Sub-zero cold tolerance of *Spartina pectinata* (prairie cordgrass) and *Miscanthus × giganteus*: candidate bioenergy crops for cool temperate climates

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Abstract

*Miscanthus × giganteus* grown in cool temperate regions of North America and Europe can exhibit severe mortality in the year after planting, and poor frost tolerance of leaves. *Spartina pectinata* (prairie cordgrass), a productive C₄ perennial grass native to North America, has been suggested as an alternative biofuel feedstock for colder regions; however, its cold tolerance relative to *M. × giganteus* is uncertain. Here, we compare the cold tolerance thresholds for winter-dormant rhizomes and spring/summer leaves of *M. × giganteus* and three accessions of *S. pectinata*. All genotypes were planted at a field site in Ontario, Canada. In November and February, the temperatures corresponding to 50% rhizome mortality (LT₅₀) were near −24°C for *S. pectinata* and −4°C for *M. × giganteus*. In late April, the LT₅₀ of rhizomes rose to −10°C for *S. pectinata* but remained near −4°C for *M. × giganteus*. Twenty percent of the *M. × giganteus* rhizomes collected in late April were dead while *S. pectinata* rhizomes showed no signs of winter injury. Photosynthesis and electrolyte leakage measurements in spring and summer demonstrate that *S. pectinata* leaves have greater frost tolerance in the field. For example, *S. pectinata* leaves remained viable above −9°C while the mortality threshold was near −5°C for *M. × giganteus*. These results indicate *M. × giganteus* will be unsuitable for production in continental interiors of cool-temperate climate zones unless freezing and frost tolerance are improved. By contrast, *S. pectinata* has the freezing and frost tolerance required for a higher-latitude bioenergy crop.

Keywords: Establishment success, leaf frost tolerance, *Miscanthus × giganteus*, perennial C₄ grasses, prairie cordgrass (*Spartina pectinata*), rhizome freezing tolerance.

Introduction

*Miscanthus × giganteus* Greef & Deuter ex Hodkinson & Renvoize is one of the most productive C₄ perennial grasses in temperate zone climates, and is therefore a leading candidate for bioenergy production at higher latitudes (Lewandowski et al., 2000; Clifton-Brown et al., 2001b; Heaton et al., 2008). The high productivity of *M. × giganteus* in temperate regions, however, depends on its ability to establish and successfully survive in climates where low winter and spring temperatures can damage or kill maladapted plants (Christian and Haase, 2001). Because *Miscanthus* cultivars are derived from material originally collected from eastern Asia (Heaton et al., 2010; Clifton-Brown et al., 2013; Sacks et al., 2013), it is not certain that they will survive low temperatures common in northern North America and Europe where winter and spring cold may be more intense than in their native range. Field trials in Germany, Denmark, and southern Ontario, Canada,
often find high mortality of *M. × giganteus* plants in the first winter after planting (Clifton-Brown et al., 2001a; Jørgensen et al., 2003; Deen et al., 2011). Poor overwinter survival of *M. × giganteus* may be explained by a modest thermal tolerance threshold for rhizomes near −4°C (Clifton-Brown and Lewandowski, 2000). Selected varieties of *M. × giganteus* parent species have slightly colder tolerance thresholds for rhizomes, with the lowest being near −6°C for *M. sinensis* (Clifton-Brown and Lewandowski, 2000). Although cultivation practices can mitigate winterkill, this limited degree of rhizome cold tolerance may restrict the use of *Miscanthus* to regions with mild winters and a low chance of hard spring frosts, for example, in the southern and central USA. Much of the northern USA, Canada and northern Eurasia may be unsuitable for *Miscanthus* cultivation if cold tolerance thresholds are universally above −6°C within the *Miscanthus* gene pool (Clifton-Brown and Lewandowski, 2000; Christian and Haase, 2001). This could be a problem for the bioenergy sector, as the high productive potential represented by *Miscanthus* would be unavailable where there is both an abundance of land and a high demand for heat. If so, other C₄ grass species may be needed to replace *Miscanthus* as the leading C₄ biofuel feedstocks at higher latitudes. One promising candidate is *Spartina pectinata* Link (prairie cordgrass).

*Spartina* is a genus of perennial C₄ grasses that may be a New World parallel to *Miscanthus* as it contains productive species whose distributions extend to high latitudes (Long and Spence, 2013). *Spartina pectinata* in particular has attracted interest as it grows up to 61°N in its native range in North America; however, its cold tolerance limits remain uncertain. Field trials in eastern South Dakota, USA, and near Essex, UK, show *S. pectinata* can yield an average of 12 t ha⁻¹ yr⁻¹ biomass above ground within 6–10 years of establishment (Potter et al., 1995; Boe et al., 2009). This is up to 50% greater than the most productive C₃ perennial grasses at similar latitudes such as reed canary grass (*Phalaris arundinacea* L.) (Lewandowski et al., 2003). On drier, marginal uplands, it achieves yields comparable to switchgrass (*Panicum virgatum* L.), but unlike switchgrass and *M. × giganteus* it also grows well on low-lying waterlogged soils and riparian land in eastern Canada and the American upper midwest (Madakadze et al., 1998; Montemayor et al., 2008; Boe et al., 2009; Gonzalez-Hernandez et al., 2009; Pyter et al., 2009). It is found throughout much of continental North America from Texas, USA, to the Northwest Territories, Canada, which represents the most northerly distribution of any candidate *C₄* biomass species (Porsild and Cody, 1980; Schwarz and Redmann, 1988). The closely related *S. gracilis* Trin. (alkali cordgrass) has a similar northern distribution as *S. pectinata* and shows ≥ 70% survival down to −29°C (Baumel et al., 2002; Schwarz and Reaney, 1989). Another related *C₄* species, *Zoysia japonica* Steud., shows temperatures corresponding to 50% rhizome mortality (LT₅₀) of −12°C to −18°C in the winter acclimated state, with significant differences between geographically disparate genotypes (Dunn et al., 1999; Rogers et al., 1975). These patterns indicate *S. pectinata* could have superior cold tolerance of overwintering rhizomes relative to *Miscanthus* genotypes, yet still maintain the high productive potential expected of candidate bioenergy feedstocks (Boe et al., 2013).

In regions with periodic spring frosts, cold tolerance of leaves is also critical for establishment and productivity of *C₄* perennial grass crops. If such tolerance is lacking, episodic chilling or frost events during the early part of the growing season could kill developing canopies, as has been observed in *Miscanthus* plantations in southern Canada (Friesen et al., 2014). In Ireland, *Miscanthus × giganteus* trials failed to re-sprout after late spring frosts killed the young canopy (Christian and Haase, 2001). Despite its superior tolerance and growth potential under chilling temperatures (7.5°C to 12°C), *M. × giganteus* leaves show weak leaf freezing tolerance compared to other varieties of *Miscanthus* (Clifton-Brown and Jones, 1997; Farrell et al., 2006; Friesen et al., 2014; Zub et al., 2012). Even if cultivars have cold tolerance, delay in the onset of canopy development, or alternatively, improper senescence, could hinder yields, for example, by preventing the exploitation of the long photoperiods of mid-to-late spring. An important requirement for the success of a bioenergy crop is the ability to convert as much sunshine as possible into biomass, but this will require tolerance of early season frost and chilling episodes that are inevitable in northern climates. In the case of *S. pectinata*, freezing tolerance of leaves is probably well developed as dormant shoots are present above the soil surface from late autumn until growth begins in early-to-mid spring (Boe et al., 2009).

Because of its success in northern latitudes, we hypothesize that rhizomes and leaves of *S. pectinata* have greater cold tolerance compared to *M. × giganteus* during the autumn, winter, and spring following planting. Here, we evaluate the rhizome LT₅₀ and degree of winterkill in rhizomes of *M. × giganteus* and *S. pectinata* grown outdoors in a common garden during the first year following planting. We evaluate whether differences in rhizome freezing tolerance are present between three populations of *S. pectinata* from midwestern and maritime North America. To evaluate cold tolerance of rhizomes collected in the autumn, winter, and spring, we used the electrolyte leakage method and re-growth assays following controlled exposure to a range of subzero temperatures. These tests assessed seasonal changes in cold hardness as well as maximum hardness levels. We also evaluated cold tolerance of field-grown leaves in the early spring by measuring photosynthesis and chlorophyll fluorescence the day after a mild frost event. Freezing tolerance of leaves was also evaluated with electrolyte leakage after controlled exposure to subzero temperatures. Finally, we evaluated shoot emergence dates and canopy height during the spring to compare early season growth of *M. × giganteus* and the three populations of *S. pectinata*.

### Materials and methods

**Plant material and field plots**

*Miscanthus × giganteus* (M161) rhizomes were extracted from a one-year-old unfertilized plot adjacent to our field site on 1 May 2013. This variety of *M. × giganteus* is the research standard at the University of Illinois and is an accession from the Chicago campus.
Botanical Gardens (Heaton et al., 2010). Spartina pectinata ‘Red River’ is an octaploid cultivar originating from an open pollination cross among populations collected from east-central Minnesota, northeastern South Dakota, and east-central North Dakota (Boe et al., 2009; Kim et al., 2010). All plantlets originated from plants grown by Millborn Seeds Inc. (Brookings, South Dakota, USA <http://www.millbornseeds.com/> and were donated by Professor D.K. Lee (Department of Crop Sciences, University of Illinois at Urbana-Champaign). Spartina pectinata ‘IL-102’ is a tetraploid cultivar collected from a natural population near Savoy, Illinois, USA (Kim et al., 2010). Spartina pectinata ‘Summerford’ was collected by the authors near Summerford, Newfoundland, Canada (49°28′3.3″ N, 54°44′50.8″ W). This is near the northern edge of the S. pectinata range in eastern North America (Supplementary Fig. S1). Plantlets were established as plugs that were then planted into a field plot at the University of Guelph Agricultural Research Station near Elora, Ontario, Canada (43.64°N, 80.40°W) on 21 June 2013 (see Friesen 2015 for details of the field plantation). Plants consisted of four blocks with 12 plants per genotype in each block. Plants of each genotype were planted in parallel rows within a block at 1 m intervals and 3 m spacing between blocks (Supplementary Fig. S2). Soil temperature at 2 cm and 8 cm depth in the plots was monitored using thermistors and Hobo Data Loggers (Onset Computer Corporation, http://www.onsetcomp.com) (see Supplementary Fig. S2 for location). Air temperature, snow depth, wind speed, and relative humidity were measured at an Environment Canada (2015) weather station 350 m from the plot.

Rhizome harvesting and freezing treatments

Twelve plants of each genotype (three per block) were extracted from the soil for rhizome cold treatments on 21 November 2013 (the autumn trial), 2 February 2014 (the winter trial), and 28 April 2014 (the spring trial). Pairs of rhizomes (including erect tillers for S. pectinata) from each plant were sorted into trays (S. pectinata) or 0.5 l plug containers (M. × giganteus), one for each temperature treatment. The soil in the trays and plugs was either soil from the field site (autumn trial), or a mixture of 40% triple mix (topsoil, sand, and compost blend), 40% coarse sand, and 20% ProMix (Premier Tech, Quebec, Canada) (winter and spring trials).

Two Thermotron S-16-8200 temperature test chambers (Thermotron Industries, http://www.thermotron.com) provided the treatments. For the autumn and spring, rhizomes were first cooled to 0.5°C from the control temperature (2°C for autumn and 4°C spring) for 2 h, then cooled to the treatment temperatures; for the winter trials, rhizomes were first placed in the chambers at 0°C. Rhizomes were cooled at 1°C h⁻¹ to the treatment temperature, held there for four hours, and then warmed to 0°C at 1°C h⁻¹. The cooling rate of 1°C h⁻¹ is a recommended cooling rate for determining the LT₅₀ of rhizomes, as it is slow enough to allow for tissue adjustments during ice crystal formation, and also to allow for thermal equilibrium between the chamber and rhizomes (Schimming and Messersmith, 1988; Peixoto et al., 2015 this issue). Rhizome, soil, and air temperature during the trials were monitored with thermocouples attached to Veritech dataloggers (Vaisala Inc., http://www.vaisala.com). Temperature treatments were −2.5°C, −6°C, −14°C, −19°C, and −29°C for all genotypes for the autumn trial, with controls held at 2°C. For the winter trial, treatment temperatures were −2.5°C, −6°C, and −14°C for M. × giganteus and −14°C, −19°C, −24°C, −29°C, −34°C, and −39°C for S. pectinata, with controls held at 0 to −1°C. Cold treatments for the spring were −2.5°C and −6°C for M. × giganteus and −6°C, −14°C, −19°C, −24°C, and −29°C for S. pectinata, with controls held at 4°C. For the winter trial, colder temperatures were selected for S. pectinata to explore the possibility that it could acclimate and survive below −30°C. The order of freezing treatments was randomized to minimize the effects of time. All rhizomes were stored with the controls prior to treatment for treatment for each trial. Rhizomes were stored in temperature-controlled plant growth chambers (Percival Scientific, http://www.percival-scientific.com); Conviron, http://www.conviron.com) in the dark at −1° to 4°C, sorted in trays/plugs in moist soil (see online supplement Supplementary Appendix S1 for specifics). For the autumn trial, all freezing treatments were completed nine days after harvest. In the winter, treatments were completed 11 days after harvest, and for the spring, eight days after harvest.

After each cold treatment, one rhizome from each pair was removed from the soil and prepared for electrolyte leakage assays by removing leaf bud scales around the rhizome tip. The rhizome was then cut 1.5 cm below the tip and the piece briefly rinsed in ddH₂O before being immersed in 10 ml ddH₂O (S. pectinata) or 15 ml ddH₂O (M. × giganteus) at room temperature (20°C). Initial electrolyte leakage (EL₁₀₀) was measured as the electrical conductivity of the solution after rhizome pieces were allowed to infuse for 24 h without agitation. Agitation did not alter the electrolyte leakage pattern (Supplementary Fig. S3). Before sampling for electrical conductivity measurements, vials were vigorously shaken by hand. Electrical conductivity was measured with a conductivity meter (Ultrameter II 4P, Myron L Company, http://www.myronl.com). Total electrolyte leakage (TEL) was measured as the electrical conductivity after vials were boiled for 2 h and cooled to room temperature. Percent relative conductivity (%RC) from freezing treatments was calculated as:

\[ \%RC = \frac{I_{EL}}{T_{EL}} \times 100\% \]

As a second assessment of rhizome viability, the second rhizome of the treated pair was allowed to sprout in a greenhouse at 25−27°C day/17−19°C night under supplemental lights which provided at least 200 μmol photons m⁻² s⁻¹ to the rhizome trays (see Friesen, 2015 for detailed methods). Rhizomes were assessed for sprouting 14−16 d after being moved into the greenhouse. Sprouting was recorded as an actively growing shoot or root from the rhizome. Rhizomes that failed to sprout typically showed signs of deterioration such as discoloration and rot, allowing us to confidently score them as dead.

To assess mortality of rhizomes at the end of the winter season, all of the unused rhizomes from spring-harvested plants on 28 April were inspected for signs of injury and death. All S. pectinata rhizomes were healthy, while numerous M. × giganteus rhizomes were discoloured and showed signs of rot. To assess the viability of the M. × giganteus rhizomes, the entire set was assessed for re-growth in the greenhouse as described above.

Cold tolerance of leaves

On 29 May and 25 June 2014, leaves were harvested from the remaining plants in the field to determine their chilling and subzero cold tolerance. The youngest fully expanded leaves were sampled, and stored in moist paper towel at 4°C until assay. Leaves were stored for 3−18 h before assay. For assay, 8 cm segments were placed into closed petri plates lined with wet filter paper and treated in either an S-16-8200 or S-1.2C-B Thermotron test chamber. Leaves were first brought to 0.5°C, cooled at 1.5°C h⁻¹ to the treatment temperature, held there for 2 h, and then warmed to 0°C at 1.5°C h⁻¹. This cooling rate mimics observed air cooling rates at the field site in the days preceding the harvests (Environment Canada, 2015). Treatment leaf temperatures were 0°C, −1°C, −4°C, −7°C, −10°C and −14°C. After treatment, 1 cm² of leaf was incubated for 24 h at 20°C in 5 ml ddH₂O to allow for electrolyte leakage. Percent relative conductivity (%RC) was then measured as described above for rhizomes with TEL determined after boiling for 45 min, followed by cooling of the extract to room temperature.

Canopy measurements

At each rhizome harvest date, samples of senesced leaves were collected from the middle of upper canopy prior to extracting rhizomes from the soil. The leaf samples were dried at 60°C for four days, pulverized and the resulting powder analysed for total % nitrogen.
Effects of a mild overnight frost, 16–17 May 2014

Photosynthesis rates and pulse-modulated fluorescence were measured on plants in the field on the morning of 17 May after a mild overnight frost. Upon arrival at the field site at 5:40 am, leaf temperature was immediately measured with a type-T thermocouple and LI-1000 datalogger (Licor Biosciences, http://www.licor.com) just before sunrise. From 9:30 am to 3:30 pm, net CO₂ assimilation rate ($A$, µmol CO₂ m⁻² s⁻¹) of young, fully expanded leaves was measured at ambient CO₂ (400 µmol mol⁻¹) at light levels from 0–1800 µmol m⁻² s⁻¹ photons (photosynthetic photon flux density, PPFD). Gas exchange and chlorophyll a fluorescence measurements were made with the LI-6400XT photosynthesis system and LI-6400 Leaf Chamber Fluorometer (Licor Biosciences). The maximum quantum yield of linear electron transport through photosystem II ($Φ_2$) and the realized yield under light ($Φ_P$) were measured using a multiphase flash in the dark and at each light intensity as the ratio of variable to maximal fluorescence [$Fv/Fm = Fv/Fm′ = Fv/Fm′_∞$ (Genty et al., 1989)]. To determine incident $Φ_{0,2max}$ $A$ was measured at 0, 10, 20, 40, 60 and 80 PPFD. Next, $A$ was measured at 450, 750, 1000, 1250, 1500 and 1800 PPFD to complete the light response curve. The leaf to air vapour pressure difference (vpdL) was held at 0.7–0.9 kPa, and leaf temperature remained close to 11°C, near the day time high of 10.5°C. All measurements followed the Licor LI-6400XT manual (http://www.licor.com/env/support/index.html?n=6400).

Statistical analyses

To estimate the exposure temperature causing 50% mortality (LT₅₀) and the %RC that corresponds to 50% rhizome death (=lethal electrolyte leakage causing 50% mortality, or LEL₅₀), data were fitted to generalized linear models (glm) with a binomial distribution ([Quinn and Keough, 2002]). First, to test for spatial heterogeneity across the plot that could affect LT₅₀ or LEL₅₀ values, differences between blocks were tested with model intercept contrasts under the following model: re-growth ~ genotype + block + season + treatment temperature or %RC. No significant effects from any block were found and the entire data set was pooled for subsequent analyses. To test for differences between genotypes in their LT₅₀ and LEL₅₀ response across all sampling dates, and for differences between dates across all genotypes, glm models of the form: re-growth ~ genotype + season + treatment temperature or %RC were performed. Different genotypes and seasons were set as the model intercept to contrast each genotype and season. To estimate the LT₅₀ and LEL₅₀ for rhizomes, fitted values from the glm models were regressed with best-fit sigmoidal curves, and the x-value (temperature or %RC) at 50% survivability ($y=0.5$) was calculated. To calculate the temperature corresponding to 50% RC (TEL₅₀) for leaves, the raw data were fitted with a best-fit sigmoidal curve, and the temperature (x-value) at 50% RC ($y=0.5$) was calculated. The TEL₅₀ was chosen to compare leaf freezing tolerance between genotypes as it is reported by other authors for grasses (Rowley et al., 1975; Bykova and Sage, 2012), but may not necessarily represent the lethal temperature threshold for leaves. All generalized linear models were performed in R statistical software (R-Core-Team, 2013) and all logistic regressions were performed with SigmaPlot version 12 (http://www.systat.com/).

For the photosynthesis data, one-way ANOVAs found no significant differences between S. pectinata ecotypes, and data was pooled to compare against M. × giganteus. Differences in incident $Φ_{0,2max}$ were tested between S. pectinata and M. × giganteus by comparing slopes of linear regressions of $A$ and PPFD up to 80 PPFD following Zar (1996). Above this light intensity, $A$ and $Φ_P$ were compared with t-tests. Intergenotypic differences in canopy height and leaf nitrogen were tested across all three accessions of S. pectinata and M. × giganteus with Holm-Sidak post hoc tests following significant one-way ANOVAs. All ANOVAs and t-tests were performed in SPSS Statistics version 20 (http://www-01.ibm.com/software/analytics/spss/). For more information on the calculation of LT₅₀, LEL₅₀, and TEL₅₀ values and statistical treatment of photosynthesis and canopy height data see Supplementary Appendix S2.

Results

Air and soil temperature

Minimum daily air temperature at the field site fell below 0°C by the end of October 2013 and did not consistently rise above 0°C until the middle of April 2014 (Fig. 1A). Minimum air temperature was below −20°C for much of January and February 2014, and fell to seasonal minimums near −30°C on four dates in later January to February (Fig. 1A). Soil temperature followed the decline in air temperature from the beginning of September until 11 November, when temperatures at 2 cm depth first fell below 0°C (Fig. 1). Following a warm front on 5 December, a cold front reduced soil temperatures to −3°C at 2 cm depth and −1°C at 8 cm depth. On 7 December, strong winds up to 41 km h⁻¹ reduced soil temperatures to their coldest point of the autumn/winter season such that on the morning of 8 December, soil temperatures across the plot ranged from −0.5°C to −6.0°C at 2 cm depth to −0.1°C to −3.4°C at 8 cm depth. Snow accumulated shortly after this time, and stayed until approximately 4 April, with mid-winter accumulations of over 40 cm (Jordan Forsyth, Elora Weather Station Manager, personal communication). Despite periodic air temperatures that fell below −25°C, soil temperatures remained near zero throughout the period of snow cover and only warmed above 0°C following complete snow melt after 6 April (Fig. 1).

Rhizome freezing tolerance and overwintering capacity

Within each genotype, we observed no difference in the LT₅₀ or LEL₅₀ of rhizomes between the November (autumn) and February (winter) trials, allowing us to pool the results for these sampling periods (Table 1, Supplementary Table S1). All accessions of S. pectinata showed a lower LT₅₀ compared to M. × giganteus (Table 2). The LT₅₀ of rhizomes from the autumn/winter harvest of the three S. pectinata genotypes was −23°C to −24°C versus −4°C for M. × giganteus (Table 2, Fig. 2). The LT₅₀ for the 28 April spring harvest of the S. pectinata genotypes was −10°C, while it remained at −4°C for M. × giganteus (Table 2, Fig. 3). When harvested on 28 April, 20% of all rhizomes from M. × giganteus were dead; most of these were close to the soil surface in the rhizome cluster. There was no rhizome mortality in any of the three S. pectinata genotypes.

The response of rhizome electrolyte leakage to treatment temperature was markedly different between S. pectinata and M. × giganteus. In M. × giganteus, %RC rose sharply as treatment temperatures fell below −3°C, while in the three S. pectinata genotypes, %RC showed a gradual increase below −10°C during the autumn and winter, and below −6°C at the time
of the spring harvest (Fig. 4). Using our LT$_{50}$ values, we estimated the %RC value that corresponded to 50% mortality (LEL$_{50}$), and observed it was near 30% in all S. pectinata genotypes and near 47% for M. × giganteus in both the autumn/winter and spring trials (Supplementary Figs S4, S5). We then estimated the temperature that corresponded to these LEL$_{50}$ values to obtain an independent estimate of the lethal cold threshold for S. pectinata and M. × giganteus rhizomes. In the autumn for both species, and winter for S. pectinata, best-fit sigmoidal regressions corresponded well to the intersection of the LEL$_{50}$ values and the LT$_{50}$ values (Fig. 4A, B). This indicates that the sigmoidal fit was a good approximation of the threshold response. However, the intersect of the LEL$_{50}$ and LT$_{50}$ values did not correspond well to the sigmoidal fit for the winter response of M. × giganteus and the spring responses for both species (Fig. 4B, C). In these three cases, a straight line connecting the two data points bracketing the threshold portion of the response provided better correspondence with the intersect of the LEL$_{50}$ and LT$_{50}$ values. The range of temperatures corresponding to the LEL$_{50}$ estimated using best-fit regressions or straight lines were near −23°C for S. pectinata harvested in November and February, and between −7°C and −10°C for S. pectinata harvested in April. For M. × giganteus, the range of temperatures corresponding to the LEL$_{50}$ estimated using best-fit regressions or straight lines was −4°C to −6°C for M. × giganteus at all sample dates (Fig. 4B, C).

**Leaf cold tolerance, emergence and canopy growth**

Cold tolerance thresholds of leaves showed the same general pattern for both spring harvest dates. For the 29 May harvest, the temperature corresponding to 50% electrolyte leakage
Fig. 7A
throughout May (on 17 May (Fig. 6) -2 the three ‘Red River’ had fully flowered and were maturing seed, however S. pectinata ‘IL-102’ and M. × giganteus had still not flowered and showed no flag leaves. On 4 November, a hard overnight frost (−5°C overnight low) killed leaves of M. × giganteus while still green. In contrast, green leaves of S. pectinata ‘IL-102’ were still viable and healthy (Henk Wichers, University of Guelph, personal observation). Leaves of S. pectinata ‘Summerford’ and ‘Red River’ had already fully senesced by this time.

Senesced leaves of M. × giganteus had greater leaf nitrogen content compared to all genotypes of S. pectinata for both the autumn (21 November 2013) and winter (2 February 2014) harvests. Autumn leaf nitrogen content ranged from 0.96% in S. pectinata ‘Red River’ to 2.02% in M. × giganteus (Table 3). Spartina pectinata ‘Summerford’ showed significantly greater leaf nitrogen content than S. pectinata ‘Red River’ but S. pectinata ‘IL-102’ was not significantly different from either genotype (Table 3). Nitrogen content of senesced leaves on 28 April 2014 ranged from 0.96% in S. pectinata ‘IL-102’ to 1.43% in M. × giganteus (Table 3).

At the autumn harvest (21 November), all genotypes of S. pectinata had senesced their leaf canopies from the previous summer and produced spikes of scale-like leaves that emerged above the soil surface before entering dormancy. These spikes appeared dormant on 19 April of the following year (2014) but by 28 April, some were opening to reveal new green leaves. Shoots of M. × giganteus had not emerged on 28 April, and had just recently emerged by 12 May (Henk Wichers, personal communication). A linear regression of M. × giganteus canopy heights from 17 May and 29 May estimated 9 May to be the emergence date (not shown). Canopy heights were significantly greater for S. pectinata ‘IL-102’ and ‘Red River’ compared to M. × giganteus throughout May (Fig. 6). By 25 June, canopy heights of S. pectinata ‘IL-102’, ‘Red River’ and M. × giganteus were all close to 80cm with only S. pectinata ‘Summerford’ being significantly lower (Fig. 6). On 25 June, S. pectinata ‘Summerford’ had visible or emerging flower spikes, however none of the other genotypes showed signs of flowering. By 6 October, S. pectinata ‘Red River’ had finished maturing seed, whereas ‘IL-102’ and M. × giganteus showed flag leaves but no visible flowers.

**Photosynthesis after a mild overnight frost**

Air temperature fell to 0.3°C early in the morning of 17 May, following 24h of air temperatures <10°C (Fig. 1A). Just before dawn on 17 May, frost was visible on leaves of both S. pectinata and M. × giganteus with leaf temperatures between 0°C and 1°C. Leaves of M. × giganteus were visibly yellow and chlorotic compared to S. pectinata, whose leaves looked healthy and similar in appearance to those later in the spring. At leaf temperatures between 9°C and 12°C, S. pectinata leaves had a greater incident ΦCO₂max of 0.051 compared to 0.026 for M. × giganteus on 17 May (Fig. 7A, inset). At every light intensity above 40 µmol m² s⁻¹, S. pectinata had a significantly higher A compared to M. × giganteus and was almost three times higher at 1800 µmol m² s⁻¹ (Fig. 7A). Photosystem II operating efficiency (Φp) was also significantly higher in S. pectinata compared to M. × giganteus.

**Growth phenology, emergence dates, and canopy height**

On 8 September 2013 both S. pectinata ‘Summerford’ and ‘Red River’ had fully flowered and were maturing seed, however S. pectinata ‘IL-102’ and M. × giganteus had still not flowered and showed no flag leaves. On 4 November, a hard overnight frost (−5°C overnight low) killed leaves of M. × giganteus while still green. In contrast, green leaves of S. pectinata ‘IL-102’ were still viable and healthy (Henk Wichers, University of Guelph, personal observation). Leaves of S. pectinata ‘Summerford’ and ‘Red River’ had already fully senesced by this time.

Senesced leaves of M. × giganteus had greater leaf nitrogen content compared to all genotypes of S. pectinata for both the autumn (21 November 2013) and winter (2 February 2014) harvests. Autumn leaf nitrogen content ranged from 0.96% in S. pectinata ‘Red River’ to 2.02% in M. × giganteus (Table 3). Spartina pectinata ‘Summerford’ showed significantly greater leaf nitrogen content than S. pectinata ‘Red River’ but S. pectinata ‘IL-102’ was not significantly different from either genotype (Table 3). Nitrogen content of senesced leaves on 28 April 2014 ranged from 0.96% in S. pectinata ‘IL-102’ to 1.43% in M. × giganteus (Table 3).

At the autumn harvest (21 November), all genotypes of S. pectinata had senesced their leaf canopies from the previous summer and produced spikes of scale-like leaves that emerged above the soil surface before entering dormancy. These spikes appeared dormant on 19 April of the following year (2014) but by 28 April, some were opening to reveal new green leaves. Shoots of M. × giganteus had not emerged on 28 April, and had just recently emerged by 12 May (Henk Wichers, personal communication). A linear regression of M. × giganteus canopy heights from 17 May and 29 May estimated 9 May to be the emergence date (not shown). Canopy heights were significantly greater for S. pectinata ‘IL-102’ and ‘Red River’ compared to M. × giganteus throughout May (Fig. 6). By 25 June, canopy heights of S. pectinata ‘IL-102’, ‘Red River’ and M. × giganteus were all close to 80cm with only S. pectinata ‘Summerford’ being significantly lower (Fig. 6). On 25 June, S. pectinata ‘Summerford’ had visible or emerging flower spikes, however none of the other genotypes showed signs of flowering. By 6 October, S. pectinata ‘Red River’ had finished maturing seed, whereas ‘IL-102’ and M. × giganteus showed flag leaves but no visible flowers.

**Photosynthesis after a mild overnight frost**

Air temperature fell to 0.3°C early in the morning of 17 May, following 24h of air temperatures <10°C (Fig. 1A). Just before dawn on 17 May, frost was visible on leaves of both S. pectinata and M. × giganteus with leaf temperatures between 0°C and 1°C. Leaves of M. × giganteus were visibly yellow and chlorotic compared to S. pectinata, whose leaves looked healthy and similar in appearance to those later in the spring. At leaf temperatures between 9°C and 12°C, S. pectinata leaves had a greater incident ΦCO₂max of 0.051 compared to 0.026 for M. × giganteus on 17 May (Fig. 7A, inset). At every light intensity above 40 µmol m² s⁻¹, S. pectinata had a significantly higher A compared to M. × giganteus and was almost three times higher at 1800 µmol m² s⁻¹ (Fig. 7A). Photosystem II operating efficiency (Φp) was also significantly higher in S. pectinata compared to M. × giganteus.
at every light intensity, being over two times higher at low light and over four times higher at the highest light intensities (Fig. 7B).

**Discussion**

*Miscanthus* has been promoted as a bioenergy crop due to high productivity in temperate climates, which is due in part to the ability of hybrid lines to maintain carbon gain under cool conditions (Beale et al., 1996; Naidu et al., 2003; Wang et al., 2008). However, to exploit northern temperate and boreal regions, a perennial feedstock such as *Miscanthus* must endure severe winter cold, episodic frost, and periodic chilling that can extend into summer. The emerging picture from this and other studies is that *Miscanthus* genotypes generally lack the necessary cold tolerance to guarantee survival in cool temperate to boreal climates (Clifton-Brown et al., 2001a; Jørgensen et al., 2003; Heaton et al., 2008; Deen et al., 2011; Rosser, 2012; Peixoto et al., 2015). Overwintering rhizomes of allopolyploids such as *M. × giganteus* harvested from the field show >50% mortality and lethal electrolyte leakage at −4°C to −6°C, even when cooled using staging procedures designed to maximize cold acclimation (Table 2; Clifton-Brown et al., 2001a; Clifton-Brown et al., 2011; Peixoto et al., 2015). We also observed that following a winter where the upper soil zone briefly chilled to an average of −3°C in December, 20%
of the *M. × giganteus* rhizomes failed to survive. These rhizomes were mainly along the upper part of the rhizome mass where the cold approached the mean LT₉₀ of −4°C recorded for *M. × giganteus* rhizomes harvested on 21 November and 2 February. In turn, following emergence, young shoots of many *Miscanthus* lines are generally intolerant of spring frost, as observed here and by Friesen et al. (2014). Allopolyploid lines were universally killed in a May 2010 frost event (Friesen et al., 2014), and *M. × giganteus* showed substantial physiological impairment following a mild 17 May 2014 frost. Frost does not generally destroy *Miscanthus* stands because the rhizomes are protected belowground [although see Christian and Haase (2001), who describe loss of first year plantlets due to frost]. It does however, set back canopy development by killing new growth and thus delaying the onset of canopy closure that is essential for exploiting the long days of late spring.

From these results, we hypothesize that the Elora field site is near the northern range limit of where *M. × giganteus* plantations would be viable in southern Ontario. In most years, we predict that frost injury would impair southern Ontario *Miscanthus* canopies, but the overwintering rhizomes would be protected by soil and snow insulation; however, in times of low snow cover, the overwintering rhizomes would periodically encounter intense cold that could kill a substantial fraction of the plantation. In the 2013/2014 winter, the December cold that lowered upper soil temperatures to −3°C was relatively mild, and rhizomes were protected from more intense cold later in the season by a robust snowpack. However, if the intense cold of January to February had occurred when there was no snow and under windy conditions, then a deeper penetration of the cold front would occur, causing widespread rhizome mortality and potential loss of the stand. Given that *Miscanthus* is a perennial that requires two to three years to reach harvestable yields and is expensive to establish (Heaton et al., 2010), periodic stand loss would be catastrophic from a grower’s perspective. In most of Canada, soil temperatures often fall to −6°C or below at 5 cm depth (Environment Canada, 2015; Peixoto et al., 2015), supporting our hypothesis that Elora is on the fringe of viable climates for biomass production of currently available *Miscanthus* lines. We therefore conclude that expansion of *Miscanthus* production into climates colder than southern Ontario will require new lines with greater cold tolerance, or non-*Miscanthus* crops such as switchgrass and *Spartina*. As demonstrated here, *S. pectinata* is suitable for colder climates because it shows superior tolerance of mid-winter cold and mild frosts, and initiates canopy development two weeks before *M. × giganteus*.

*Spartina pectinata* shows much greater rhizome and leaf freezing tolerance than *M. × giganteus*, with a midwinter LT₉₀ near −24°C, and a spring leaf TEL₉₀ near −10°C. These are among the coldest viability thresholds reported for C₄ grasses.
and comparable to cold acclimated C₄ grasses such as winter oats (Avena sativa) (Rowley et al., 1975; Rowley, 1976; Webb et al., 1994) and its sister species S. gracilis, which remains viable to −27°C in the winter-hardened state (Schwarz and Reaney, 1989). Leaves of S. pectinata exhibited no physiological signs of stress following a mild frost on 17 May, in contrast to M. × giganteus where photosynthesis and quantum yields were well below peak values. In Canada, there are few recorded soil temperatures cold enough to kill any of our S. pectinata accessions in their winter-hardened state outside the Arctic (Environment Canada, 2015). The ability of S. pectinata to acclimate to cold autumn temperatures is also superior to cold tolerant switchgrass varieties, which exhibit LT₅₀ values of −19°C and −22°C at the end of November (Hope and McElroy, 1990).

Little variation was observed in the cold tolerance thresholds of the three accessions of S. pectinata used in this study, indicating little interpopulation variation in this species. With the procedures used here, it would be possible to test this hypothesis by rapidly screening the cold tolerance thresholds of many genotypes using the electrolyte leakage test, indicating little interpopulation variation in this species. The ability of S. pectinata to acclimate to cold autumn temperatures is also superior to cold tolerant switchgrass varieties, which exhibit LT₅₀ values of −19°C and −22°C at the end of November (Hope and McElroy, 1990).

By maintaining an active canopy late into the growing season, frost tolerance in the autumn can contribute to seasonal productivity as it does in the spring, but is also important for complete senescence of leaves and culms, with the result that sugars and nutrients can be fully translocated back to rhizomes. In Miscanthus, failure to completely senesce is evident by frequent frost kill of leaves while still green, trapping the nutrients inside the dead leaves (Patrick Friesen, personal observation). Loss of nutrients in senesced leaves complicates Miscanthus cultivation by contributing to leaching loss of nutrients from the stand, which increases fertilizer requirements and could reduce regional water quality (Mann and Tolbert, 2000; Heaton et al., 2009). Miscanthus × giganteus leaves had almost twice the leaf nitrogen content of S. pectinata leaves when senesced. By contrast, the 1.1% to 1.5% nitrogen concentrations of S. pectinata leaves were more typical of autumn senesced leaves in a wide range of species (Aerts, 1996; Bausenwein et al., 2001; Lee et al., 2003).

Spartina has been called ‘a New World parallel to Miscanthus’ (Long and Spence, 2013), a conclusion we support, but add that in many ways, S. pectinata is superior to Miscanthus. In addition to showing superior tolerance of deep winter cold and spring frost relative to Miscanthus, S. pectinata is able to begin canopy development two weeks earlier than Miscanthus, and maintains higher photosynthetic capacity and quantum yield during cold fronts in May. This allows for superior harvesting of photons during the long photoperiods of mid-to-late spring, which are crucial if maximum yields are to be achieved at higher latitude sites. At higher latitudes, the long days of spring and summer partially compensate for the longer growing seasons of lower latitudes. While S. pectinata can achieve respectable biomass yields of 12 t ha⁻¹ yr⁻¹ (Potter et al., 1995; Boe et al., 2009), Miscanthus is clearly superior in terms of overall growth potential, with typical yields that exceed 20 t ha⁻¹ yr⁻¹ (Heaton et al., 2008). This growth differential of S. pectinata and Miscanthus was observed here. The canopy heights of M. × giganteus and S. pectinata were equivalent on 25 June 2014, and by July M. × giganteus had a larger canopy. S. pectinata, however, is unimproved, and reported yields are likely well below peak yields that may arise with genetic improvement (Boe et al., 2013). If greater growth could be achieved, the earlier canopy development of S. pectinata would be a clear benefit that would add to its value as a bioenergy feedstock. Given the lack of differences in cold tolerance between genotypes and the greater growth of ‘Red River’ and ‘IL-102’ that we observed relative to ‘Summerford’, there does not appear to be a tradeoff between cold tolerance and growth capacity in S. pectinata. Such a tradeoff is an important consideration during breeding, and efforts to improve S. pectinata growth will need to consider this possibility. The procedures used here would allow for rapid screening of cold tolerance in new varieties, thus ensuring that cold-tolerant lines of S. pectinata are fully capable of exploiting the superior productivity enabled by the C₄ photosynthetic pathway.

Supplementary material

Supplementary Appendix S1. Description of rhizome harvest procedure.

Supplementary Appendix S2. Calculation of LT₅₀, LEL₅₀, and TEL₅₀ values and statistical analyses of photosynthesis, canopy height, and leaf nitrogen data.

Supplementary Table S1. Re-growth of rhizomes (%) for autumn harvest.

Supplementary Table S2. Re-growth of rhizomes (%) for winter harvest.

Supplementary Table S3. Re-growth of rhizomes (%) for spring harvest.

Supplementary Fig. S1. Spartina pectinata ‘Summerford’ collection information.

Supplementary Fig. S2. Field site plot map.

Supplementary Fig. S3. Agitation test for rhizome electrolyte leakage.

Supplementary Fig. S4. Electrolyte leakage corresponding to 50% rhizome mortality (LEL₅₀) for autumn/winter harvest.

Supplementary Fig. S5. Electrolyte leakage corresponding to 50% rhizome mortality (LEL₅₀) for spring harvest.

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