develop an early canopy, thereby enabling it to exploit the long photoperiods occurring in late spring (Beale & Long, 1995; Naidu *et al.*, 2003; Heaton *et al.*, 2008a; Dohleman *et al.*, 2009). At cool-temperate latitudes, however, the early season is punctuated with episodic frost and snow events, which could harm early growth and eliminate any advantage associated with early canopy development. To avoid such injury, exposed tissues of *Miscanthus* require both chilling and frost tolerance. Winter dormant rhizomes are tolerant of subzero temperatures down to -6ºC to -14ºC in a genotype specific manner (Chapter 3), but the chilling tolerance and early spring performance of different *Miscanthus* hybrids is less well characterized. Farrell *et al.* (2006) showed that in the dark, *Miscanthus* shoots can tolerate temperatures as low as -6ºC to -9ºC. However, studies has not examined how *Miscanthus* shoots would perform under realistic low temperature and high light conditions that would typically follow a spring cold front in a northern climate. In general, $C_4$ plants do not tolerate chilling temperatures in the spring and early summer when sunlight is present (Long, 1983; Long *et al.*, 1983; Stirling *et al.*, 1991). This is one of the factors that restricts most $C_4$ plants from regions where spring temperatures are below 14ºC (Long, 1983; Sage *et al.*, 1999).

Episodic chilling in the spring is one of the main causes of crop injury in temperate regions (Andrews *et al.*, 1995; Fryer, 1998; Long & Spence, 2013). Low temperature associated with high light on the leaves is particularly stressful as it causes an imbalance between the production of chemical energy (ATP and NADPH) by the photosynthetic light reactions and their consumption by the Calvin-Benson cycle (Osmond, 1994). If the energy captured from sunlight cannot be utilized by photosynthetic processes, it must be dissipated before the excess energy forms reactive oxygen species (ROS) that can damage the leaf (Asada, 1999; Murata *et al.*, 2012). Primarily, this excess energy can be thermally dissipated by carotenoids of the xanthophyll cycle (VAZ) in a process called flexible photoinhibition (also referred to as dynamic or regulated non-photochemical quenching), which is a photo-protective mechanism of the leaf (Demmig-Adams *et al.* 1987, 2004; Niyogi *et al.* 1997). In the presence of light, the pH gradient in thylakoid increases and stimulates the conversion of violaxanthin (V) to zeaxanthin (Z) (Demmig-Adams 1990; Demmig-Adams *et al.* 2004). Zeaxanthin is a carotenoid of the xanthophyll cycle responsible for thermal dissipation. When light is low or absent, Z is de-epoxidated to the non-protective form (V) and does not compete with the photochemical pathway for light energy. The rapid inter-conversion between the protective and non-protective form of this mechanism, which is regulated by the pH gradient, is the reason why it is called flexible or regulated non-photochemical quenching. Chilling conditions slow photosynthetic capacity and creates an imbalance between light absorption and energy use, which leads to an increase in the pH gradient promoting the formation of Z.

If light energy cannot be used for photochemistry or dissipated as heat, damage to the photosystem reaction centers will occur. These include the disruption of D1 proteins in PSII, and damage to the thylakoid membrane and PSI polypeptides in leaves, creating chilling lesions which

reduce photosynthetic capacity and leaf fluorescence (Long *et al.*, 1994; Tjus *et al.*, 1999). In maize for example, cold temperature and high light radiation resulted in increased disruption of thylakoid polypeptides causing injury to the chloroplast (Nie & Baker, 1991).This process is called sustained or chronic photoinhibition (Demmig-Adams & Adams-III 2006; Ensminger *et al.* 2006; Murata *et al.* 2007). The damaged PSII can trap Z in the protective form, becoming a ΔpH-independent photo-protection response (Demmig-Adams *et al.* 2006). Reversal of sustained photoinhibition is relatively slow (hours) since it involves the regeneration of damaged PSII complexes (Melis 1999; Murata *et al.* 2007). PSII repair is slowed by chilling temperatures if not altogether halted (Allakhverdiev *et al.* 2005; Huang *et al.* 2010a, 2010b).

Schreiber (1983) first proposed the use of chlorophyll fluorescence as a rapid and non-invasive method to estimate the quantum efficiency of PSII and its relationship to carbon assimilation. Based on his work, chlorophyll fluorescence has been used in many studies to access a wide range of environmental effects, including both slow and rapid chilling (Hendrickson *et al.*, 2005; Baker, 2008). Korneyev and Holaday (2008) propose a more detailed set of fluorescence parameters to evaluate flexible and sustained photoinhibition, which I incorporate here to screen chilling tolerance in 12 different *Miscanthus* genotypes and two switchgrass genotypes. In the present study, I compare the low temperature tolerance of new leaves early in the growing season during a period that was punctuated by a lethal frost event, thereby allowing me to examine survival and recovery patterns of the *Miscanthus* lines. I used pulse amplitude modulated (PAM) fluorescence to analyse the light energy partitioning in the photosystem II (PSII) in leaves of field-grown plants in the beginning of the growing season just after sprouting in early May. By estimating the quantum yields of energy conversion, I evaluated the variation in sensitivity of *Miscanthus* and switchgrass lines to episodic chilling early in the growing season.

## 4.3 - Materials and methods

*Plant material*

Genotypes of *Miscanthus* and switchgrass were grown at the Elora Research Station as described by Kludze *et al.* (2013) and Friesen *et al.* (2014). At the experimental site, plants were grown in 24 plots (6 rows x 4), with one genotype per plot (each plot had 6 x 4 plants). I studied 14 genotypes with shoots available on 4 May 2010. Nine genotypes were hybrids of *M. sinensis* and *M. sacchariflorus*: six Amuris diploids (M114, M115, M143, M144, M145, M147); one tetraploid Msa/Msi (M118); and two triploid (Nagara and Illinois). The study also included three genotypes of *M. sinensis* (*M. sinensis* 13, *M. sinensis* 14, *M. sinensis* 15), and two genotypes of switchgrass, *Panicum virgatum* (Alamo and Kanlow; Table 4.1).

*Chlorophyll fluorescence and leaf gas exchange measurements*

Chlorophyll fluorescence was measured using a PAM 2100 chlorophyll fluorometer (Heinz Waltz, Effeltrich, Germany). The field measurements were made on days with clear skies with maximum sunshine: 4 May, 10 May, 19 May, 27 May, and 29 July of 2010. For all measurements, one new, fully expanded leaf from 10 different plants of each genotype was chosen randomly. Leaf temperature was measured and recorded at each fluorescence log by a thermocouple installed on the leaf clip of the chlorophyll fluorometer. Air temperature was provided by Environment Canada (https://weather.gc.ca). At the time of the measurements, the air temperature/leaf temperature were 15ºC/21ºC on 4 May; 7ºC/12ºC on 10 May; 18ºC/25ºC on 19 May; 26ºC/31ºC on 27 May; and 17ºC/20ºC on 29 July 2010. Sampled leaves were from the upper canopy position receiving full sunshine on the measurement dates. A quantum sensor LI-190 installed on a light meter LI-250A

(LiCor, Lincoln, NE, USA, www.licor.com) monitored the photosynthetic active radiation delivered by the sunlight.

I measured (Fm-Fo)/Fm, which is the maximum quantum efficiency of PSII measured on dark acclimated leaves, and (Fm'-Fo')/Fm' for the potential quantum efficiency of PSII photochemistry on leaves under light irradiance (ϕPSII; Figure 4.1) (Genty *et al.*, 1989). The minimal fluorescence (Fo') in irradiance acclimated leaves is measured by concomitantly shading the leaf from the sun and illuminating it for at least 3 seconds with a weak far-red light that promotes electron flow to PSI, so that the available PSII electron acceptors are "open" (=in oxidized state; Kornyeyev & Holaday, 2008). The term Fv and Fv' has been used as the variable fluorescence and are respectively Fm-Fo and Fm'-Fo'.

Before dawn, the maximum PSII quantum yield for the leaf (Fv/Fm) was measured in 10 dark-adapted leaves on each genotype. Around 9am, leaves were cut, placed on trays with moist cloths and exposed for 90 minutes to full sunlight (1300 to 1800 µmol $m^{-2}$ $s^{-1}$). Then, another fluorescence measurement was made on these irradiance acclimated leaves (Fv'/Fm'). Such conditions precluded high rates of leaf gas exchange. Immediately after the measurement of irradiance-acclimated fluorescence, leaves were dark acclimated for 50-60 minutes at ambient temperature. Dark acclimation is used to oxidize the Qa acceptors (making them available to accept excited electrons) and to promote the de-epoxidation of the xanthophyll cycle pigments (i. e. reverse pigments to the non-protective form; Demmig-Adams & Adams-III, 2006). After dark acclimation, fluorescence was measured on dark acclimated tissues ($Fv_{pi}/Fm_{pi}$; Figure 4.1).

Photosynthesis data were collected at the same time the fluorescence measurements were performed. The net photosynthetic rate ($A_{max}$) was measured using the LiCor 6400 (LiCor, Lincoln,

NE, USA) at ambient temperature and $CO_2$ level. Data for M115, M147, M118, Nagara, Illinois, and *M. sinensis* 15 on 4 May 2010, 10 May 2010, and 19 May 2010, were published by Friesen *et al.* (2014). The remaining data were generously provided by Patrick Friesen (unpublished data). On 4 May and 10 May 2010, photosynthetic data for the Switchgrasses were not collected. On 19 May 2010 photosynthetic data for *M. sinensis* 13 and switchgrass Kanlow were also not collected.

*Quantum yield estimation*

For this study, I adapted the formulas suggested by Hendrickson *et al.* (2005) and Kornyeyev & Holaday (2008) to the conditions found in the field to estimate the quantum yields of the different light partitions.

At predawn, the estimation of the quantum yield associated with photoinactivated PSII reaction centers that has not recovered during the night ($\phi NF_n$) was estimated according to Hendrickson *et al.* (2005):

$$\Phi NF_n = 1 - \frac{Fv / Fm}{Fv_{\max} / Fm_{\max}} \qquad \text{Equation1}$$

The highest Fv/Fm measured for each genotype in the entire experiment was considered $Fv_{max}/Fm_{max}$ for that genotype. During the day, solar radiation may over energize the electron transport chain causing PSII reaction centers to become photoinactivated. The total sustained photoinhibition ($\phi NF$) at the time of the measurement is estimated based on the difference between $Fv_{max}/Fm_{max}$ to $Fv_{pi}/Fm_{pi}$, on the assumption that $Fv_{pi}/Fm_{pi}$ is completely dark-adapted and the regulatory system (flexible photoinhibition or $\phi REG$) is fully relaxed (Figure 4.1). This eliminates

the need to use the irradiance acclimated coefficient $\frac{Fv'/Fm'}{Fv_{pi}/Fv_{pi}}$ suggested by Kornyeyev *et al.* (2001) in the estimation of ϕNF. This leads to the formula suggested by Hendrickson *et al.* (2005):

$$\Phi NF = 1 - \frac{Fv_{pi}/Fm_{pi}}{Fv_{\max}/Fm_{\max}} \qquad \text{Equation 2}$$

Assuming that $Fv_{pi}/Fm_{pi}$ was measured when the flexible photoinhibition is completely relaxed, the estimation of ϕREG is basically the difference between $Fv_{pi}/Fm_{pi}$ and Fv'/Fm' as a fraction of $Fv_{max}/Fm_{max}$:

$$\Phi REG = \frac{Fv_{pi}/Fm_{pi} - Fv'/Fm'}{Fv_{\max}/Fm_{\max}} \qquad \text{Equation 3}$$

The remaining quanta absorbed are partitioned as the quantum yield of fluorescence emission and constitutive energy dissipation (ϕf.D). Therefore, ϕf.D is estimated as:

$$\Phi f,D = \left(1 - \frac{Fv_{\max}}{Fm_{\max}}\right)\left(\frac{Fv'/Fm'}{Fv_{\max}/Fm_{\max}}\right) \qquad \text{Equation 4}$$

In summary:

$$\Phi PSII + \Phi NF + \Phi REG + \Phi f,D = 1$$

Equation 2 and 3 differ from the estimation of ϕNF and ϕREG proposed by Kornyeyev & Holaday (2008). These authors considered these calculations in healthy leaves to estimate the sustained photoinhibition that was accumulated during the day. Ignoring the sustained photoinhibition ($\phi NF_n$) present in the leaves at night can lead to erroneous conclusions of the total

sustained photoinhibition of the leaves at the time of the measurement during the day. For example, on 10 May 2010 the ϕNF estimated according to Kornyeyev & Holaday (2008) would be the lowest in this study. However, on 10 May 2010 at predawn, the leaves were so photoinhibited that the small diurnal increase in ϕNF was irreversible and resulted in death of the leaves. Contrasting with this, on 27 May 2010 when leaves were healthy and temperature was warm, ϕNF estimated according to Kornyeyev & Holaday (2008) would be much higher. The calculations proposed by Kornyeyev & Holaday (2008) are effective in estimating the increase in the quantum yield of sustained photoinhibition after predawn, but not the total quantum yield of sustained photoinhibition present on light irradiated leaf. On 19 May 2010, $Fv_{pi}/Fm_{pi}$ was higher than Fv/Fm meaning that ϕNFn estimated in the predawn was lower than ϕNFd during the day. If estimated according to Kornyeyev & Holaday (2008), ϕNF would be negative, which does not make sense.

*Statistics*

The experimental design was a random distribution of each genotype per plot, and repeated measurement over time were performed. Because Fv/Fm was not measured in the same leaves that Fv'/Fm' and $Fv_{pi}/Fm_{pi}$ were measured, these parameters cannot be analysed together. Thus, to estimate ϕNF, ϕREG, or ϕf,D just the mean of each parameter (Fv/Fm, Fv'/Fm', and $Fv_{pi}/Fm_{pi}$) are used, and statistical tests are not appropriate.

All the fluorescence measurements had N=10, except for switchgrass Alamo that only had four plants alive. Because quantum yields are proportional data, thus constrained between 0 and 1, the GAMM was performed assuming beta distribution. The photosynthesis data were analysed assuming a normal distribution and homogeneous variance of the error that was confirmed by the model validation. Fluorescence and photosynthesis data were analyzed using date, leaf temperature

and genotypes as the independent variables. Temperature has a strong effect on the photosynthetic rate, but it had to be used an interaction between leaf temperature and date because on 19 May 2010 and 29 July 2010 the air temperature was similar, but there was a considerable difference between measurements (Table 4.1). The fluorescence and photosynthetic measurements were not performed at the same leaf at each date, so the plant effect was considered to be random and nested within its corresponding genotype. In addition, it was considered a temporal autocorrelation between the populations of the sampled plants on each collection time. Data were analysed using a generalized additive model (GAMM), applying a cubic spline on the date variable. The post hoc comparison was performed by sequential testing. All statistics were made using the package "GAMLSS" (Rigby & Stasinopoulos, 2005) on R statistics software (R-Development-Core-Team 2006).

## 4.4 - Results

Weather conditions during the experiment are shown in Figure 4.2, and are available on the Environment Canada website for the Elora Station (Environment Canada). This weather station is located 900 m away from the field site. The first measurement, on 4 May 2010, followed 4 days with mean temperatures around 16ºC and minimum not below 9ºC. Then, the temperature lowered until it reached -2ºC at pre-dawn on 10 May 2010. On this day, air temperature did not rise above 12ºC. After this cold event the air temperature increased and on 19 May, it reached a maximum of 23ºC and a minimum of 6ºC at pre-dawn. The mean temperature on 19 May was still around 14ºC. On 27 May 2010, temperatures were already similar to the summer temperatures in that year in Elora-ON, with maximum of 29ºC and minima of 21ºC. In late July, when the last measurement

was performed, the air temperature had a slight drop compared to the days that preceded it and lowered to 11ºC at dawn with a mean of 17ºC.

Fv/Fm showed significant differences between genotypes on 4 May 2010 and 19 May 2010 ($p < 0.05$; Table 4.2). In general, each switchgrass plant had only two or three leaves, and only 4 of 24 Alamo switchgrass plants re-sprouted. *Miscanthus sinensis* had large leaves, but those showed evidence of necrosis and chlorosis. The *Miscanthus* hybrids had green leaves, apparently younger than the *M. sinensis* leaves with no apparent sign of chlorosis or necrosis. This indicates that *M. sinensis* had started building a canopy earlier than the other hybrids. Predawn Fv/Fm varied from a minimal of 0.48 and 0.50 (for Kanlow and M114) to 0.68 - 0.65 (for M118, Illinois, Nagara; and Alamo; Table 4.2). Net photosynthetic rate at light saturation ($A_{max}$) varied from 19.6 and 19.2 µmol $m^{-2}$ $s^{-1}$ (Illinois and Nagara) to 12.6 µmol $m^{-2}$ $s^{-1}$ (M118; Table 4.1).

On 10 May 2010, a strong cold front lowered night air temperature to -2ºC, resulting in leaf frost (Figure 4.2; Figure 4.3a). Leaves initially looked healthy when frosted, but after a few hours of sunshine, leaves of the *Miscanthus* hybrids and Switchgrasses were curled and had a dull dark green color, which indicated leaf injury and cytosolic leakage (Figure 4.3b). Figure 4.3a shows *Miscanthus x giganteus* Illinois at dawn and Figure 4.3b shows a plant of the same genotype by noon. *M. sinensis* genotypes dealt better with high light and low temperature. Figure 4.3c shows the *M. sinensis* 15 with leaves apparently. The post irradiance $Fv_{pi}/Fm_{pi}$ of *M. sinensis* 15 was significantly higher than all other genotypes, being equivalent to the mean plus three standard deviations of all plants in this measurement day (Table 4.2). *M. sinensis* 15 was the only genotype that can be considered to have had positive net photosynthetic rate (Table 4.1). Figure 4.3d shows how leaves of the *Miscanthus* hybrids were more stressed compared to the leaves of *M. sinensis*.

Before dawn, the Fv/Fm was on average 0.16 with maximum of 0.24 for *M. sinensis* 13 and 14 (Table 4.2; Figure 4.4). The lowest predawn Fv/Fm was 0.11 for M114, M147, Alamo, M145, and M143. The values measured for Fv'/Fm' and $Fv_{pi}/Fm_{pi}$ were very low and variable.

Air temperature warmed on the following week (19 May 2010; Figure 4.2) and a new set of leaves replaced those killed on all *Miscanthus* hybrids and Switchgrasses. Although the *M. sinensis* plants still had a few old leaves, measurements were made on the new ones (second top most expanded leaf). On 19 May 2010, the average predawn Fv/Fm was 0.34 with M144, Illinois, and Nagara having the highest predawn Fv/Fm (0.49, 0.46, and 0.43), while M143, M114, M115, and Kanlow having the lowest (0.20, 0.22, 0.25, and 0.25; Table 4.2; Figure 4.4). At the time of the light measurements, the air temperature was near 18ºC and leaf temperature was around 25ºC. Many leaves had $Fv_{pi}/Fm_{pi}$ higher than Fv/Fm (Table 4.2). Even though temperature was similar to the measuring temperature on 4 May 2010, the $A_{max}$ on 19 May was lower (Table 4.1); The lowest $A_{max}$ measured were 2.4, 2.9, and 3.7 µmol $m^{-2}$ $s^{-1}$ (M147, Alamo, and M144) and the highest were 13.9 and 11.4 µmol $m^{-2}$ $s^{-1}$ (Illinois and *M. sinensis*14; Table 4.1; Figure 4.5).

On 27 May 2010, two weeks after the cold front had killed most leaves, plants of *Miscanthus* hybrids had built up a short but closed canopy and leaves were around 30-60 cm long. Nagara and Illinois had a visibly larger canopy than the other lines. On *M. sinensis* the old leaves had senesced, and the new leaves had a healthy appearance but had poor canopy in comparison to the *Miscanthus* hybrids. The switchgrass had less and shorter leaves then the *Miscanthus* lines. At the time of measurements, the air temperature was around 26ºC and leaf temperature was around 31ºC. Their Fv/Fm was comparable to summer values and showed no signs of stress (Table 4.2). The average predawn Fv/Fm was 0.76 and there was very little difference between genotypes

(standard deviation 0.01; Table 4.2; Figure 4.4). Nagara, M147 and Illinois had the highest $A_{max}$ (45.4, 43.8, and 43.6 µmol $m^{-2}$ $s^{-1}$; Table 4.1; Figure 4.5). The Switchgrasses had the lowest $A_{max}$ values (22.6 and 31.4 µmol $m^{-2}$ $s^{-1}$).

In the summer, the mean temperature in July 2010 was 20ºC, but on the day of the last measurement (29 July 2010) it was 17ºC with a low of 12ºC. At the time of the measurements, leaf temperature was around 20ºC. Plants were healthy and were around 2.5 meters tall (Figure 4.6). The average predawn Fv/Fm was 0.77 and it also had minimal variability (Table 4.2). $A_{max}$ varied from 28.0 µmol $m^{-2}$ $s^{-1}$ (M118) to 10.5 µmol $m^{-2}$ $s^{-1}$ (M144; Table 4.1).

Overall, the *Miscanthus* Nagara and Illinois and *M. sinensis* had lower photoinhibition at predawn ($\phi NF_n$) than the remaining genotypes (Figure 4.4). Except for 10 May 2010, when the temperature drastically decreased for at least 24 hours prior to the measurement, M144 also had low $\phi NF_n$ rates (Figure 4.4b). Once leaves received sunshine, the accumulation of sustained photoinhibition ($\phi NF$) was more prominent in the Illinois hybrid, even though it maintained high $A_{max}$ rates on all dates (Figure 4.5). M147 had high $Fv_{pi}/Fm_{pi}$ on 27 May 2010 indicating low increase of $\phi NF$ from predawn (Figure 4.5 c). However, this genotype had high $\phi NF$ values on the colder measuring dates. *M. sinensis* had moderate $\phi NF$ rates over all season. Although in the spring Switchgrasses were very photoinhibited, in the summer (29 July 2010) the switchgrass had high $\phi PSII$ and low $\phi NF$ (Figure 4.5h). On 19 May 2010, the day photoinhibition was lower than at night because even though plants received sunlight, the warm temperature allowed them to recover the $Fv_{pi}/Fm_{pi}$ to a higher value than Fv/Fm.

Table 4.1 - Net photosynthetic rate measured in parallel to the fluorescence measurements on the five measurement dates. Data for M115, M147, M118, Nagara, Illinois, and *M. sinensis* 15 on 4 May 2010, 10 May 2010, and 19 May 2010 from Friesen *et al.* (2014). Remaining photosynthetic data were provided by Patrick Friesen (unpublished data). The leaf temperature when photosynthesis was measured is indicated in parenthesis, mean ±SE. Different letters indicate significant differences between genotypes on that measurement date. Mean and St Dev in the last two rows are the mean and standard deviation photosynthetic values for all plants measured on that date. All values are in μmol $m^{-2}$ $s^{-1}$. Mean ± standard deviation. N=5, except for switchgrass Alamo that N=4.

| Genotypes | 4 May 2010 (21ºC±0.6) | 10 May 2010 (13º±0.2) | 19 May 2010 (25ºC±0.4) | 27 May 2010 (31ºC±0.3) | 29 July 2010 (20ºC±0.2) |
|---|---|---|---|---|---|
| M114 (2n) | 15.54±1.7$^{ab}$ | -0.35±0.05$^{b}$ | 6.33±1.4$^{b}$ | 33.66±3.0$^{bc}$ | 19.41±1.7$^{b}$ |
| M115 (2n) | 14.56±1.3$^{b}$ | -0.17±0.08$^{b}$ | 4.46±1.3$^{b}$ | 33.03±1.5$^{bc}$ | 17.03±1.2$^{b}$ |
| M143 (2n) | 16.07±1.4$^{a}$ | -0.39±0.05$^{b}$ | 7.97±1.8$^{ab}$ | 35.54±2.1$^{bc}$ | 22.05±1.6$^{ab}$ |
| M144 (2n) | 15.41±1.9$^{ab}$ | -0.32±0.05$^{b}$ | 6.04±0.7$^{ab}$ | 40.96±2.3$^{ab}$ | 10.50±2.2$^{c}$ |
| M145 (2n) | 17.28±0.8$^{a}$ | -0.37±0.04$^{b}$ | 10.91±1.9$^{a}$ | 37.28±1.4$^{b}$ | 26.39±1.9$^{a}$ |
| M147 (2n) | 15.00±1.3$^{b}$ | 0.02±0.07$^{b}$ | 2.38±1.0$^{b}$ | 43.79±1.4$^{a}$ | 21.14±1.7$^{ab}$ |
| M118 (4n) | 12.61±1.4$^{b}$ | -0.35±0.04$^{b}$ | 4.55±2.3$^{b}$ | 37.51±1.2$^{b}$ | 27.97±1.9$^{a}$ |
| Nagara (3n) | 19.15±0.3$^{a}$ | -0.15±0.02$^{b}$ | 6.55±2.0$^{ab}$ | 45.36±1.8$^{a}$ | 25.85±2.5$^{a}$ |
| Illinois (3n) | 19.57±1.4$^{a}$ | -0.55±0.06$^{b}$ | 13.85±1.4$^{a}$ | 43.61±1.5$^{a}$ | 26.30±1.4$^{a}$ |
| *M. sinensis* 13 (2n) | 17.58±2.2$^{a}$ | 0.03±0.05$^{b}$ | N/A | 40.99±0.9$^{a}$ | 26.94±2.4$^{a}$ |
| *M. sinensis* 14 (2n) | 12.90±2.2$^{b}$ | -0.23±0.05$^{b}$ | 11.37±0.9$^{a}$ | 33.69±1.9$^{bc}$ | 25.72±1.2$^{a}$ |
| *M. sinensis* 15 (2n) | 13.10±1.6$^{b}$ | 0.66±0.04$^{a}$ | 9.75±1.2$^{ab}$ | 34.03±1.5$^{bc}$ | 25.24±1.3$^{a}$ |
| Alamo switchgrass (4n) | N/A | N/A | 2.91±2.1$^{b}$ | 22.62±5.0$^{d}$ | 23.37±1.4$^{ab}$ |
| Kanlow switchgrass (4n) | N/A | N/A | N/A | 31.44±2.0$^{c}$ | 19.13±1.8$^{b}$ |
| Mean | 15.73 | -0.18 | 7.06 | 36.68 | 22.65 |
| St Dev | 2.32 | 0.31 | 3.72 | 6.06 | 4.85 |

Table 4.2 - Mean quantum yield values measured for the five studied dates. Abbreviations: Gen, genotype; Fv/Fm, predawn maximum quantum yield of PSII; Fv'/Fm', irradiance acclimated potential quantum yield of PSII; $Fv_{pi}/Fm_{pi}$, dark recovered post irradiation maximum quantum yield of PSII. Mean and St Dev shown in the last two rows of the table are the mean and standard deviation values of the quantum yield of all plants measured on that date. Different letters indicate significant differences between genotypes on that measurement date. N = 10 except for Alamo where n = 4.

| | 04 May 2010 | | | 10 May 2010 | | | 19 May 2010 | | | 27 May 2010 | | | 29 July 2010 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Gen | Fv/ Fm | Fv'/ Fm' | Fvpi/ Fmpi | Fv/ Fm | Fv'/ Fm' | Fvpi/ Fmpi | Fv/ Fm | Fv'/ Fm' | Fvpi/ Fmpi | Fv/ Fm | Fv'/ Fm' | Fvpi/ Fmpi | Fv/ Fm | Fv'/ Fm' | Fvpi/ Fmpi |
| M114 (2n) | 0.51$^{b}$ | 0.11 | 0.46$^{a}$ | 0.14 | 0.04 | 0.10$^{b}$ | 0.22$^{c}$ | 0.13 | 0.33 | 0.75 | 0.09 | 0.57$^{b}$ | 0.78 | 0.19 | 0.55 |
| M115 (2n) | 0.63$^{a}$ | 0.15 | 0.50$^{a}$ | 0.16 | 0.06 | 0.09$^{b}$ | 0.25$^{c}$ | 0.04 | 0.35 | 0.76 | 0.20 | 0.58$^{b}$ | 0.77 | 0.14 | 0.57 |
| M143 (2n) | 0.52$^{b}$ | 0.09 | 0.47$^{a}$ | 0.12 | 0.02 | 0.11$^{b}$ | 0.20$^{c}$ | 0.11 | 0.33 | 0.77 | 0.16 | 0.59$^{ab}$ | 0.75 | 0.18 | 0.54 |
| M144 (2n) | 0.65$^{a}$ | 0.14 | 0.50$^{a}$ | 0.11 | 0.01 | 0.01$^{c}$ | 0.49$^{a}$ | 0.08 | 0.41 | 0.75 | 0.10 | 0.58$^{b}$ | 0.74 | 0.16 | 0.46 |
| M145 (2n) | 0.58$^{ab}$ | 0.10 | 0.45$^{a}$ | 0.11 | 0.01 | 0.02$^{c}$ | 0.29$^{bc}$ | 0.11 | 0.38 | 0.76 | 0.18 | 0.58$^{b}$ | 0.75 | 0.12 | 0.50 |
| M147 (2n) | 0.64$^{a}$ | 0.23 | 0.54$^{a}$ | 0.11 | 0.01 | 0.02$^{c}$ | 0.33$^{bc}$ | 0.06 | 0.28 | 0.76 | 0.17 | 0.72$^{a}$ | 0.78 | 0.18 | 0.46 |
| M118 (4n) | 0.68$^{a}$ | 0.26 | 0.54$^{a}$ | 0.21 | 0.02 | 0.03$^{c}$ | 0.40$^{ab}$ | 0.14 | 0.47 | 0.77 | 0.15 | 0.61$^{ab}$ | 0.78 | 0.13 | 0.53 |
| Nagara (3n) | 0.65$^{a}$ | 0.20 | 0.62$^{a}$ | 0.15 | 0.01 | 0.02$^{c}$ | 0.43$^{ab}$ | 0.13 | 0.44 | 0.78 | 0.19 | 0.60$^{ab}$ | 0.79 | 0.15 | 0.54 |
| Illinois (2n) | 0.67$^{a}$ | 0.19 | 0.57$^{a}$ | 0.18 | 0.01 | 0.05$^{c}$ | 0.46$^{a}$ | 0.08 | 0.37 | 0.77 | 0.17 | 0.53$^{b}$ | 0.78 | 0.08 | 0.41 |
| *M. sin*13 (2n) | 0.63$^{a}$ | 0.15 | 0.50$^{a}$ | 0.24 | 0.05 | 0.10$^{b}$ | 0.42$^{ab}$ | 0.12 | 0.36 | 0.76 | 0.10 | 0.55$^{b}$ | 0.78 | 0.13 | 0.57 |
| *M. sin*14 (2n) | 0.58$^{ab}$ | 0.12 | 0.52$^{a}$ | 0.24 | 0.02 | 0.07$^{bc}$ | 0.39$^{ab}$ | 0.09 | 0.28 | 0.74 | 0.14 | 0.52$^{b}$ | 0.76 | 0.14 | 0.59 |
| *M. sin*15 (2n) | 0.61$^{a}$ | 0.11 | 0.51$^{a}$ | 0.16 | 0.04 | 0.18$^{a}$ | 0.25$^{c}$ | 0.15 | 0.37 | 0.74 | 0.14 | 0.56$^{b}$ | 0.76 | 0.17 | 0.53 |
| Alamo (4n) | 0.65$^{a}$ | 0.19 | 0.49$^{a}$ | 0.11 | 0.01 | 0.03$^{c}$ | 0.37$^{b}$ | 0.03 | 0.36 | 0.73 | 0.13 | 0.59$^{ab}$ | 0.78 | 0.21 | 0.55 |
| Kanlow (4n) | 0.49$^{b}$ | 0.05 | 0.34$^{b}$ | 0.18 | 0.01 | 0.04$^{c}$ | 0.25$^{c}$ | 0.13 | 0.36 | 0.75 | 0.14 | 0.54$^{b}$ | 0.77 | 0.22 | 0.63 |
| Mean | 0.61 | 0.15 | 0.50 | 0.16 | 0.02 | 0.06 | 0.34 | 0.10 | 0.36 | 0.76 | 0.15 | 0.58 | 0.77 | 0.16 | 0.53 |
| St Dev | 0.09 | 0.07 | 0.07 | 0.09 | 0.03 | 0.04 | 0.15 | 0.06 | 0.08 | 0.02 | 0.06 | 0.07 | 0.02 | 0.06 | 0.08 |

Figure 4.1 - Schematic of chlorophyll fluorescence signals and the quantum yield partitioning. At predawn, the maximum and minimal fluorescence values are Fm and Fo, respectively. The highest (Fm-Fo)/Fm measured in the entire experiment was considered the ($Fm_{max}$-$Fo_{max}$)/$Fm_{max}$. The difference between ($Fm_{max}$-$Fo_{max}$)/$Fm_{max}$ and (Fm-Fo)/Fm is the quantum yield of the photoinhibition that could not be recovered overnight (ϕNFn). Once the leaf is irradiated, it emits the steady state fluorescence Fs. A saturating light pulse at this condition will result in a fluorescence signal Fm'. Soon after the leaf is briefly irradiated with a weak far-red light to move the electrons to PSI opening the PSII reaction centers, resulting in a weaker than steady state fluorescence signal Fo'. The relative difference between Fm' and Fo' is the potential quantum yield of photochemistry in light irradiated leaves (ϕPSII). Once in the dark again, the regulated photoprotective system of the leaf is relaxed, lowering the $Fo_{pi}$ and increasing the $Fm_{pi}$ in comparison to the light irradiated values. Thus, the difference between the dark recovered and light irradiated quantum yield, is the ϕREG. The quantum yield that could not be recovered from pre-dawn is the photoinhibition accumulated during the day (ϕNFd). The remaining light in the system is dissipated as fluorescence or by other constitutive systems (ϕf,D).

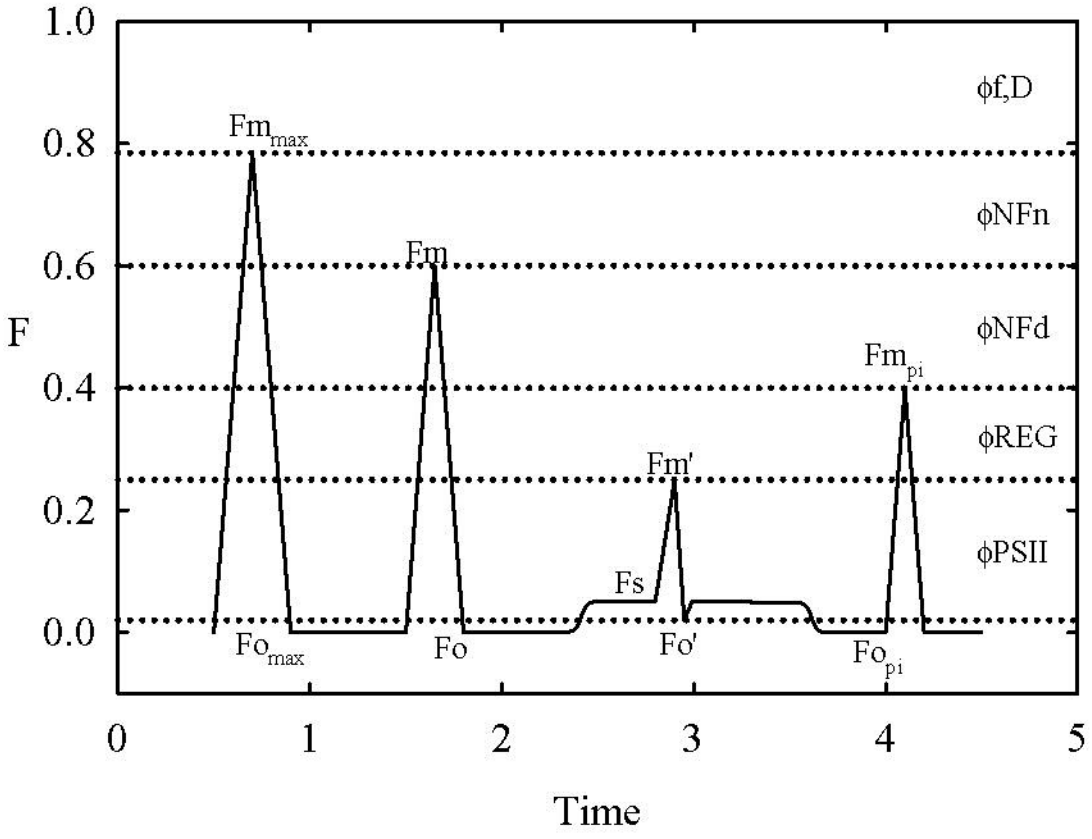


Figure 4.2 - Mean day air temperature and minimal day air temperature measured at the Elora weather station. The dotted lines indicate the field measurement dates.

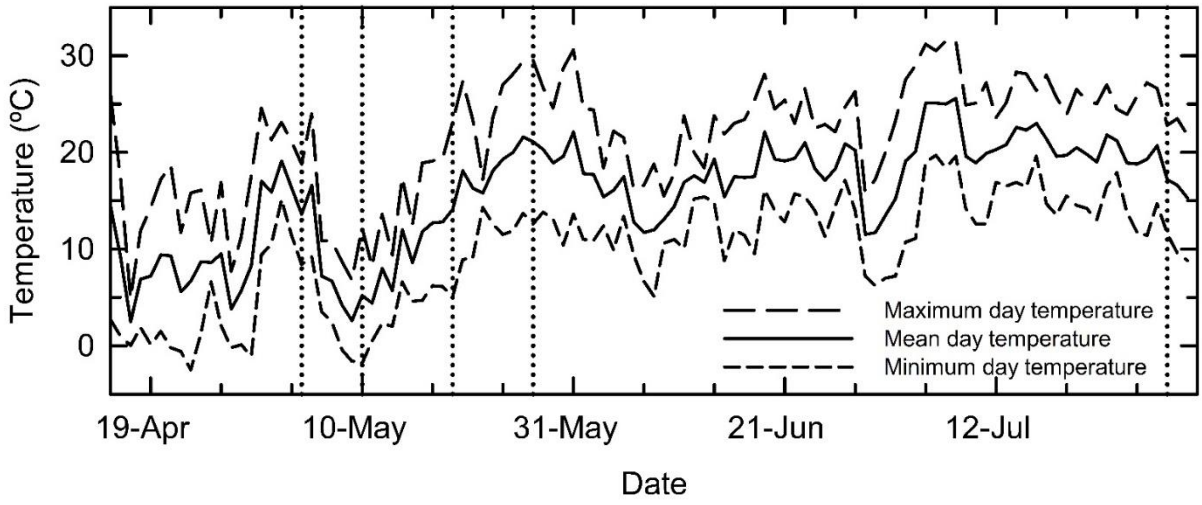


Figure 4.3 - Pictures from 11 May 2010 documenting leaf injury. **(a)** Leaves of *Miscanthus x giganteus* "Illinois" before dawn when air temperature was -2 ºC (note ice crystals on the leaves). At 2pm, after 6:30 hours of exposure to full sunlight under temperatures between 3ºC and 13ºC. **(b)** *Miscanthus x giganteus* "Illinois" appeared stressed while **(c)** *Miscanthus sinensis* 15 apparently dealt better with the stressful conditions. **(d)** Leaves from each of the *Miscanthus* genotypes at around 2pm, where numbers beside shoots indicate genotype. #1 for M114,#2 for M115, #3 for M143, #4 for M144, #5 for M145, #7 for M147, #8 for M118, #10 for Nagara, #12 for Illinois, #13 for *M. sinensis* 13, # 14 for *M. sinensis* 14, # 15 for *M. sinensis* 15

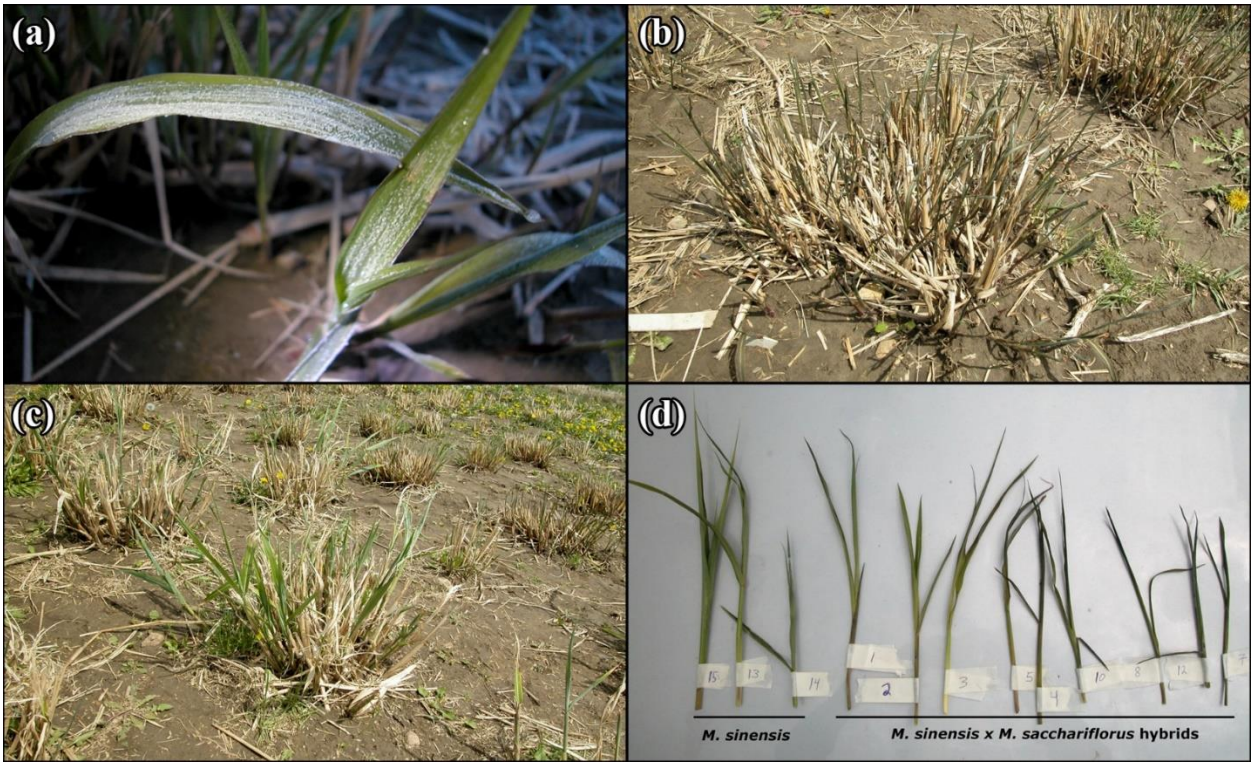


Figure 4.4 - Predawn quantum yield of photochemistry and quantum yield of sustained photoinhibition (ϕNFn) estimated according to Hendrickson *et al.* (2005). Dotted vertical lines indicate the measurement dates: 4 May 2010, 10 May 2010, 19 May 2010, 27 May 2010, and 29 July 2010.

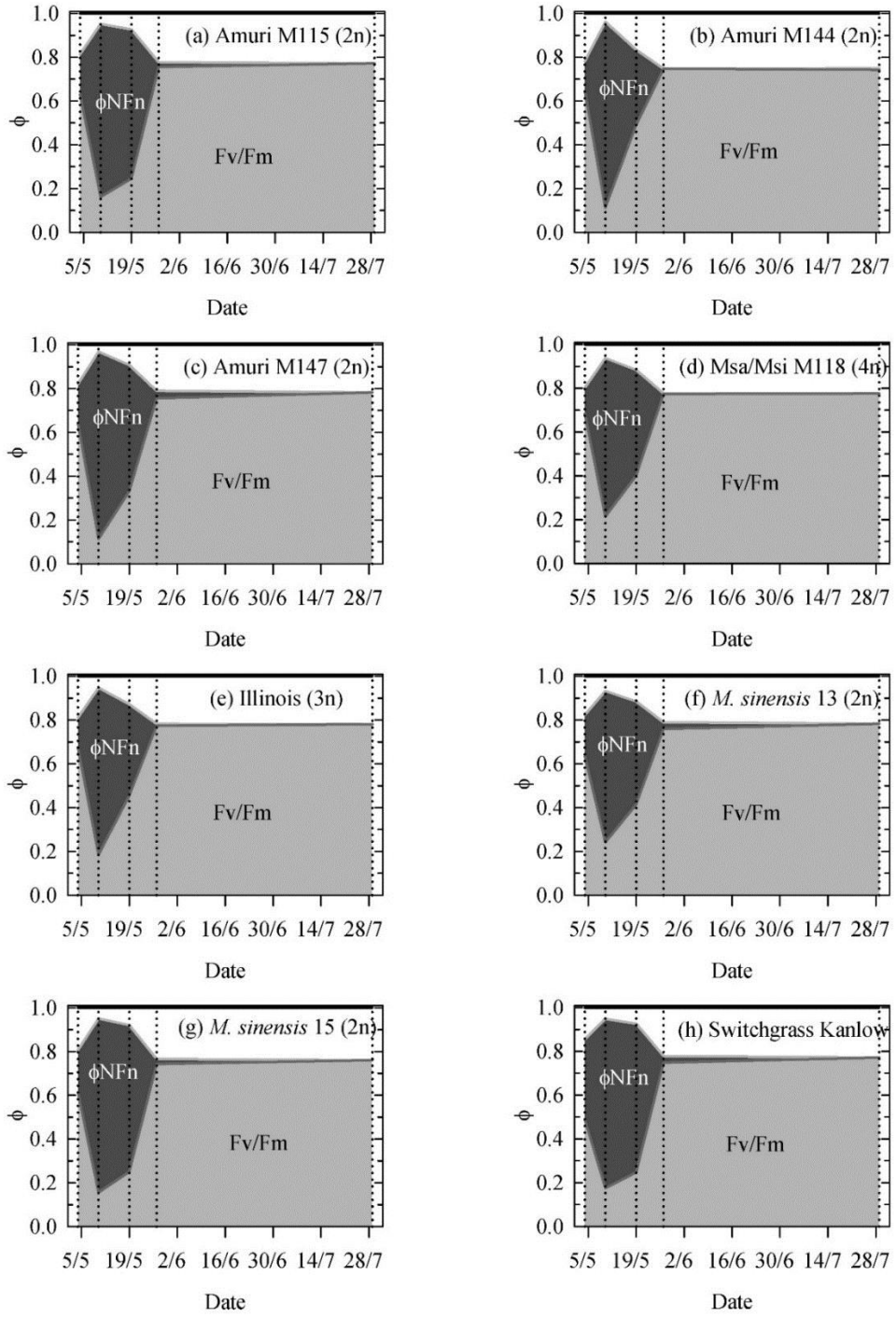


Figure 4.5 - Irradiance acclimated potential quantum yield of photochemistry (ϕPSII), and estimated quantum yield of sustained photoinhibition (ϕNF), flexible photoinhibition (ϕREG), and fluorescence and constitutive dissipation (ϕf,D). Dotted vertical lines indicate the measurement dates: 4 May 2010, 10 May 2010, 19 May 2010, 27 May 2010, and 29 July 2010. Open circles are the net photosynthetic rate ($A_{max}$).

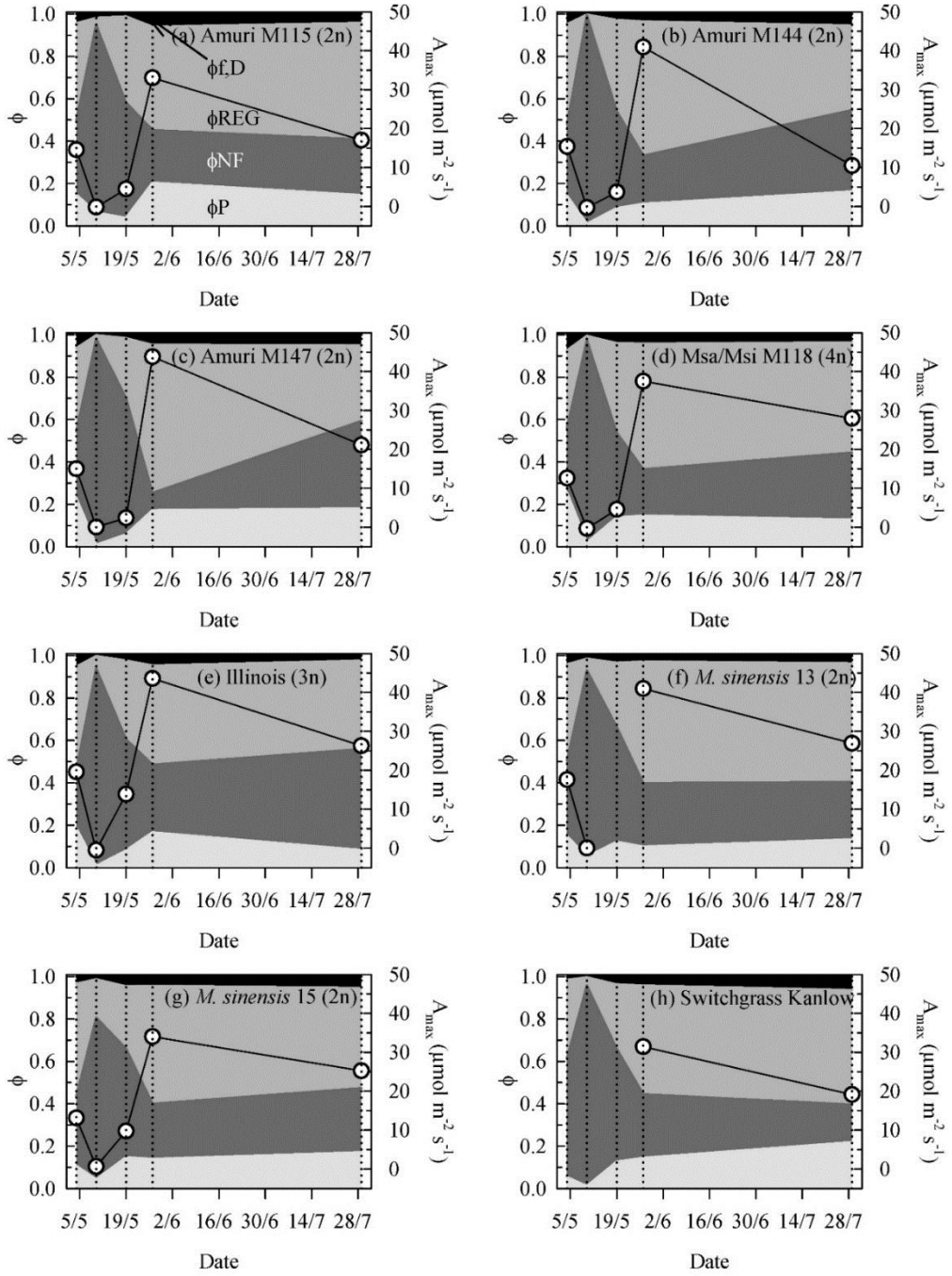
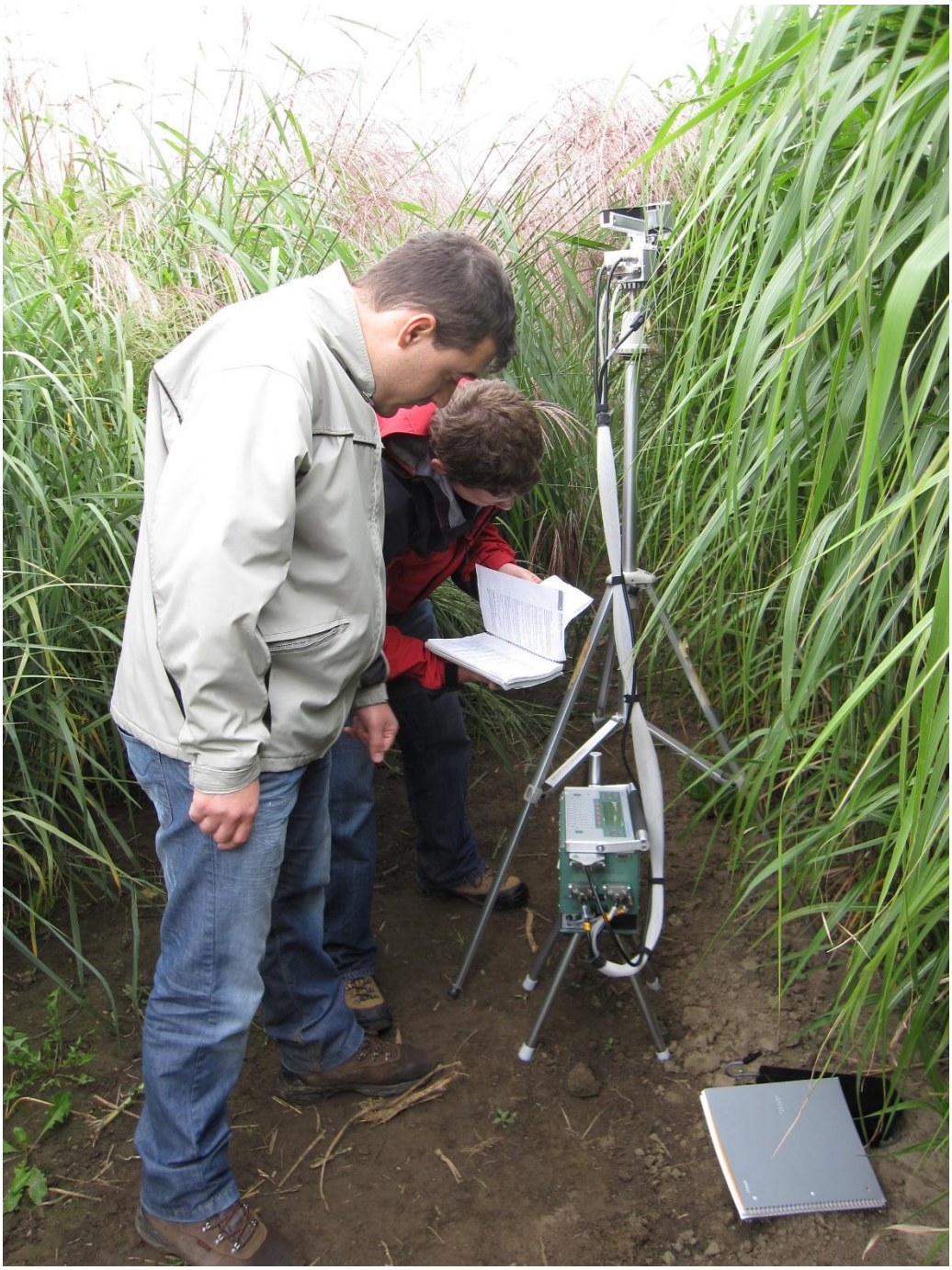


Figure 4.6 - Picture showing the *Miscanthus* field on 29 July 2010. Plants were about 2.5 m tall. In the foreground the author Murilo Peixoto and Patrick Friesen are measuring the photosynthetic rate using the LiCor 6400. Picture from Sage *et al.* (2014), with permission.



## 4.5 - Discussion

In the field, *Miscanthus* and switchgrass leaves showed high sensitivity to low temperature and high solar irradiation resulting in minimal Fv/Fm that preceded death of the leaves during the frost on 10 May 2010. However, most *Miscanthus* lines showed fast growth of new leaves after the killing frost. The triploids (Nagara and Illinois) showed high net photosynthetic rates ($A_{max}$) at the warmer temperatures. Also, these two genotypes along with the tetraploid (M118) and the *M. sinensis* lines (diploids) had the highest Fv/Fm under non-stressful conditions; however, only *M. sinensis* 15 had leaf survivorship after the 10 May 2010 frost. In contrast, switchgrass Alamo and Kanlow had consistently low Fv/Fm and $A_{max}$ until the summer.

On the warm days, one week after the chilling episode of 10 May 2010, the triploid Illinois, diploid M145, and *M. sinensis* 14 and 15 already had recovered and developed a photosynthetically active canopy with $CO_2$ assimilation rates near or above 10 µmol $m^{-2}$ $s^{-1}$ at 25ºC leaf temperature. Leaf temperature on 19 May 2010 was above the leaf temperature when the measurements were performed on 4 May 2010 and similar to the leaf temperature on 29 July 2010. However, because on 19 May 2010 leaves had just sprouted, and on the preceding days air temperature was lower than 13ºC, the photosynthetic rate was lower than on 4 May and 29 July. One week later on 27 May, temperature was warmer and both photosynthetic rates and quantum yield of photochemistry were the highest measured. The fast recovery of photosynthetic activity could also be noticed in the fluorescence measurements. On 19 May 2010, the increased quantum yield of photochemistry resulted in higher post irradiance $Fv_{pi}/Fm_{pi}$ than the predawn Fv/Fm measured for some plants. Spring maize also shows higher post irradiance Fv/Fm than predawn Fv/Fm when exposed to 25ºC for 10 hours after a chilling day in field (5.5ºC to 19.2ºC; Andrews *et al.*, 1995). According to

Kornyeyev (2003), PSII reaction centers can be repaired in a matter of one or two hours at warmer conditions. The recovery of damaged PSII reaction centers has a high demand for ATP, which can be produced by cyclic electron flow (Allakhverdiev *et al.* 2005; Takahashi *et al.* 2009; Huang *et al.* 2010b). A high $\phi P:\phi CO_2$ ratio was observed on young *Miscanthus* and maize leaves (during the first warm days of spring), which is an indication of high rates of cyclic electron transport producing ATP (Fryer 1998; Farage *et al.* 2006). This suggests that at high temperature during the day, PSII reaction centers are rapidly repaired in *Miscanthus* hybrids. If this is true, on 19 May 2010 the rate of repair exceeds photodamage, resulting in higher $Fv_{pi}/Fm_{pi}$ than Fv/Fm.

*Miscanthus* has been considered a chilling tolerant genus (Beale *et al.*, 1996; Naidu *et al.*, 2003; Farage *et al.*, 2006). After 7 days with temperature below 10ºC, the triploid *Miscanthus x giganteus* (Mxg) showed maximum quantum yield of $CO_2$ assimilation ($\phi CO_{2,max}$), similar to the summer values (June and July; Beale *et al.*, 1996). Also, *Miscanthus* grown at 25º/20ºC day/night temperature showed little reduction in Fv/Fm when exposed to 12º/5ºC for six days with a day light intensity of 550 ± 50 µmol $m^{-2}$ $s^{-1}$ (Friesen *et al.*, 2014). However, in the field a soft overnight frost with minimal air temperature reaching -0.3ºC severely damaged the Illinois line (Friesen *et al.*, 2015). In that study, *Miscanthus* leaves were not completely killed during the day which was cloudy temperatures were not greater than 10ºC (Friesen *et al.*, 2015). The present study reports that *Miscanthus* and switchgrass leaves were killed under more extreme conditions: air temperature never rose above 12ºC with clear skies light intensities were above 1300 µmol $m^{-2}$ $s^{-1}$ during the day. This condition appears to be more realistic for higher latitudes where day temperatures commonly drop below 10ºC and spring-summer days are often sunny following cold fronts (Shabbar & Bonsal, 2003).

Because triploid *Miscanthus* shows a smaller impact of chilling on $A_{max}$ than diploids and tetraploids, authors have considered that triploid lines have improved chilling tolerance compared to diploids and tetraploids (Purdy *et al.*, 2013; Friesen *et al.*, 2014; Głowacka *et al.*, 2014). After plants grown at 25º/20ºC were exposed for six days to 12º/5ºC night/day temperature, the triploids Nagara and Illinois (M116 and Mxg, respectively) had less reduction in $A_{max}$ compared to the diploid M115 and the tetraploid M118 (Friesen *et al.*, 2014). Purdy *et al.* (2013) showed that while triploid *Miscanthus* sustain high photosynthetic rates after chilling, diploids and tetraploids shows lower $A_{max}$ but higher expression of cold shock genes. Here I observed that the extreme stress caused by a cold front with high light intensities caused the death of the *Miscanthus* hybrids while the diploid *M. sinensis* was able to maintain leaves, and sustained both a the photosynthetic rate higher than respiration and the highest post-irradiation $Fv_{pi}/Fm_{pi}$ amongst all genotypes studied. *M. sinensis* had larger and more developed leaves compared to the other lines in the first day of the experiment (4 May 2010) indicating that leaves may had had sprouted earlier in the spring. Also, *M. sinensis* apparently sprouted leaves earlier in the spring because in the first field measurement on 04 May 2010, the leaves of *M. sinensis* were larger and better developed. Triploid *Miscanthus* senesce later than the diploid lines in the fall, indicating a faster response of nutriend mobilization by diploids to the lowering temperatures at the end of the growing season (Jørgensen, 1997; Clifton-Brown & Lewandowski, 2000a). Thus, it is possible that chilling triggers low temperature protection mechanisms in diploid *Miscanthus*, while triploid genotypes invest in maintaining high photosynthetic rates even at chilling temperature. That could explain the higher photoprotection and early senescence of the diploid lines, which results in higher low temperature tolerance of the rhizomes in the winter (Chapter 3; Clifton-Brown & Lewandowski, 2000a).

One of the greatest source of photoprotection in the leaves are the pigments of the xanthophyll cycle (Demmig-Adams, 1990). It was observed that *Miscanthus x giganteus* genotype Illinois grown at 10ºC had a 6-fold greater zeaxanthin content compared to plants grown at 14ºC, indicating a great photoprotective system in conditions similar to those observed in early spring (low temperature growth) (Farage et al., 2006). The ability of *M. sinensis* to maintain leaves under the chilling stress on 11 May 2010 and the inability of *M. x giganteus*, may be associated with even greater content of photoprotective pigments, which requires further study. Controversially, in growth chambers with controlled temperature, Friesen *et al.* (2014) showed a negative relationship between $A_{max}$ and ϕNF after six days of chilling. However, the low light level in the growth chamber (550 µmol photons $m^{-2}$ $s^{-1}$) compared to solar irradiance (1300 µmol photons $m^{-2}$ $s^{-1}$) may not have caused enough stress to significantly overcome the photoprotection granted by zeaxanthin (reflected in the ϕREG) resulting in low levels of sustained photoinhibition (ϕNF). Here, I observed that during the day the triploid *Miscanthus* had greater ϕNF and smaller ϕREG than the diploids. Interestingly, the overnight recovery of the photoinhibition was greater in the triploids that started the day with higher Fv/Fm than most diploids, which could be a result of greater energy availability at night in the triploids.

While greater photoprotection possibly allowed *M. sinensis* to start a canopy earlier, the greater $A_{max}$ allowed triploid *Miscanthus* to have a larger canopy once the temperature warmed on 27 May 2010. Early establishment of a leaf canopy is important for improving productivity supported by high radiation use efficiency of *Miscanthus* (Beale & Long, 1995; Dohleman *et al.*, 2012). $C_4$ plants have a theoretical efficiency of conversion of incident solar energy of 6%, contrasting with $C_3$ plants that have only 4.6% (Heaton *et al.*, 2008a; Zhu *et al.*, 2008). For the

entire growing season in the Midwest USA, *Miscanthus x giganteus* had 1.5% to 2.0% of conversion of solar radiation while switchgrass had only 0.7% to 0.9% (Dohleman *et al.*, 2012). In this region, shoots emerge in April, when mean temperature is around 11ºC, and plants reach 2 meters by late May to the beginning of June, when they reach canopy closure (Pyter *et al.*, 2007; Dohleman *et al.*, 2009; Anderson *et al.*, 2011). That allows for an increase in the efficiency in the solar energy conversion to 4.4% between June and August (Dohleman *et al.*, 2012).

In Canada, chilling events in the spring are particularly frequent (Shabbar & Bonsal, 2003). If the canopy is killed in the spring by a cold frost, the late formation of a canopy will decrease conversion efficiency as leaves will be exposed later to solar radiation in the growing season, removing the $C_4$ conversion advantage. It is thus desirable to prioritize improvement in frost tolerance of young *Miscanthus* shoots as is observed in other cold-adapted $C_4$ species such as *Spartina pectinata*. This would allow the effective start of canopy development to occur 2-4 weeks earlier to increase the capture of solar irradiation.

In the present study, I report the sensitivity of *Miscanthus* hybrids to sustained photoinhibition caused by day temperatures below 10ºC and clear skies delivering 1300 µmol of photons $m^{-2}$ $s^{-1}$ or more. Amongst all the *Miscanthus* and Switchgrasses hybrids studied here, only the *M. sinensis* genotypes had consistently lower sustained photoinhibition on the cold spring days, and they were the only genotypes that could maintain some living leaves after frost/chilling events. On the other hand, once the temperature warmed *M. sinensis* had a poor canopy and the triploid *Miscanthus* rapidly built a large canopy by achieving high photosynthetic rates. These results demonstrate that there is a potential to increase frost tolerance in the *Miscanthus* genetic-pool,

which can be exploited by breeding or genetic engineering to optimize the production of *Miscanthus* in colder climates.

# Chapter 5 - Comparative photosynthetic responses in upland and lowland sugarcane cultivars grown under different temperatures

## 5.1 - Abstract

Sugarcane is the most productive and one of the most important crops in the world. In Hawaii, upland varieties have been selected for growth at the low temperatures found in high altitudes. Although this is a crop adapted to tropical regions, there is a significant interest in extending the ranges in which this crop can be cultivated, and to determine what can be achieved by improving photosynthesis acclimation. Also, productivity can be further increased if this plant can rapidly acclimate to seasonal temperature changes. In this study, I compare lowland and upland Hawaiian sugarcane grown at 32ºC/26ºC and 21ºC/18ºC day/night temperature. Although these plants grow well in different climates, when grown at the same moderate temperature no difference in photosynthetic rate (*A*) was observed. Also, growth at these two moderate temperatures resulted in no change in *A*. However, after plants were briefly exposed to 45ºC, *A* at 20ºC declined considerably and could not recover to the same rate measured at 20ºC before exposure to 45ºC, indicating injury related to heat stress. This decline was more pronounced in plants grown at 21ºC/18ºC than for plants grown at 32ºC/26ºC. This indicates the thermal acclimation that occurs in both lines enhances tolerance to extremes, rather than altering the thermal optimum associated with optimal rates *A*.

**Abbreviations:** *A*, net photosynthetic rate; $A_{max}$, maximum net photosynthetic rate at saturated $CO_2$ concentration and light; T, temperature; Ci, intercellular $CO_2$ concentration; PPFD,

photosynthetic photon flux density; CE, carboxylation efficiency; $\phi CO_2$, quantum yield of $CO_2$ assimilation, $\phi P$, quantum yield of photosystem 2.

**Note:** The term $\phi PSII$ is a source of confusion between authors and the different bibliographies cited here use this term for two different parameters. The present chapter uses the term $\phi P$ for the quantum yield of photosystem II as suggested by Kornyeyev & Holaday (2008). According to these authors $\phi P$ is Fq'/Fm' and $\phi PSII$ is Fv/Fm', where Fq' is Fv'-Fs, and Fv' is Fm'-Fo'. In this case $\phi PSII$ is the instantaneous maximum potential quantum yield of photosystem 2. Kramer *et al.* (2004) uses a different nomenclature for each of these parameters. Baker (2001, 2008) avoid using $\phi$, calling these parameters simply Fq'/Fm' or Fv'/Fm'.

## 5.2 - Introduction

Sugarcane is one of the leading bioenergy crops in the world, due in large part to its high productivity and large capacity to store non-structural carbohydrates in vegetative tissues. However, it is a plant of tropical origin, generally noted to be chilling sensitive (Tai & Lentini, 1998; Du *et al.*, 1999b; Friesen *et al.*, 2014). This sensitivity largely limits the area of sugarcane cultivation to subtropical latitudes, where temperatures are above the threshold for cold inhibition (Bacchi & Souza, 1980; Grantz, 1989). This is problematic for temperature zone nations that could potentially exploit sugarcane as a renewable source of energy. There is, however, a potential that the sugarcane genetic complex has cold tolerant capabilities. For example, in Florida, Hawaii and Louisiana-USA, varieties of sugarcane have been breed for cold tolerance (Tai & Miller, 1996; Eggleston *et al.*, 2004). One of the breeding varieties is from high altitude in Tucumán, Argentina (1400-2200 m above sea level), but upland sugarcane is also found in Hawaii (Moore, 1987) and Japan (Nagatomi & Degi, 2007). For example, upland sugarcane is grown in Kula, Hawaii, located

at 1420 meters above sea level, where monthly average air temperature range between 22ºC and 13ºC. Studies on cold induced gene expressions in sugarcane could provide tools for breeders and genetic engineering programs to increase sugarcane cold tolerance (Belintani *et al.*, 2012), but this also requires physiological examination to identify cold tolerance attributes. In this study, I compare the photosynthetic response to temperature of upland and lowland Hawaiian sugarcane, to better understand the photosynthetic tolerance to suboptimal temperature and the acclimation of photosynthesis to cool temperature in sugarcane. The study focus on non-chilling temperatures below the thermal optimum, as chilling and photosynthetic depression at cold but non injurious conditions may differ.

Chilling injury is often associated with lesion to photosynthetic membranes, repair mechanism (e. g. photosystem II) and protein denaturation (Berry & Björkman, 1980; Öquist & Martin, 1986; Long *et al.*, 1994; Uemura *et al.*, 1995). By contrast, inhibition of photosynthesis at non-injurious suboptimal temperatures may involve depression of the activity of certain processes of the photosynthetic apparatus. In $C_4$ plants, the leading hypotheses concerning limitations at suboptimal temperatures are that Rubisco is limiting, and that PPDK (pyruvate phosphate di-kinase) becomes labile and this imposes limitation in PEP (phosphoenol pyruvate) regeneration (Sage *et al.*, 2014). At supraoptimal temperatures it is possible that Rubisco activase (Crafts-Brandner & Salvucci, 2002) or the electron transport rate (von Caemmerer & Furbank, 1999) are the limiting step. Using a combination of whole leaf gas exchange and leaf fluorescence, it is possible to investigate how these processes respond to non-stressful temperatures in lowland and upland Hawaiian sugarcane varieties.

## 5.3 - Materials and methods

*Plant material*

Upland and lowland Hawaiian sugarcane were provided by Dr. Albert Arcsinas (Hawaiian Agricultural Research Center, Kunia, HI, USA). The stem of a single plant was divided in two: one was planted at 32ºC/26ºC and the other at 21ºC/18ºC day/night temperature, in two temperature controlled glasshouses. Samples were planted in March 2011 and maintained in the glasshouse of the Earth and Sciences Building in University of Toronto, under 14 hours photoperiod. High pressure sodium lamps delivered up to 200 ± 50 PAR (photosynthetic active radiation) in addition to natural illumination, which reached 1500 µmol $m^{-2}$ $s^{-1}$ on sunny days. Plants were positioned randomly inside the glasshouse and rotated every week to minimize the within room effects. Plants were grown in 20 liter pots with a soil mixture containing 40% topsoil, 40% coarse sand, and 20% ProMix (Premier Tech, Quebec, Canada). Commercial fertilizer (30-10-10 NKP Miracle-Gro, Scotts Company LCC) was applied weekly at the manufacturer's suggested concentration. In addition to the commercial fertilizer, a modified Johnson-Hoagland's fertilizer (Sage & Pearcy, 1987) was provided biweekly to provide a full complement of essential nutrients. On 1 June 2011, the fertilization regime was doubled: commercial fertilizer was applied twice a week and Johnson-Hoagland's solution was applied weekly.

*Gas exchange and chlorophyll fluorescence measurements*

All photosynthetic measurements were carried between 18 July 2011 and 31 August 2011. The photosynthesis response to temperature (*A* vs T) was measured using a null-balance gas exchange system. In this system, an infra-red gas analyzer (LiCor-6262, Lincoln, NE, USA) was used to

measure $CO_2$ and water vapor concentrations. Mass flow controllers (model 840, Sierra Instruments, Monterrey, CA, USA) supplied a mixture of $N_2$, $O_2$ (21%), and $CO_2$ at 380 µmol $mol^{-1}$. This system is further described by Makino & Sage (2007). The leaf was placed in a leaf chamber illuminated with 2000 µmol $m^{-2}$ $s^{-1}$ PPFD (photosynthetic photon flux density) at 20ºC. The photosynthetic measurements were taken after the leaf temperature ($T_{leaf}$), $CO_2$ concentration ($CO_2$ sample), and water vapour pressure ($H_2O$ sample) leaving the chamber were stable (steady state). Measurements were then taken at 20º, 15º, and 10ºC. Next, leaf temperature was returned to 20ºC and the measurements were taken at 20º, 25º, 30º, 35º, 40º, and 45ºC after steady state was reached. In a different test, one set of measurements were taken first at 20º, 25º, 30º, 35º, 40º, and 45ºC, and then at 20º, 15º, and 10ºC. To avoid photoinhibition, at 20ºC and 15ºC the light level was reduced to 1800 µmol $m^{-2}$ $s^{-1}$ PPFD, and at 10ºC it was 1500 µmol $m^{-2}$ $s^{-1}$ PPFD, which were previously determined to be sufficient to saturate the photosynthetic apparatus at these temperatures.

The net photosynthetic response to intercellular $CO_2$ concentration (*A* vs Ci) and to incident light (*A* vs PPFD) was measured using the LiCor 6400 gas exchange system (LiCor, Lincoln, NE, USA). The Li-6400-40 fluorometer and leaf chamber allowed for simultaneous measurements of photosynthesis and leaf chlorophyll fluorescence. The fluorescence parameters measured in the dark were Fo, Fm, and for light irradiated leaves it was Fs and Fm'. Fv/Fm (Fv= Fm-Fo) is a measurement of the maximal quantum yield of photochemistry, while Fq'/Fm' (Fq'=Fm'-Fs) gives the light irradiated quantum yield of photochemistry (ϕP). *A* vs $C_i$ and *A* vs PPFD measurements were conducted at 15º, 25º, and 35ºC. For these measurements, the leaf was placed on the leaf chamber at 380 µmol $mol^{-1}$ $CO_2$. Leaves at 25ºC and 35ºC received 2000 µmol $m^{-2}$ $s^{-1}$ PPFD and leaves at 15ºC initially received 1800 µmol $m^{-2}$ $s^{-1}$ PPFD. For the *A* vs $C_i$ response, the light was maintained at the initial level, with measurements taken when the leaf chamber was supplied with

different concentrations of $CO_2$ in the following sequence 380, 550, 750, 1000, 380, 300, 200, 100, 60, and 40 µmol $mol^{-1}$ of $CO_2$. For the light responses, the $CO_2$ supply was maintained at 380 µmol $mol^{-1}$, and light was changed in sequence to 2000, 1800, 1500, 1100, 750, 500, 250, 100, 50, and zero µmol $m^{-2}$ $s^{-1}$ PPFD. All gas exchange/chlorophyll fluorescence measurements were conducted on the youngest fully expanded leaf. The vapor pressure deficit between the air in the chamber and the leaf was maintained between 1.5 and 2.0 kPa.

The leaf absorbance was measured using a Taylor integrating sphere (Idle & Proctor, 1983; Long *et al.*, 1996) associated to a quantum sensor (LI-190, LiCor Inc.). For determination of blue light absorption, a filter was installed in the light inlet of the integrating sphere, allowing the passage only of light on the bandwidth of 464 nm. This process was repeated using a red light absorbance filter that only allows passage of light on the bandwidth of 634 nm. Those are near the peaks for light absorption of chlorophyll (Idle & Proctor, 1983).

Carboxylation efficiency (CE) was determined as the initial slope of the *A* vs $C_i$ response at $C_i$ < 90 µmol $mol^{-1}$. $\phi CO_2$ (intrinsic quantum efficiency of $CO_2$ assimilation) was determined by dividing the gross photosynthesis rate ($A_{gross}$) by the absorbed PPFD:

$$\phi CO_2 = \frac{A_{gross}}{(0.1 x \text{Blue}_{abs} x PPFD) + (0.9 x \text{Red}_{abs} x PPFD)}$$

where, $A_{gross} = A - Rd$ (Rd is dark respiration); $Blue_{abs}$ is the absorbance for blue light, while $Red_{abs}$ is the absorbance for red light (von Caemmerer, 2000). The light incident on the leaf is composed of around 10% of blue light and 90% of red light from the LED lamps on the LI-6400-40 leaf chamber (Naidu & Long, 2004).

*Statistical analysis*

Twenty plants from each of the two varieties of sugarcane were equally divided between temperature treatments (21ºC or 32ºC). From the 10 plants of each variety and growth temperature, five were randomly chosen for each measurement. Because the response of the photosynthetic rate (*A*) to temperature (T) has a Gaussian shape, and the *A* response to intercellular $CO_2$ concentration (Ci) and to light (photosynthetic photon flux density - PPFD) are hyperbolic, the data was analyzed using a generalized additive mixed model (GAMM). The fluorescence responses (Fq'/Fm') to light (PPFD) was also analyzed using a GAMM but assuming a beta distribution, because Fq'/Fm' is proportional data bounded between 0 and 1. For the temperature responses of *A,* a cubic spline was applied on T. On the *A* vs Ci, a cubic spline was applied to Ci, and for *A* vs PPFD and Fq'/Fm' vs PPFD, a cubic spline was applied to PPFD. Carboxylation efficiency was also analyzed separately using a generalized linear model assuming the beta distribution. Except for the *A* vs T analysis, temperature was considered as a factor with three levels (15ºC, 25ºC, and 35ºC) because in these measurements temperature was not a continuous variable. All analysis were performed using R statistics software (R-Core-Team, 2013) and using the package "gamlss" (Rigby & Stasinopoulos, 2005).

## 5.4 - Results

The response of net photosynthetic rate (*A*) to temperature for lowland and upland Hawaiian sugarcane was similar in both cultivars and growth temperatures ($p > 0.05$, Figure 5.1). Lowland sugarcane grown at 21ºC (LC, Figure 5.1) had a thermal optimum of *A* near 30ºC with mean *A* of 43 µmol $m^{-2}$ $s^{-1}$, while plants from the same variety grown at 32ºC (LW, Figure 5.1), and upland sugarcane grown at 21ºC and 32ºC (UC and UW, respectively; Figure 5.1) had a thermal optimum

near 35ºC with a mean *A* around 45 µmol $m^{-2}$ $s^{-1}$. At 10ºC, *A* ranged between 10–12 µmol $m^{-2}$ $s^{-1}$, with the smallest values for plants grown at 32ºC. At 40ºC, the mean *A* varied between 38–44 µmol $m^{-2}$ $s^{-1}$ in each variety from each growth condition but increasing temperature to 45ºC caused a drop in *A* to 15–22 µmol $m^{-2}$ $s^{-1}$.

In a separate trial, the leaf was first measured at 20º, 25º, 30º, 35º, 40º and 45ºC, and then temperature was lowered to 20º, 15º and 10ºC (Figure 5.2). After exposure to 45ºC, all sugarcane plants had *A* at 20ºC lower than before they were exposed to 45ºC (Figure 5.2). On sugarcane plants grown at 21ºC the reduction in *A* was greater than on plants grown at 32ºC after exposed to 45ºC, such that when temperature returned to 20ºC, *A* was not greater than 5 µmol $m^{-2}$ $s^{-1}$ (Figure 5.2a, c), and much less at 10º and 15ºC than when *A* was first lowered to 15º and 10ºC in an A vs T response curve (Figure 5.1).

The two sugarcane varieties also showed no difference in the photosynthetic response to intercellular $CO_2$ partial pressure (Ci, $p > 0.05$, Figure 5.3). At all temperatures measured, photosynthesis reached its maximum ($A_{max}$), or very close to its maximum, at ambient $CO_2$ concentration (indicated by the arrows on Figure 5.3). For both sugarcane varieties from both growth temperatures, $A_{max}$ at 15ºC was around 11-15 µmol $m^{-2}$ $s^{-1}$, at 25ºC it was around 30-35 µmol $m^{-2}$ $s^{-1}$, and at 35ºC it was around 41-49 µmol $m^{-2}$ $s^{-1}$ (Figure 5.4). The carboxylation efficiency (CE), which is measured as the initial slope of the *A* vs $C_i$ curve, showed little difference between the sugarcane varieties and growth temperature (Table 5.1; Figure 5.4). The CE measured at 25ºC and 35ºC for both varieties and growth temperatures was similar, but it was greater than the CE measured at 15ºC ($p < 0.05$; Figure 5.4).

The light responses of photosynthesis showed no difference between the sugarcane varieties and the growth temperature ($p > 0.05$; Figure 5.5). At 15ºC, the light saturation point was between 1100-1500 PPFD (µmol of photons $m^{-2}$ $s^{-1}$), except for lowland variety grown at 21ºC, where the light saturation point was 1800 PPFD. As PPFD increased, PSII reaction centers were being occupied and the excess of light was driven to non-photochemical processes. Due to high irradiation, a smaller fraction of the absorbed light was used by photochemistry, thus lowering the Fq'/Fm' (Figure 5.6). Similar response was observed by Kubien & Sage (2004a) in *Muhlenbergia glomerata*. The fluorescence response to light showed no difference between varieties and growth temperatures ($p > 0.05$). As PPFD approached the light saturation point, the Fq'/Fm' curve flattened (Figure 5.6).

With increasing light, $\phi CO_2$ changes were proportional to changes on $\phi P$ (Fq'/Fm'), maintaining the $\phi P$:$\phi CO_2$ ratio near 12 (Table 5.1, Figure 5.7). This ratio was maintained at all three temperatures in on both sugarcane varieties and growth temperatures, and followed the theoretical response.

Table 5.1 - The ratio of quantum yield of PSII (ϕPSII) and quantum efficiency of $CO_2$ assimilation ($\phi P:\phi CO_2$) and the carboxylation efficiency at light saturation (CE) of lowland and upland sugarcane grown at 21ºC and at 32ºC. Differences between variety and growth temperature was not significant but differences between measuring temperature are significant ($p < 0.05$). Superscript letters indicate significant differences between values on that parameter. Mean ± standard error. N = 5.

| Variety/growth | Measuring temperature | $\phi P/\phi CO_2$ | CE ($\Delta A\ \Delta Ci^{-1}$) |
|---|---|---|---|
| Lowland 21ºC grown | 15ºC | 12.9±0.2 | 0.170±0.049[b] |
| | 25ºC | 12.4±0.2 | 0.411±0.044[a] |
| | 35ºC | 12.1±0.2 | 0.356±0.017[a] |
| Lowland 32ºC grown | 15ºC | 13.4±0.2 | 0.171±0.033[b] |
| | 25ºC | 13.1±0.1 | 0.368±0.059[a] |
| | 35ºC | 12.7±0.1 | 0.317±0.032[a] |
| Upland 21ºC grown | 15ºC | 12.7±0.2 | 0.169±0.001[b] |
| | 25ºC | 12.4±0.2 | 0.354±0.042[a] |
| | 35ºC | 12.3±0.1 | 0.393±0.063[a] |
| Upland 32ºC grown | 15ºC | 12.9±0.2 | 0.222±0.036[b] |
| | 25ºC | 12.4±0.2 | 0.475±0.041[a] |
| | 35ºC | 12.1±0.2 | 0.412±0.007[a] |

Figure 5.1 - Net photosynthetic responses to temperature in two varieties of sugarcane grown at 21ºC and 32ºC day temperature. Photosynthesis was measured first at the lower temperatures and then at the warmer temperatures. No difference between the sugarcane varieties or growth temperature was significant ($p < 0.05$). Mean ± SE, N = 5.

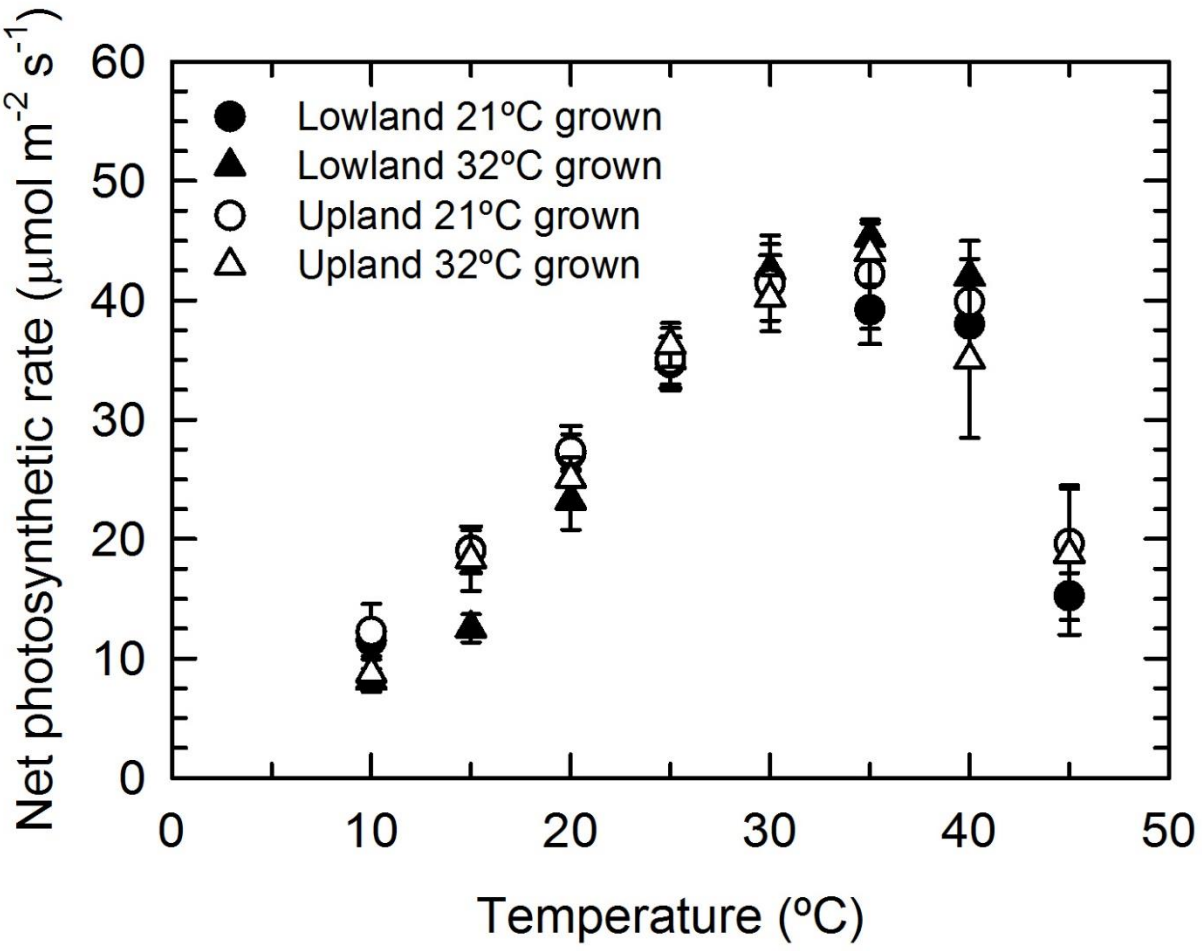


Figure 5.2 - Net photosynthetic responses to temperature in two varieties of sugarcane grown at 21ºC and 32ºC day temperature. Measurement started at 20ºC and was gradually raised to 45ºC (closed symbols). After measured at 45ºC temperature was lowered to 20ºC and gradually reduced to 10ºC (open symbols). No difference between the two sugarcane varieties was significant, but differences between growth temperatures was significant ($p < 0.05$). Symbols represent the mean value, error bars are standard error. N = 4.

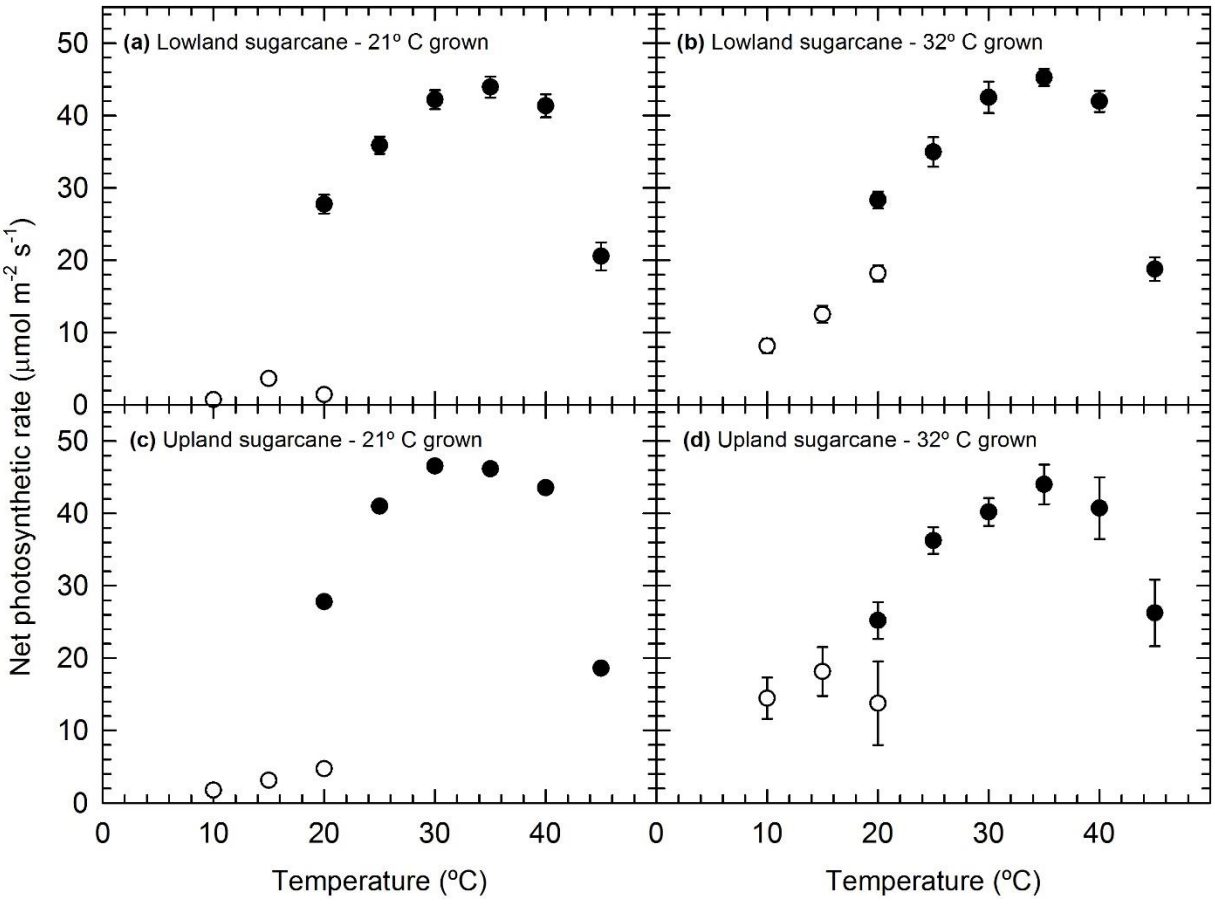


Figure 5.3 - The response of net photosynthetic rate to intercellular $CO_2$ concentration on lowland and upland sugarcane grown at 21ºC and at 32ºC, measured at 15º, 25º, and 35ºC. No significant difference was detected between the sugarcane varieties or the growth temperatures ($p < 0.05$). Arrows indicate measurement at ambient level of $CO_2$ (400 mbar). Symbols represent mean, error bars are standard error. N = 5.

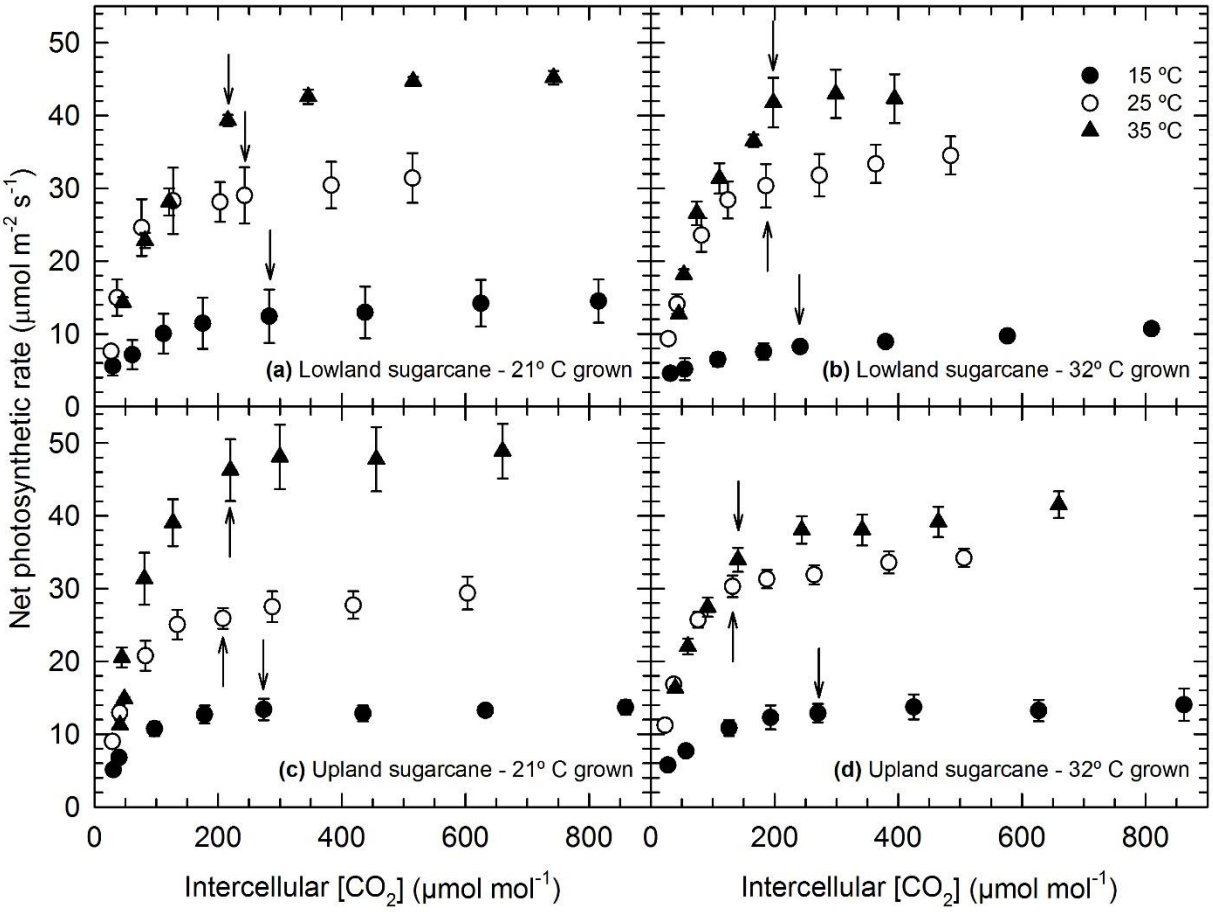


Figure 5.4 - The carboxylation efficiency (CE; filled symbols, solid lines) and the photosynthetic rate at light ant $CO_2$ saturation ($A_{max}$, open symbols, dashed lines) measured at 15º, 25º, and 35ºC. Arrows show that the CE difference between 15ºC and 25ºC indicates a PPDK limitation while between 25ºC and 35ºC the limitation PEPCase should be limiting. The CE was determined by pooling the low $CO_2$ data from 5 samples. $A_{max}$ is mean ± SE, N = 5.

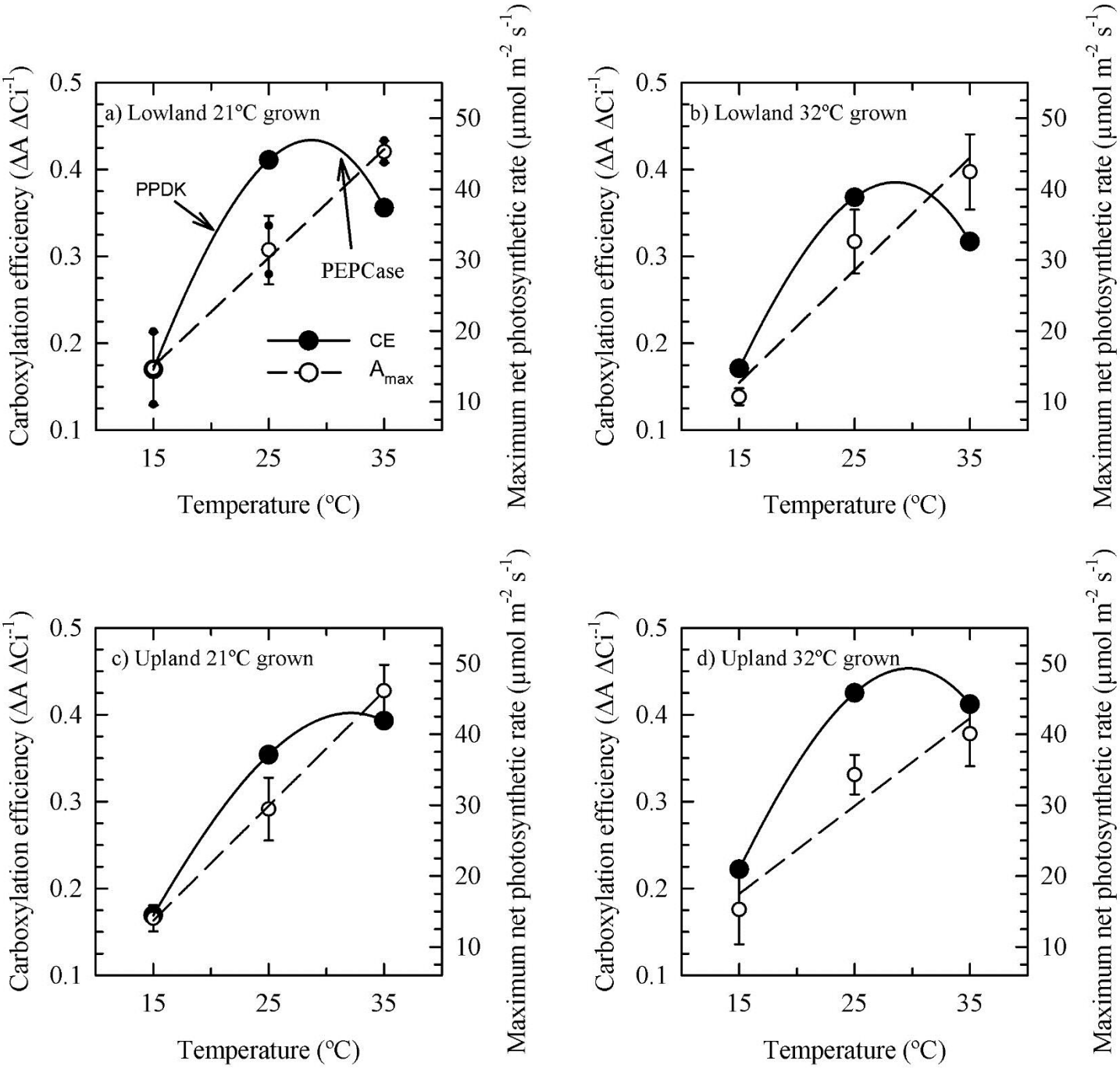


Figure 5.5 - The response of net photosynthesis rate to incident photosynthetic photon flux density (PPFD). Measurements were taken in two varieties of sugarcane grown at 21ºC and 32ºC and measured at 15º, 25º, and 35ºC. No significant difference was detected between the sugarcane varieties or the growth temperatures ($p < 0.05$). Symbols represent mean, error bars are standard error. N = 5.

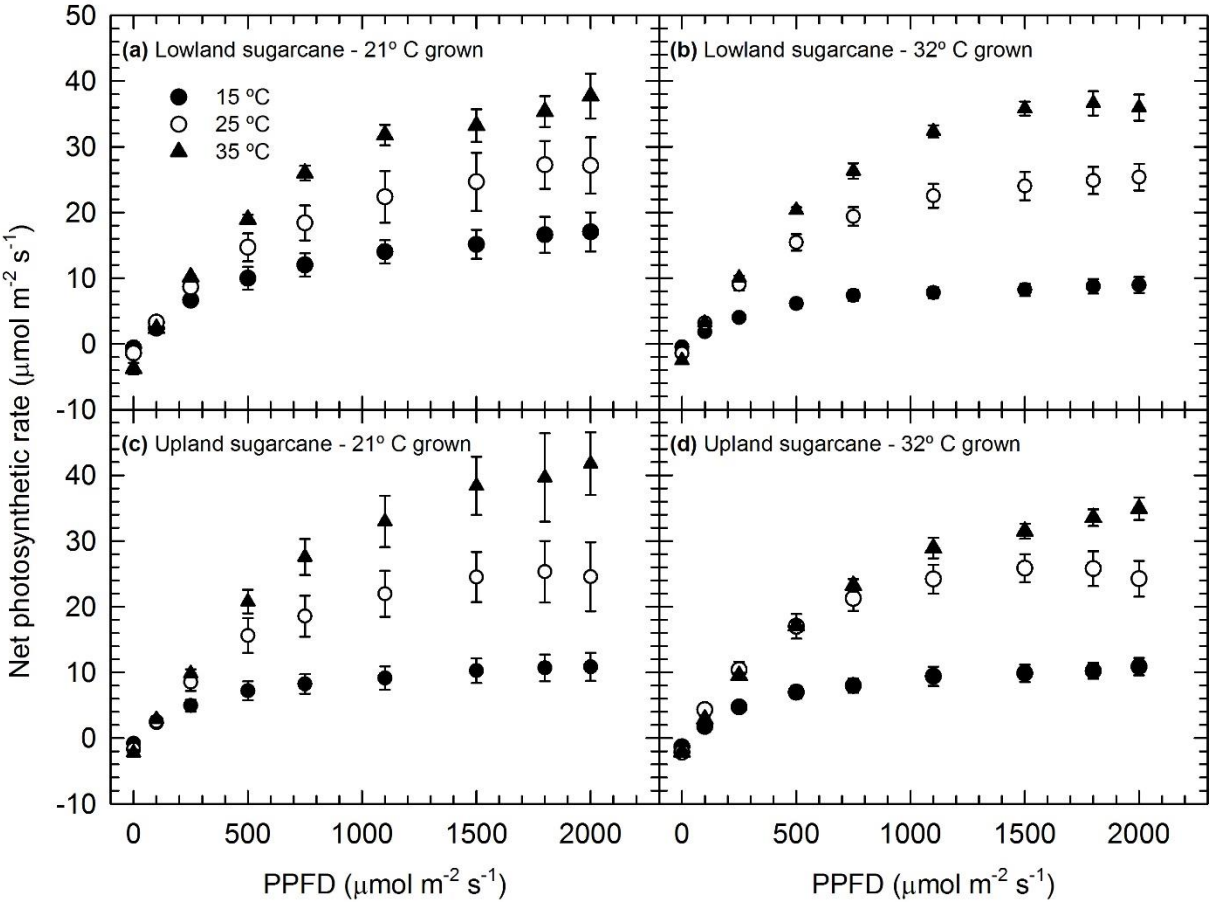


Figure 5.6 - The response of the quantum yield of PSII (Fq' /Fm'; Fv/Fm if PPFD is zero) to incident photosynthetic photon flux density (PPFD) in the leaf of two varieties of sugarcane grown at 21ºC and 32ºC and measured at 15º, 25º, and 35ºC. Fq'/Fm' and Fv/Fm measurements were taken in parallel to the net photosynthesis measurements in Figure 5.5. No significant difference was detected between the sugarcane varieties or the growth temperatures $p < 0.05$). Symbols represent mean, error bars are standard error. N = 5.

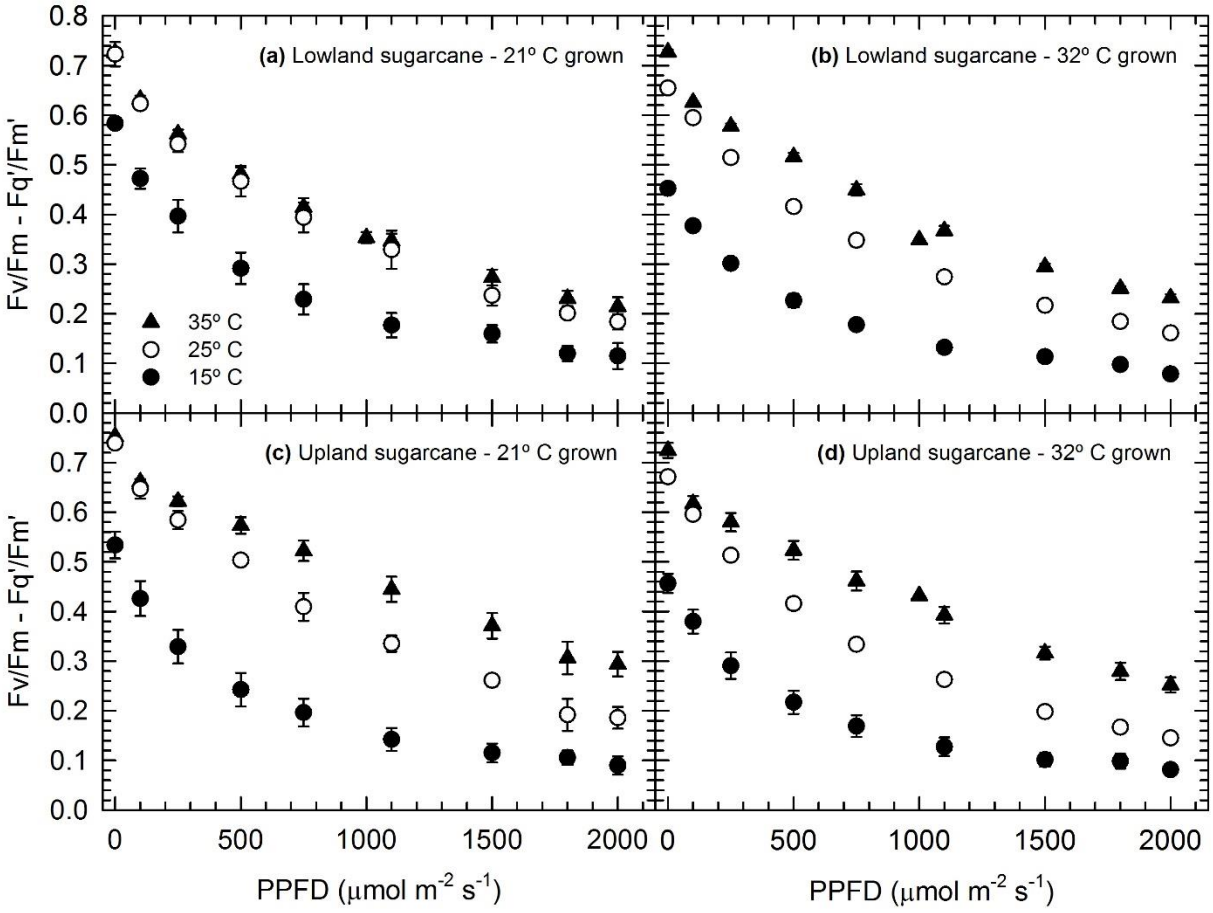
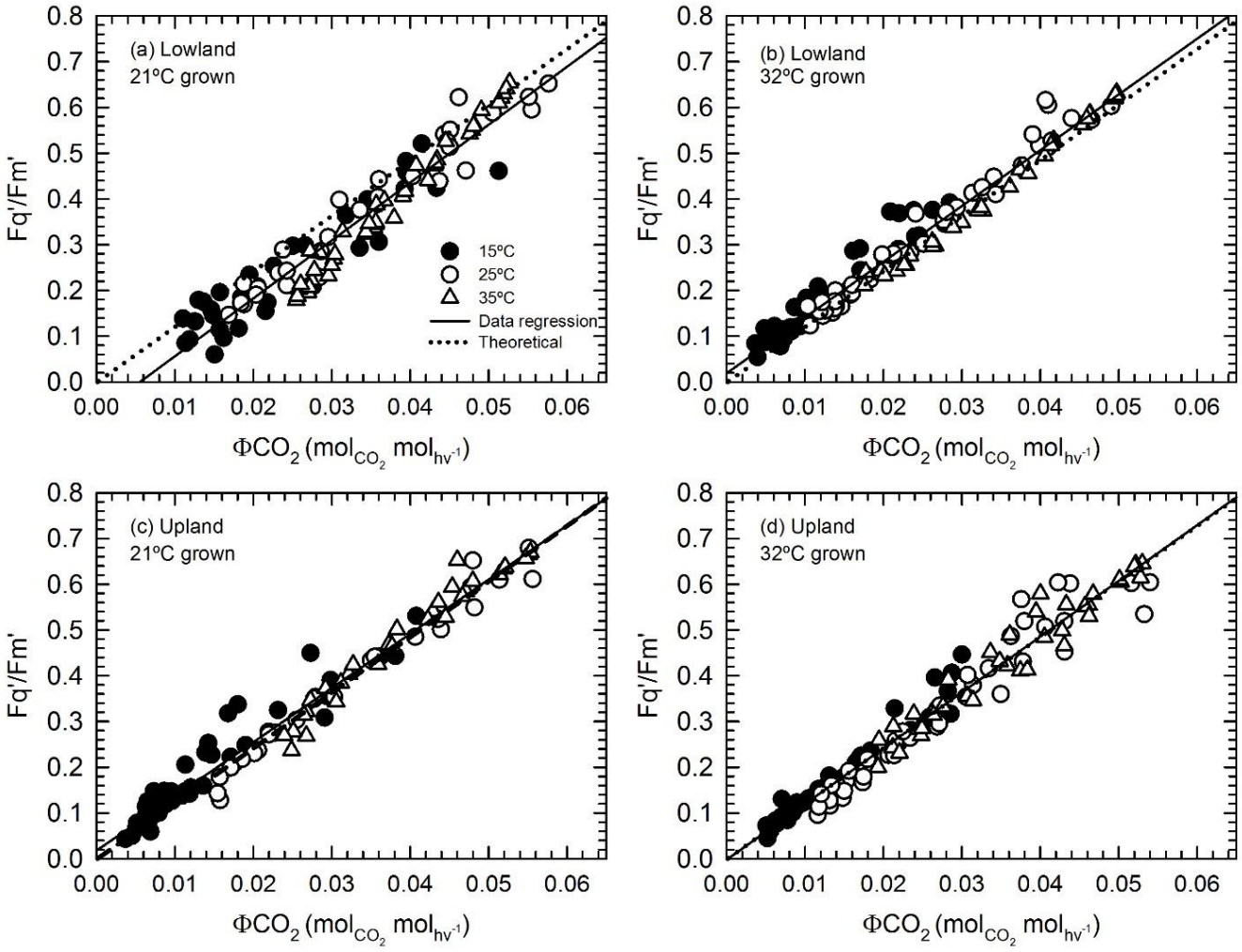


Figure 5.7 - The quantum yield of photosystem II (ϕP = Fq'/Fm') as a function of the quantum yield of $CO_2$ assimilation (ϕ$CO_2$) for lowland and upland Hawaiian sugarcane grown at 32º/26ºC and 21º/18ºC. The theoretical line assumes that 12 photons are used per $CO_2$ fixed (Sage *et al.*, 2011).



## 5.5 - Discussion

The two types of Hawaiian sugarcane analyzed in this study showed little difference in photosynthetic response (*A*) to different temperature, light levels, or $CO_2$ levels. Growing these sugarcane plants at 21º/18ºC or 32º/26ºC day/night temperature was not sufficient to alter most of the photosynthetic responses, indicating low acclimation potential at non-stressful temperatures. However, the photosynthetic values at 20ºC and below declined considerably after the leaf was exposed to 45ºC. In plants grown at 21º/18ºC this drop was substantial, with *A* value collapsing to near zero. This demonstrates that acclimation to cooler growth temperature resulted in a loss of heat tolerance in sugarcane, rather than an improvement in *A* at lower growth temperature. Similar results were observed in *Miscanthus* and *Muhlenbergia*, where acclimation altered *A* at the thermal optimum and above, but not at suboptimal temperatures (Naidu *et al.*, 2003; Kubien & Sage, 2004b)

Because upland plants are adapted to colder climates, it was hypothesized that they would have improved photosynthetic responses and acclimation to low temperatures relative to lowland plants. My data do not support this hypothesis. Differences between chilling sensitive and chilling tolerant $C_4$ plants are not always observed when plants are grown at non-chilling temperatures (Du *et al.*, 1999b; Friesen *et al.*, 2014). However, such differences can arise after plants are exposed to at least a few hours of chilling. The chilling adapted sugarcane NiF4 and the chilling sensitive sugarcane Badira grown at 30º/25ºC had similar *A* when measured at 10ºC or 30ºC (Du *et al.*, 1999b). After being chilled at 10ºC for 4 and 52 hours, NiF4 could maintain photosynthetic rate, but in Badira the $CO_2$ assimilation rate suffered a considerable decline when measured at 10ºC and 30ºC (Du *et al.*, 1999b). Friesen *et al.* (2014) also showed no changes in the reduction of *A* between

*Miscanthus* genotypes and upland Hawaiian sugarcane grown at 25ºC when exposed for 24 hours to 12ºC. However, *Miscanthus x giganteus* showed the smallest decrease in *A* after seven days at this chilling temperature compared to the lowland sugarcane. My data show that lowland and upland Hawaiian sugarcane have the same thermal performance if grown at non-stressful temperatures, and that growth at 21ºC and 32ºC day temperature was not enough to show differences due to acclimation down to at least 10ºC during short term measurement exposure.

Variation in warmer growth temperature between 25ºC and 35ºC was shown to trigger photosynthetic acclimation in other $C_4$ plants. The $C_4$ *Panicum coloratum* (NAD-ME grass), *Cenchrus ciliaris* (NADP-ME grass), and *Flaveria bidentis* (NADP-ME dicot) grown at 25ºC had higher *A* than plants grown at 32ºC on measurements made at temperatures between 25ºC and 37ºC (Dwyer *et al.*, 2007). Above 40ºC, *A* was similar for plants grown at both temperatures. In general, $C_4$ plants show significant decrease in *A* if grown at stressful temperatures. Even though the cold adapted *Miscanthus x giganteus* did not show significant differences in *A* on plants grown at 25ºC and 14ºC, when grown at 10ºC the *A* measured at 25ºC was much lower (Farage *et al.*, 2006). In addition, plants grown at 10ºC had a 6-fold increase in zeaxanthin content (a photoprotective pigment) compared to the ones grown at 14ºC (Farage *et al.*, 2006). For warm adapted $C_4$ plants, temperatures below 20ºC are generally stressful. Maize (*Zea mays*) grown at 14ºC show low *A* and $\phi P$ values compared to plants growth at 25ºC when measured at temperatures between 5ºC and 38ºC (Naidu *et al.*, 2003; Naidu & Long, 2004). A similar response was observed for *Atriplex lentiformis* (Pearcy, 1977) and *Tidestromia oblongifolia* (Berry & Björkman, 1980; Björkman *et al.*, 1980). This shows the low capacity of $C_4$ plants to adjust to low temperatures. In fact, Yamori *et al.* (2014) observed that in general $C_4$ plants grown at different temperatures have a narrower variation of thermal optimum than $C_3$ plants (33º-39ºC vs 16º-36ºC).

In the present study, differences in carboxylation efficiency (CE) were not observed between upland and lowland sugarcane grown at the two different temperatures. The CE is given by the initial slope of the *A* vs $C_i$ curve, at very low intercellular $CO_2$ pressure, where PEP carboxylation is considered to be limiting photosynthesis (von Caemmerer, 2000; Sage *et al.*, 2014). The steep decline in the CE observed between 25ºC and 15ºC indicates that PPDK might be limiting the carboxylation of PEP and thus photosynthesis at the lower temperature and low $CO_2$ levels (Table 5.1; Figure 5.4) (von Caemmerer, 2000; Friesen *et al.*, 2014). It has been shown *in vitro* PPDK had a steep reduction in activity below 20ºC while this reduction was much lower in PEPCase in chilling tolerant and chilling sensitive sugarcane (Du *et al.*, 1999a). At low temperature $C_4$ photosynthesis is believed to be limited either by PPDK or by Rubisco. In general, warm adapted $C_4$ plants are observed to have PPDK with low lability and/or low activity under low temperature, just like what observed here (Sugiyama & Boku, 1976; Long, 1983, 1999; Du *et al.*, 1999a; Sage & McKown, 2006). However, cold adapted $C_4$ plants can overcome this limitation either by producing more stable isoforms of PPDK, like *Bouteloua gracilis* (Pittermann & Sage, 2000), or by producing large amounts of PPDK, as in *Miscanthus x giganteus* (Naidu & Long, 2004; Wang *et al.*, 2008b).

In cold adapted plants, at suboptimal temperatures, Rubisco imposes a strong limitation and low acclimation potential to cold temperatures was observed on this enzyme (Sage & McKown, 2006). It was observed small changes in the Rubisco capacity in warm and cold grown cold adapted species such as *Muhlenbergia montana* (Pittermann & Sage, 2001), *Muhlenbergia glomerata* (Kubien & Sage, 2004b), *Miscanthus x giganteus* (Naidu & Long, 2004; Wang *et al.*, 2008a). Also, *Spartina anglica* showed little changes in the Rubisco capacity after plants grown at 27ºC were transferred for one week to 20ºC and then for two weeks to 10ºC (Matsuba *et al.*, 1997).

One of the indications of Rubisco limitation at low temperature is that $CO_2$ leakage from the bundle sheath increases, which can be estimated by the increase of the ratio $\phi P:\phi CO_2$ (Oberhuber & Edwards, 1993; Pittermann & Sage, 2001; Kubien *et al.*, 2003). At low temperature, Rubisco is not able to maintain a high *A*, which lowers the quantum yield of $CO_2$ assimilation ($\phi CO_2$). Thus, if Rubisco is unable to assimilate $CO_2$ at the same rate that $CO_2$ is pumped to the bundle sheath the $CO_2$ pressure in the bundle sheath increases, resulting in increased leakage of $CO_2$. Enzymes of the $C_4$ cycle are believed to maintain a relatively high rate of $CO_2$ transport to the bundle sheath, functioning as a sink for cyclic electron transport, and keeping the light irradiated quantum yield of photochemistry ($\phi P$) elevated (von Caemmerer, 2000; Kubien *et al.*, 2003). As a result $\phi P:\phi CO_2$ should rise at low temperature, which was not observed here. This indicates that Rubisco has either low control over *A* in sugarcane, or its relative degree of control is inconsistent (Du *et al.*, 1999a, 1999b).

An increase in the $\phi P:\phi CO_2$ ratio results an increase in the slope of the $\phi P$ vs $\phi CO_2$ regression (Edwards & Walker, 1983; von Caemmerer & Furbank, 1999). In theory, if all $CO_2$ pumped to the bundle sheath by the $C_4$ cycle is fixed by the $C_3$ cycle (no $CO_2$ loss), 5 ATP is required for each $CO_2$ fixed by NADP-ME $C_4$ plants. Assuming that 2 quanta are required for the formation of each ATP, the minimal ratio $\phi P:\phi CO_2$ should be 10 (von Caemmerer & Furbank, 1999). Due to $CO_2$ leakage from the bundle sheath or other alternative sinks of electrons, high energy loss generally occur below 20ºC in $C_4$plants, causing an increase in the $\phi P:\phi CO_2$ ratio, that can reach 20-25 (Sage & Kubien, 2003; Kubien & Sage, 2004b; Sage *et al.*, 2011). Interestingly, both sugarcane varieties studied here exhibited a $\phi P:\phi CO_2$ ratio of 12 even at 15ºC. In the cold adapted $C_4$ plants *Muhlenbergia glomerata*, *Miscanthus x giganteus* and *Cyperus longus*, the $\phi P:\phi CO_2$ ratio had a smaller influence of temperature in warm grown plants than in cold grown

plants (Kubien & Sage, 2004b; Farage *et al.*, 2006). Maize, which is a warm adapted $C_4$, shows a steeper slope for the relationship $\phi P$ vs $\phi CO_2$ than what was observed here, indicating that this plant has a higher energetic demand for $CO_2$ assimilation (Fryer, 1998). As observed here, growth at 25ºC and 35ºC resulted in no change in $\phi P:\phi CO_2$ in *Flaveria bidentis* which also had $\phi P:\phi CO_2$ ratio near 12 (Dwyer *et al.*, 2007). However, Kubien *et al.* (2003) showed evidence of $CO_2$ leakage from the bundle sheath in *Flaveria bidentis*. The findings that sugarcane does not show indication of $CO_2$ leakage from the bundle sheath, while *Flaveria* does, is well supported by the fact that the bundle sheath in most grasses has its outer lamellae suberized, what imposes a barrier to the leakage of $CO_2$, while in $C_4$ dicots this suberin is absent (Hattersley & Browning, 1981; Mertz & Brutnell, 2014).

The photosynthetic rate of plants grown at 21ºC and at 32ºC showed a significant difference when measured at 20ºC before and after exposure to 45ºC for a short period of time (around 15 minutes; $p < 0.05$). Above the thermal optimum, *A* is believed to be limited by Rubisco activase (Crafts-Brandner & Salvucci, 2002) or electron transport rate (Sage & Kubien, 2007; Sage *et al.*, 2011). The inability to maintain the same *A* measured at 20ºC before the brief exposure to 45ºC is a sign of injury on the system. It has been suggested that at high temperatures Rubisco activase denaturates resulting in reduction in *A* (Crafts-Brandner & Salvucci, 2002; Salvucci & Crafts-Brandner, 2004). On the other hand, high temperature can cause injury to the thylakoid membrane impairing the pH gradient build up through this membrane as well as interrupting the electron flow through the photosystems (Berry & Björkman, 1980; Sage & Kubien, 2007). It was shown for some plants that high temperature alters the fluidity of the thylakoid membrane such that protons may leak out of the lumen, impairing ATP production (Havaux, 1996; Bukhov *et al.*, 1999, 2000). In response, it was observed that plants grown at high temperatures showed more heat stable

thylakoid membrane (Terzaghi *et al.*, 1989; Gombos *et al.*, 1994). Although it has not yet been studied in $C_4$ plants, the better performance of *A* after exposure to 45ºC in leaves grown at 32ºC compared to leaves grown at 21ºC indicates either Rubisco activase or the electron transport chain can acclimate, maintaining its stability at higher temperatures.

Sugarcane is the most productive crop globally (Moore *et al.*, 2014) and one of the most important bioenergy crops in the world (Chum *et al.*, 2014a, 2014b). It is possible to further increase the productivity of this crop if this plant can rapidly acclimate to the changes in temperature, which can be chronic or seasonal. In this study I show that upland and lowland Hawaiian sugarcane did not show any difference in photosynthesis acclimation when grown at moderate temperatures. However, this study shows that high temperature causes damage to the photosynthetic apparatus, possibly due to injuries in the thylakoid membrane or on Rubisco activase. Sugarcane showed ability to acclimate to high temperatures decreasing the levels of these injuries caused by heat stress.

# Chapter 6 - Conclusion

In this thesis, I studied the physiological aspects of a bioenergy crop being considered for cool climates. The need for sustainability, reducing dependence on oil, and the necessity to avoid climate change have led society to consider the merits of bioenergy. Here, I studied two of the leading bioenergy crops: *Miscanthus* and sugarcane. Sugarcane is a well-established crop in Brazil, but it has low productivity in temperate regions (Inman-Bamber, 2014). Thus, acclimation to cooler climates is essential for the geographic expansion of this crop. *Miscanthus x giganteus* exhibits high yields in Midwest USA, Germany, Italy, Austria and Portugal (Vyn *et al.*, 2012). However, the northern range for this crop is still unknown. The objectives of this thesis were to investigate the freezing tolerance of *Miscanthus* rhizomes and the tolerance of *Miscanthus* shoots to episodic chilling events. Also, I investigated the acclimation of photosynthesis in lowland and upland sugarcane to moderate temperatures.

*Development of methods to test low temperature tolerance in rhizomes*

Before investing in a *Miscanthus* crop in cool climate regions, it is important to investigate the lowest temperature that the available genotypes can survive. The capacity of *Miscanthus* to overwinter and re-sprout in the following spring depends on the survivability of the rhizomes. To evaluate the lowest temperature at which *Miscanthus* rhizomes can survive, I first performed a series of trials to develop a reliable protocol. In these trials, I examined the temperature that ice nucleation occurs, and the best cooling rate and incubation time at the treatment temperature. I also improved the electrolyte leakage method to facilitate fast screening of freezing injury in large samples size. Finally, two freezing procedures were evaluated, one that tests the in situ low

temperature tolerance of the rhizomes, and another that allows for further low temperature acclimation, by decreasing the temperature in stages, allowing for incubation at each temperature. In this method, I observed that the *Miscanthus* diploid line M115 had a greater potential to acclimate compared to the triploid *Miscanthus x giganteus* Illinois. Lowering temperature in stages appears to be more realistic because the soil temperature is well buffered and should not suffer rapid declines. Also, I show that diploid *Miscanthus* rhizomes are not vulnerable if there is a warm event followinf a fast reduction in temperature, which is common in the Canadian winter.

*Winter cold tolerance in field grown* Miscanthus *hybrids rhizomes*

After the cooling methods were developed and tested, I studied the thermal ranges that diploid, triploid and tetraploid *Miscanthus* lines can tolerate. Here, I confirmed that rhizomes of diploid *Miscanthus* hybrids have a superior ability to acclimate to sub-zero temperature relative to allopolyploid hybrids. It was estimated that before sub-zero acclimation occurs, rhizomes of all hybrids are killed at temperatures below -6.5ºC. However, when sub-zero acclimation occurs, the diploid rhizomes can tolerate temperatures as low as -14ºC, while allopolyploids showed no significant changes in rhizome low temperature tolerance. These cold tolerance thresholds indicate diploid lines maintain mechanisms allowing for survival to severe winter cold at higher latitudes. By examining the soil temperature across Canada provided by Environment Canada, I estimated that diploid *Miscanthus* lines have the potential to be grown up to 60ºN latitude.

*Photoinactivation and the effects of episodic chilling events in* Miscanthus

Chilling events are common in the Canadian spring, and can impair the light harvesting system in developing canopy of the newly emerged plant, causing loss of energy and nutrients

invested in the leaf, and delaying the formation of an efficient canopy to capture solar irradiation. Chilling tolerance in *Miscanthus* was previously tested in temperature-controlled chambers (Farrell *et al.*, 2006; Friesen *et al.*, 2014), but light levels in these chambers are low (500 µmol of photons $m^{-2}$ $s^{-1}$), compared to sunlight (> 1300 µmol of photons $m^{-2}$ $s^{-1}$). In Chapter 4, I studied the development of leaves and the effects of chilling episode in 9 *Miscanthus* hybrids, three *M. sinensis* lines, and two switchgrass (*Panicum virgatum*) lines in the field. A cold front lowered air temperature to a minimum of -2ºC, and frost formed on the leaves. Following the overnight frost, exposure to sunshine with air temperature no higher than 12ºC resulted in severe injury in the leaves of all *Miscanthus* hybrids and switchgrasses. Only leaves of *Miscanthus sinensis* were alive by the end of the day, showing positive photosynthetic rate and a fair value of post irradiance quantum yield of photochemistry. In the following days, air temperature gradually increased to a mean air temperature of 14ºC one week later. By this time, all plants had sprouted new leaves that already had predawn quantum yield of photochemistry (Fv/Fm) around 0.34, with the maximum values for the triploids Nagara and Illinois above 0.45. Most genotypes already recovered a photosynthetically competent canopy. Two weeks after the frost event, when air temperatures were between 21ºC and 29ºC, all genotypes showed maximum quantum yield of photochemistry and photosynthetic rate, showing rapid recovery potential. All *Miscanthus* hybrids had a short but closed canopy, while *M. sinensis* and the switchgrasses had a relatively poor canopy. At non-stressful temperatures, the triploid *Miscanthus x giganteus* Illinois had consistently high predawn quantum yield of photochemistry. However, when exposed to sunlight this plant had the highest photosynthetic rates but also the highest photoinactivation of the PSII reaction centers. In contrast, *M. sinensis* showed better frost protection, but poor growth capacity compared to the other

genotypes. Although *Miscanthus* hybrids showed high sensitivity to chilling events on days with clear skies, *M. sinensis* demonstrated the potential the genus has to increase chilling tolerance.

*Comparative acclimation of photosynthesis in lowland and upland sugarcane*

Sugarcane has been the focus of the bioenergy industry for many years. In Brazil, this plant achieved the highest productivity in the world. There has been much interest in extending the ranges this crop can be cultivated. Thus, it is important to test the capacity for adaptation and acclimation of this plant. Here, I used an upland and a lowland lines of sugarcane that are grown in Hawaii. Plants from these genotypes were grown at two different moderate temperatures: 21º/18ºC and 32º/26ºC day/night temperature. Although it was expected that upland sugarcane would perform better at low temperatures, no difference in photosynthetic performance was observed between this and the lowland line. Also, growth at the two temperatures showed no difference in the photosynthetic performance. However, after exposed to 45ºC, the plants showed a decline in photosynthetic rate at cooler temperatures and the plants grown at 21ºC were more affected and could not recover the photosynthetic rate at 20ºC after exposed to 45ºC. That indicates that lesions either in the thylakoid membrane or on the stability of Rubisco activase could be limiting photosynthesis following exposure to the highest temperatures.

*Perspectives in the use of Miscanthus in Ontario, Canada*

The use of bioenergy crops in marginal lands reduces competition between these crops and food production, loss of biodiversity and other priorities for farmland use (Tilman *et al.*, 2006; Fargione *et al.*, 2008; Searchinger *et al.*, 2009). In Ontario, it is estimated that 25% of existing marginal lands will be needed if *Miscanthus* and switchgrass were to substitute for the use of coal in the

production of electricity in the province (Kludze *et al.*, 2013). Even if using productive lands to grow a biofuel crop, the same energetic yields as corn can be achieved with *Miscanthus* using half the land invested in corn, decreasing environmental impact and minimizing the impact on food production (Kim & Dale, 2011; Kludze *et al.*, 2013).

The use of land in Ontario and in the rest of Canada for bioenergy crops will depend on the crops survivability and productivity. Here, I show that *Miscanthus* rhizomes can survive to temperatures as low as -14ºC while the more productive triploid hybrids just survive to -6.5ºC. Even though that indicates that *Miscanthus* can be grown in regions up to 60ºN, it is also important to consider the frequency of episodic chilling events in the region because of the sensitivity of *Miscanthus* leaves to temperatures below 10ºC under high sunlight. In conclusion, from the bioenergy crops currently considered for cold climates, *Miscanthus* is the most productive and should be used in the regions where the climate permits its high yield. The *Miscanthus* lines currently available will likely be restricted to southern Canada, but here I demonstrate the potential in the *Miscanthus* genepool to increase the low temperature tolerance for the genotypes with high photosynthetic rates and quantum efficiency. Meanwhile, other less productive crops, but more cold tolerant, such as upland switchgrass and *Spartina* can be considered for regions beyond *Miscanthus* reach (Lee *et al.*, 2014).

*Perspectives in the use of sugarcane*

Sugarcane is the most productive bioenergy crop in the world and in Brazil, it reaches its maximum productivity. Currently, the production of ethanol from sugarcane exclusively comes from sucrose, which wastes two thirds of the bioethanol potential of the plant's biomass (lignocellulose). New technologies have been implemented to produce ethanol out of this biomass that is wasted (Soccol

*et al.*, 2010). Because the conversion rate of lignocellulose to ethanol is more efficient than glucose, breeding programs have been aiming for the production of "energy cane", which is a type of sugarcane that invest in the production of cellulose and hemicellulose (Jones, 2011; Lee *et al.*, 2014).

Because of the good results achieved by sugarcane on tropical and subtropical regions, there is an interest in expanding the reaches of this crop to temperate zones. By using the lowland and upland Hawaiian sugarcane as an example, I showed little variation on thermal responses and acclimation potential on both lines. Thus, the use of sugarcane in temperate zones is still risky. As a solution, breeding sugarcane with *Miscanthus* is an alternative to increase low temperature tolerance and photosynthetic rates at low temperature. That is already been made, resulting in a hybrid named "Miscane". For this hybrid, it is also important to inherit the perennial trait of *Miscanthus*, in order to avoid the winter.

In conclusion, there is much potential to grow bioenergy grasses in cooler climate zones where they are currently limited by cold tolerance (*Miscanthus*) or by productivity (switchgrass and *Spartina*). By utilizing efficient and fast screening methods, such as those improved upon here, it will be possible to screen many lines of candidate $C_4$ perennials for good cold tolerance and high productivity. By doing so, plant scientists should provide new product for agricultural sector, one which will contribute to the reduction in $CO_2$ emissions and climate change.

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