Improved experimental protocols to evaluate cold tolerance thresholds in *Miscanthus* and switchgrass rhizomes

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**Abstract**

This study investigates protocols to evaluate cold tolerance thresholds for overwintering rhizomes of perennial bioenergy grasses. Protocols examined include the temperature at which ice formation occurs, cooling rate, incubation time at the treatment temperature, and the electrolyte leakage (EL) method to assess mortality thresholds. Using these protocols, we assessed low temperature injury in two genotypes of *Miscanthus* and two genotypes of lowland switchgrass (*Panicum virgatum*). Ice formed near 0°C in the rhizomes cooled at 1°C h⁻¹, but at variable temperatures at cooling rates of 3 and 5°C h⁻¹. Rhizome temperature followed chamber temperature at a cooling rate of 1°C h⁻¹, whereas at faster cooling rates, there was a lag in rhizome temperature that affected treatment exposure time. A 1°C h⁻¹ cooling rate is thus suitable. In rhizomes incubated for <4 h at the treatment temperature, EL values were variable, while there was no change in EL when samples were incubated 4–20 h. A continuous, steady rate of cooling at 1°C h⁻¹ demonstrated the *Miscanthus* and lowland switchgrass varieties exhibited lethal levels of electrolyte leakage below −6°C. Continuous cooling does not allow for subzero acclimation and reflects thermal tolerances of sampled tissue in situ. To allow for maximum acclimation at subzero temperatures, a prolonged, staged-cooling procedure was adopted. This procedure showed diploid *Miscanthus* rhizomes could acclimate and adjust their tolerance limit to −12°C, while a triploid Illinois line showed little acclimation and was still killed below −6°C.

**Abbreviations**

EL = Electrolyte leakage  
RC = Relative conductivity  
LT₅₀ = Temperature at which the sample has a 50% mortality  
LEL₅₀ = Percentage of electrolyte leakage at which the sample has a 50% mortality

**Keywords:** cold acclimation, cold tolerance, deacclimation, electrolyte leakage, LT₅₀, Miscanthus, relative conductivity, switchgrass

**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 determine cold tolerance thresholds. This is particularly necessary in the case of perennial C₄ grasses, which can show superior productivity in the long warm summers of high latitudes; however, the more productive genotypes 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 45N (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 efficient and comprehensive methods for assessing cold tolerance. The objective of this study was to evaluate protocols to assess cold tolerance of overwintering rhizomes from *Miscanthus* and switchgrass genotypes 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, allowing for comprehensive screens of a greater variety of plants. For remote locations, portable test chambers can be used, thus enabling field studies in situ away from established laboratories. 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, which reduces costs and space requirements (Bykova & Sage, 2012; Gusta & Wisniewski, 2013). LT₅₀ assessments can be slow and cumbersome given the need to regrow treated plants to establish viability. Regrowth of exposed tissues is widely used to determine the LT₅₀ in studies of cold tolerance (Palta et al., 1982; Gilmour et al., 1988; McKay, 1992; Boorse et al., 1998; Ryvppö et al., 2008; Skinner & Garland-Campbell, 2014). If whole plants are used, large chamber volumes may be required to accommodate potted plants, 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; Ryvppö 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 LT₅₀ 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; Coiner, 2012; Peixoto et al., 2015). Given their 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 corresponding to a mortality limit, thus allowing for rapid screening of Miscanthus lines using small tissue samples.

In this study, we evaluate regrowth and electrolyte leakage protocols used to determine cold tolerance thresholds of Miscanthus and switchgrass (Panicum virgatum) genotypes. Among the protocols investigated are cooling rate, ice nucleation temperature, time of electrolyte leakage incubation, and tissue acclimation time. Cold treatments were conducted using a series of computer-controlled, temperature-test chambers that allow for precise regulation of subzero temperature exposure down to −40 °C.

Materials and methods

We divided this study into a series of six experiments. Experiment 1 evaluates the cooling rate and the temperature of ice nucleation in cold-acclimated versus nonacclimated samples (Table 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 nonacclimated samples. Experiments 5 and 6 use two different protocols to evaluate cold acclimation in rhizomes: the continuous cooling rate and a staged-cooling rate.

Plant material

Rhizomes of four Miscanthus and two switchgrass (Panicum virgatum) genotypes were obtained from New Energy Farms (http://newenergyfarms.com) in Leamington-ON, Canada. The Miscanthus hybrids were ‘Nagara’ (M116, 3n; Kludze et al., 2013; Friesen et al., 2014), ‘Illinois’ (M161, 3n; Heaton et al., 2008; Arundale et al., 2014; Friesen et al., 2014), ‘Amuri M115’ (2n; Friesen et al., 2014), and ‘Polish P01’ (unknown ploidy; Deen et al., 2011), from crosses between Miscanthus sacchariflorus and Miscanthus sinensis. The switchgrass genotypes were the tetraploid lowland Alamo and Kanlow (Casler et al., 2011), obtained from the same rhizome stock as Deen et al. (2011). Plants were grown at either the University of Toronto in pots in a greenhouse or outdoor platform (experiments 1–5), or in field plots in Leamington-ON managed by New Energy Farms (Experiment 6).

Plants used in experiments 1–5 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-Howard’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 h. 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, at which time the pots with dormant rhizomes were covered with plastic tarp and each pot wrapped with a heating tape connected to a thermostat placed in a centrally positioned pot under the tarp. This arrangement maintained pot temperatures between 0°C and −3°C during the cold of winter.

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. Preliminary tests indicated that individual rhizomes separated from the rhizome mass had similar responses to temperature treatment as whole rhizome masses (data not shown). Rhizomes sampled for treatment were cut to either 5 cm in length (*Miscanthus* genotypes Illinois, Nagara, and Polish) or due to shorter size, 3 cm in length (*Miscanthus* M115 and the two switchgrass lines). Individual rhizomes were then placed in one of a series of trays that corresponded to the individual temperature treatments, and covered with a 1-cm layer of moist soil (50% Pro-mix, 25% loam, and 25% sand) to avoid dehydration of the samples. The tray was then enclosed with clear plastic wrap and stored at 0°C in a Thermotron 2800 temperature-test chamber (Thermotron Industries, Holland, MI, USA http://www.thermotron.com) until temperature trials commenced. All temperature treatments were conducted using two Thermotron 8200 and one Thermotron 2800 programmable temperature-test chambers. Tissue viability after freezing was evaluated with electrolyte leakage (EL) and rhizome regrowth assays. The EL protocols were adapted from Zhang & Willison (1987), Murray et al. (1989), and Steffen et al. (1989).

After each experimental temperature test, the rhizomes were removed from the tray and allocated to either the EL or regrowth assessments. Rhizomes used for the EL assessment were trimmed to 1 cm in length by cutting off the basal and distal portions. After rinsing with distilled water, they were placed in vials with 7 ml of double de-ionized water (ddH2O) and incubated for 24 h at room temperature (21°C) to allow electrolytes to diffuse out of the tissue. Rinsing did not alter tissue electrolytes and electrolytes leaking out of the cut ends did not appreciably increase the relative conductivity of the solution, which was below 15% in the non-frozen, control samples. Samples were not shaken during incubation. After 24 h, the electrolyte conductivity of the treated sample (EL_mean) was 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_IL.htm) at room temperature. This meter enabled rapid sample assay (2–3 samples per minute) because incubation solutions could be poured in and out of the sample well quickly, and the meter equilibrated within 5 s of sample addition. The solution was returned to the vial containing the sample.

Table 1 List of experiments performed identifying the acclimation condition of the rhizomes, the test applied in the experiment, and the genotypes (with ploidy) used. Tests were performed in four *Miscanthus* genotypes (M) and two switchgrass (*Panicum virgatum*) genotypes (S). The superscript numbers on each genotype indicate references where these genotypes were also studied

| Experiment number | Rhizome acclimation | Test | Genotypes (ploidy) |
|-------------------|---------------------|------|--------------------|
| 1                 | Cold acclimated     | Cooling rate; Nucleation temperature. | (M) Illinois (3n)<sup>1,2</sup> (M) Nagara (3n)<sup>1,3</sup> (M) Polish (unknown)<sup>4</sup> |
| 2                 | Cold acclimated     | Cooling rate; Post-treatment incubation temperature. | (M) Illinois (3n)<sup>1,2</sup> (M) Nagara (3n)<sup>1,3</sup> (M) Polish (unknown)<sup>4</sup> |
| 3                 | Non-acclimated      | Post-treatment diffusion agitation. | (M) M115 (2n)<sup>1,4</sup> (M) Illinois (3n)<sup>1,2</sup> (M) Nagara (3n)<sup>1,3</sup> (M) Polish (unknown)<sup>4</sup> |
| 4                 | Cold acclimated     | Incubation time at the treatment temperature. | (M) Illinois (3n)<sup>1,2</sup> (M) Nagara (3n)<sup>1,3</sup> (M) Polish (unknown)<sup>4</sup> |
| 5                 | Cold acclimated     | Continuous cooling rate | (M) M115 (2n)<sup>1,4</sup> (M) Illinois (3n)<sup>1,2</sup> (S) Alamo (4n)<sup>5</sup> (S) Kanlow (4n)<sup>5</sup> |
| 6                 | Cold acclimated     | Staged cooling rate Subzero acclimation Winter warming event de-acclimation. | (M) M115 (2n)<sup>1,4</sup> (M) Illinois (3n)<sup>1,2</sup> |

<sup>1</sup>Friesen et al. (2014); <sup>2</sup>Heaton et al. (2008); <sup>3</sup>Kludze et al. (2013); <sup>4</sup>Deen et al. (2011); <sup>5</sup>Casler et al. (2011). The ploidy of Miscanthus Polish genotype is unknown.
and boiled for 1 h to kill all cells causing complete leakage of electrolytes. When the temperature of the resultant solution reached room temperature, the total electrolyte content was measured (ELtotal). Relative conductivity (RC) was calculated as:

\[
RC \% = \frac{EL_{treat}}{EL_{total}} \times 100\%
\]

To measure regrowth, treated rhizomes were planted in the soil media described above and kept in a greenhouse at a day/night temperatures near 24 C/18 C 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 sprouting rate of untreated 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.

Experiments 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 1). The purpose of these experiments was to evaluate effects of cooling rate on cold tolerance of Miscanthus rhizomes and when ice nucleation occurs in the rhizome. In Experiment 1, the response of cold-acclimated and nonacclimated rhizomes to cooling rate was evaluated, and in Experiment 2, the post-treatment temperature for electrolyte diffusion was analyzed. In each experiment, a sampled rhizome was first cooled from 0 C to −15 C. The chamber temperature was maintained for 4 h at the target temperature, and then raised back to 0 C. For Experiment 1, the cooling and thawing rates were the same and set to be either 1, 3 C, or 5 C h⁻¹. The same procedure was used in Experiment 2, but the rates tested were 1 and 3 C h⁻¹ plus the control (rhizomes were kept at 4 C). In Experiment 1, the temperature of individual rhizomes was measured using copper/constantan, 0.125-mm-thick thermocouples imbedded 3 mm into the rhizome. The thermocouple outputs were monitored by the Thermotron 8200 datalogger or a Veriteq Spectrum 1700 thermocouple data logger (Veriteq Instruments Inc., Richmond- BC, Canada, http://www.vaisala.com).

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

Published EL studies typically incubated samples in a bathing solution at either room temperature (21 C) or 4 C to allow for the efflux of electrolytes into the bathing solution (Knowles & Knowles, 1989; Murray et al., 1989; Campos et al., 2003; Ebeling et al., 2008). To evaluate whether incubation temperature is significant, pairs of rhizomes from the same plant were taped together and included in the trays used in Experiment 2. After the cold treatment, one rhizome of each pair was placed in a vial filled with 7 ml of ddH2O and incubated for 24 h at 4 C, and the other was incubated for 24 h at 21 C prior to the electrolyte leakage measurement.

In Experiment 3, the effects of agitating the bathing solution after treatment were evaluated. Because the EL measurement of the samples was repeated at various times, this experiment was designed as a repeated measures with 8 replicates. Nonacclimated rhizomes of the Miscanthus hybrids Amuri M115 and Illinois were frozen using a continuous cooling rate at 5 C h⁻¹ to −20 C and incubated for 10 h at this temperature, and then thawed at 5 C h⁻¹. This procedure was performed to promote high electrolyte leakage. After the cold treatment, 16 rhizomes of each genotype were placed in vials with ddH2O for electrolyte diffusion. From these vials, 8 per genotype were randomly chosen for incubation on a G10 Shaker (New Brunswick Scientific Co. Inc. New Brunswick, NJ, USA, http://newbrunswick.ependorf.com/) and agitated at 150 rpm, while the other 8 samples of each genotype were left unshaken. Electrolyte conductivity was measured after 2, 4, 10, 20, 24, 28, and 32 h of incubation of the samples in vials with 7 ml of ddH2O, and all samples were then boiled for 1 h. After another 24 h to cool to room temperature, total electrolyte conductivity was measured.

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 non-ideal treatment lengths, the time spent at a treatment temperature was evaluated. Cold-acclimated and nonacclimated plants from genotypes Nagara, Illinois, and Polish were used with 5 replicates for each treatment temperature at each sampling time. Nonacclimated rhizomes were cooled to −2, −8, or −12 C. Cold-acclimated rhizomes were exposed to −2, −8, −12, −14, −18, and −22 C. In all treatments, rhizomes were continuously cooled to the treatment temperature at a rate of 1 C h⁻¹. Once the treatment temperature was reached, rhizomes were sampled after 4, 8, 12, 16, and 20 h of incubation. After sampling they were placed in a second programmable freezer, set at the respective treatment temperature, and thawed to 21 C at 1 C h⁻¹ after which EL was assessed, as described above.

Experiment 5: Evaluation of continuous cooling rate

This experiment uses the most common artificial cooling protocol (continuous cooling) to evaluate the electrolyte leakage caused by the treatment temperature. It also compares two Miscanthus genotypes and two lowland switchgrass (Panicum virgatum) genotypes (Table 1). Five rhizomes of each genotype were cooled from 0 C to the treatment temperature at the cooling rate of 1 C h⁻¹ and incubated at this temperature for 16 h before being thawed at the same cooling rate. The treatment temperatures were 0, −5, −10, −15, and −20 C. Due to shortage of rhizomes, the switchgrasses were not tested at 0 C. The order at which each treatment was performed was randomized.

Experiment 6: Evaluation of staged-cooling rate, acclimation potential, and de-acclimation potential

This experiment was designed to test whether an artificial cooling protocol can induce greater cold tolerance of subzero temperatures. 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 and analyzed for their cold tolerance under different cooling rates and acclimation potentials. The results showed that the cold tolerance of these Miscanthus genotypes improved significantly when exposed to lower temperatures for longer periods. This suggests that an appropriate cooling protocol can be used to enhance the cold tolerance of Miscanthus genotypes, which could be beneficial for agricultural practices.
and another Thermotron 8200 precooled to the treatment temperature, and represented one thermal stage. Sampled rhizomes were placed in a pair of rhizomes from each plant was sampled. Each 24-h incubation led to an increase in metabolic activity in the nonfrozen state, or deharden. One rhizome of the pair while the other was planted to determine mortality using regrowth assays.

Episodic warming above freezing temperatures can occur at temperate latitudes and could allow for enhanced acclimation due to an increase in metabolic activity in the nonfrozen state, or might weaken the cold acclimation state if rhizomes deharden. To test whether warming events affect the cold tolerance limits of Miscanthus rhizomes, a subset of samples treated at −10°C were warmed to 4°C at the rate of 1°C h⁻¹. Samples were kept at 4°C for 24 h and then cooled to −10°C in stages as described above, allowing 24 h of incubation at each stage. The rhizomes were then sampled after the −10°C stage for EL assay.

Data from Experiment 6 were used to determine LT₅₀ and LEL₅₀ (percentage of electrolyte leakage at which the sample has 50% mortality). 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 in the R statistical package (R-Core-Team, 2013) for each (a) temperature or (b) RC, to estimate the temperature (LT₅₀) or RC (LEL₅₀) at which rhizomes have 50% chance of mortality.

Statistical analysis

All experiments were analyzed using a generalized linear mixed model (GLMM) on the beta or binomial family using R Statistical software (R-Core-Team, 2013). 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 ‘gamsls’ (Rigby & Stasinopoulos, 2005). In experiments 5 and 6, (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).

Results

Nucleation temperature and cooling rate

Cooling rate had a large effect on the time required to complete an experiment to a nadir temperature of −15°C (Fig. 1). A freezing trial using a cooling rate of 1°C h⁻¹ required 34 h (15 h to cool down, 4 h at −15°C, 15 h to raise temperature back to 0°C); at 3°C h⁻¹, a trial lasted 14 h while at 5°C h⁻¹, trials took about 10 h to complete. At 1°C h⁻¹, Miscanthus rhizomes exhibited exotherms near −1°C (Fig. 1c). For the cooling rates of 3 and 5°C h⁻¹, exotherms occurred at variable temperatures ranging from −1 to −11°C (Fig. 1a, b). Exotherms indicate when ice formation occurs (Akyurt et al., 2002; Gusta et al., 2009). Therefore, the results indicate that in Miscanthus rhizomes cooled at 3°C h⁻¹ and faster, water may supercool before ice crystals are formed. Rhizome temperature lagged behind chamber temperature by 1–2 h at the faster cooling rates (Fig. 1a, b), but it was close to chamber temperature at 1°C h⁻¹ (Fig. 1c). As a result, at the faster cooling rates rhizomes are not being incubated at the nadir temperature for the full 4 h.

In experiments 1 and 2, cooling at 1°C h⁻¹ resulted in higher relative conductivity (RC) than observed with faster cooling rates (P < 0.05; Fig. 2). No significant differences in RC among the Miscanthus genotypes Nagara, Illinois, and Polish were detected (P > 0.05), while cold-acclimated rhizomes had lower RC than the nonacclimated rhizomes (Fig. 2).

Post-treatment electrolyte diffusion: temperature, time, and agitating the bathing solution

In Experiment 2, the EL was similar in rhizomes incubated at either 4°C or 21°C (P > 0.05; Fig. 2), demonstrating no effect of incubation temperature on EL values. We observed that readings at 21°C stabilized more rapidly, which was due to the conductivity meter and the bathing solution starting out at equal temperatures. As a result, samples incubated at 21°C were assayed more rapidly than samples incubated at 4°C. Following sample incubation at 21°C, we were able to assay two to three samples per min, enabling us to assay over 100 samples per h, which allowed for timely measurement of large number of replicated samples. 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 h at room temperature (P > 0.05;
Fig. 3). There was also no difference between genotypes. Relative conductivity reached its maximum at 24 h and samples began to rot by 32 h, demonstrating the hazards of lengthy incubation time.

**Relationship between incubation time and nadir temperature**

Incubation periods between 4 and 20 h at the nadir temperature had no significant effect on RC ($P > 0.05$; Fig. 4). In this experiment, differences between genotypes were also not significant ($P > 0.05$), and thus, the genotype results were pooled. Differences between cold-acclimated and nonacclimated rhizomes were significant, as was the interaction between temperature and acclimation status. For instance, cold-acclimated rhizomes treated at a nadir temperature of $-8 \, ^\circ \text{C}$ had an RC near 30%, while nonacclimated rhizomes at the same temperature had more than double the RC (about 70%). At $-12 \, ^\circ \text{C}$, nonacclimated rhizomes had almost complete leakage of electrolytes while cold-acclimated rhizomes exhibited an RC around 50%.

**Continuous cooling rate and the comparison between cold tolerance of Miscanthus and switchgrass**

In the continuous cooling rate experiment, Miscanthus hybrids did not increase EL after treatment at $-5 \, ^\circ \text{C}$ when compared with rhizomes maintained at 0 $^\circ \text{C}$ (Fig. 5a). Because of shortage in the number of rhizomes, the switchgrasses were not treated at 0 $^\circ \text{C}$ (Fig. 5b). For all the genotypes tested, the EL at $-5 \, ^\circ \text{C}$ was 12–14%. The EL increased substantially when rhizomes were treated at $-10 \, ^\circ \text{C}$ and ranged from 47 to 72% at this treatment temperature: Miscanthus M115 had EL of 47%, Miscanthus Illinois 64%, switchgrass variety Alamo was 63%, and switchgrass variety Kanlow had 72% of EL. At $-15 \, ^\circ \text{C}$, M115 had 77% EL, and the EL in Illinois and both switchgrasses 82–83%. At $-20 \, ^\circ \text{C}$, M115 had 83% of EL, while all other genotypes had 90–93%. Even though M115 had lower EL than the other genotypes at most temperatures, this difference was not sufficient to resolve significant differences between the genotypes.

**Evaluation of staged-cooling rate and potential to acclimate (Experiment 6)**

Experiment 6 assessed whether cold tolerance is enhanced upon prolonged exposure to successively lower subzero temperatures. Because cooling occurred in multiple 24 h stages, this experiment required 22 days for the $-22 \, ^\circ \text{C}$ treatment to be completed. One of six Illinois rhizomes was alive after 24 h at the nadir temperature of $-10 \, ^\circ \text{C}$, and that was the lowest temperature a rhizome from this genotype could survive (Fig. 6a). At $-10 \, ^\circ \text{C}$, M115 had survivability of 83% before and after the freeze–thaw cycle. Even at $-12 \, ^\circ \text{C}$ and $-14 \, ^\circ \text{C}$, M115 had survivability of 66% and 33%, respectively. The estimated LT$_{50}$ was $-12.8 \, ^\circ \text{C}$ for M115 and $-6.3 \, ^\circ \text{C}$ for Illinois.
When analyzing the RC corresponding to the survivability responses, M115 rhizomes had significantly higher survivability at a common RC than Illinois (Fig. 6b). Consequently, the LEL50 was 28.6% for M115, and 18.6% for the Miscanthus Illinois genotype. Injury as indicated by RC value was analyzed for each genotype as a function of temperature (Fig. 6c). At \(-7^\circ C\) and below, Illinois had consistently higher RC than M115, indicating greater cold sensitivity in the Illinois line. The RC value from the Illinois rhizomes rose sharply above \(0^\circ C\) as temperatures declined below \(-10^\circ C\); in M115, the rise in RC was more gradual at stages below \(-10^\circ C\) (Fig. 6c). A 24-h warming treatment from \(0^\circ C\) to \(4^\circ C\) had little effect on RC in M115 samples subsequently harvested after 24 h at \(-10^\circ C\) (Fig. 6). In Miscanthus Illinois, survivability was already minimal before the thawing event so no difference in mortality could be resolved after recooling to \(-10^\circ C\). However, EL increased from 28% to 47% after samples were thawed and refrozen to \(-10^\circ C\).

**Discussion**

In this study, we evaluate protocols for high-throughput trials of cold tolerance in C4 grass varieties being developed for bioenergy production at higher latitudes. We then used our procedures to determine cold tolerance limits in two Miscanthus and two switchgrass (Panicum virgatum) varieties. To test whether cold tolerance could be experimentally enhanced in the two Miscanthus genotypes, we introduced stages in the cooling rate to promote maximum acclimation to severe cold. The results identified clear guidelines that could improve uniformity between trials and provide robust estimates of cold tolerance in Miscanthus and switchgrass.
Artificial freezing trial

Rapid cooling can cause erroneous tolerance estimates for a variety of reasons. For one, it can promote harmful ice crystal growth in the protoplast, whereas slower cooling can allow for more natural ice growth in the apoplast (Boorse et al., 1998; Griffith & Yaish, 2004; Ruelland et al., 2009). In Solanum acaule leaves, the temperature at which ice nucleation occurs influences the LT₅₀: if nucleation occurred at −1 C, the LT₅₀ was −7 C; if nucleation occurred at −2 C, the LT₅₀ was −3 C (Rajashekar et al., 1983). Alternatively, rapid cooling could delay ice formation and thus produce inaccurate results. In experiments 1 and 2, some rhizomes cooled at 3 and 5 C h⁻¹ formed ice crystals at lower, yet variable, temperatures than those cooled at 1 C h⁻¹. As well, the treatments using a faster cooling rate of 3 C h⁻¹ and/or 5 C h⁻¹ had less damage (based on RC values) than the 1 C h⁻¹ cooling rate. These results likely reflect exposure time at the treatment temperature, which was reduced in the 3 and 5 C h⁻¹ trials due to lags in tissue equilibration with the ambient temperature. For example, at 3 and 5 C h⁻¹, samples remained at the nadir

Fig. 5  Effects of temperature on relative conductivity of rhizomes cooled in at a continuous cooling rate for two Miscanthus varieties and two switchgrass varieties. Rhizomes were cooled at 1 C h⁻¹ and incubated at the treatment temperature for 16 h. For the switchgrass (Panicum virgatum), no treatment at 0 C was conducted. Assuming rhizomes cannot recover if they suffer more than 20% injury (LEL₅₀ = 20%; Peixoto et al., 2015), LT₅₀ for all genotypes was between −5 and −6 C. There was no difference between genotypes or species. Regression lines are the best fit logistic regression. Mean ± SE (n = 5).

Fig. 6  The effects of staged-cooling rate on Miscanthus genotypes M115 (filled circles, long dashed lines) and Illinois (M161; open triangles, short dashed lines). (a) Survivability of Miscanthus rhizomes as a function of temperature. Vertical lines indicate 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 survival probability as a function of temperature (panel b) or RC (panel c). (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 panels (a) and (c), the stars (filled symbol for M115 and open symbol for M161) indicate results for the incubation at −10 C after the freeze–thaw cycle. For M115, the LT₅₀ = −13 C and LEL₅₀ = 29% of RC. For Illinois, the LT₅₀ = −6 C and LEL₅₀ = 19% of RC. (n = 6). Significant P-values are <0.002 because the statistical model used Laplace approximation.
temperature for just 2–3 h, not the 4 h in the experimental protocol. Based on our results, we recommend a relative slow cooling (and thawing) rate of 1 C h⁻¹ because this allows for similar chamber and rhizome temperatures, which generates slightly higher RC values than at 3 and 5 C h⁻¹ and is closer to rhizome cooling rates that might occur in the field. Field cooling rates are highly variable depending upon wind speed, radiation load, and air temperature change, but as an indication of the faster rates that can be expected, we observed maximum cooling rates at 1 cm soil depth to be approximately 10 C in a 24-h period above 0 C, and 2–3 C per 24 h below 0 C (Friesen et al., 2015; Peixoto et al., 2015). These measurements were conducted in Miscanthus stands near Leamington-ON and Elora-ON.

Previous studies often incubate samples (usually shoot material) at the nadir temperature for 5 to 10 min (for example, Ball et al., 2006; Márquez et al., 2006) and 1–4 h (Murray et al., 1989; Steffen et al., 1989; Farrell et al., 2006; Bykova & Sage, 2012). Incubation time is important in freezing trials because the equilibrium between the cell membrane and the extracellular solution can require hours to occur (Gusta & Wisniewski, 2013). Short incubation periods at the nadir temperature can thus give imprecise results. In the case of wheat and Brassica varieties, for example, short-term freezing studies with no incubation period at the nadir temperatures produced estimates of LT₅₀ below —17 C, while when plants were allowed to incubate for over a day at a nadir temperature between —8 C and —9 C, the LT₅₀ increased by 9 C or more (Gusta et al., 1997; Waalen et al., 2011). Hope & McElroy (1990) also used no incubation time at the nadir temperature for crowns of upland switchgrass (Panicum virgatum). They determined an LT₅₀ for upland switchgrass derived from the variety Pathfinder was near —20 C, which was well below the —6 C value we estimated in lowland varieties Kanlow and Alamo using 16-h incubation at the nadir temperature. Their study is notable as it is the only controlled freezing study of overwintering switchgrass we could find in the literature. While upland switchgrass varieties can be expected to have substantial cold tolerance, the rapid protocols used by Hope & McElroy (1990) may not have allowed for tissue adjustments. Here, we observed no difference in RC of samples incubated between 4 and 20 h at the nadir temperatures, either in acclimated or nonacclimated rhizomes. Exposure times as low as 4 h would be suitable where lengthy experimental times are a concern.

In this study, we used the same rate for cooling and thawing within a given trial due to limitations in time and resources. Thus, while we emphasize effects of cooling rate, our treatments also include variable thawing rates, which could contribute to rhizome injury. Rapid thawing promotes uncontrolled water flux across the cell membrane which can cause lysis due to cell expansion (Steponkus, 1984; Gusta et al., 2009). Rapid thawing may also create gas bubbles in solution, leading to cavitation in xylem conduits and cellular disruption (Steponkus, 1984; Ball et al., 2006). While our recommendations regarding cooling (and warming) rates should hold for chilling tolerance assessments in grass rhizomes, the physiological question of how chilling versus warming rate separately affect tissues will require follow-up study. Such studies could also evaluate responses to actual rates of temperature change in the field. Rhizomes and other belowground tissues generally experience relatively slow cooling as the season progresses, due to the insulating properties of the soil and litter (Peixoto et al., 2015). By contrast, warming rates can be relatively sudden, for example, a warm rain may thaw soil in a few hours (Friesen et al., 2015).

**Electrolyte diffusion**

Prior studies have used 4 C (Murray et al., 1989; Sheppard et al., 1993; Ebeling et al., 2008) or room temperature (21 C) for the conditions at which electrolytes are allowed to diffuse into the bathing solution following sample thawing (Knowles & Knowles, 1989; Fan & Blake, 1994; Tarhanen, 1997; Campos et al., 2003). Incubation at 4 C could help preserve the tissue and avoid warm temperature effects, but can be more difficult if refrigeration capacity is limited. The results of Experiment 2 showed that Miscanthus rhizomes had similar RC values following electrolyte efflux at 4 C or 21 C, so incubation temperature should not be a major concern for the RC estimates. By contrast, incubation at 4 C versus 21 C does affect experimental logistics. We observed that if the bathing solution is at the same temperature as the conductivity meter, as was the case at room temperature, the conductivity readings stabilized quickly, which allowed for faster measurements. Thus, incubating thawed rhizomes at room temperature is the recommended option.

Another concern is whether samples need to be agitated during this incubation period. Many experiments report the use of a shaker for leaching of electrolytes. Thalhammer et al. (2014) suggest incubating leaves of Arabidopsis thaliana on a shaker at 150 rpm for 24 h. Płażek et al. (2012) used a shaker at 100 rpm for 2 h for Miscanthus stolon disks. Agitating the samples for leaching of electrolytes might be used where there is a need to shorten the incubation time (Kaplan & Guy, 2005); 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. In Experiment 3, the samples were treated to extreme conditions (~20 C) to kill most cells and then RC determined after agitation or no-agitation of the rhizomes during the post-thaw incubation period. Miscanthus rhizomes showed no difference between shaken and nonstirred treatments.
demonstrating no need for agitation during the leaching period. Of note, incubation of the samples in the bathing solution for <24 h was not enough time for complete diffusion of the electrolytes out of the tissue.

Artificial cooling experiments, subzero acclimation and de-acclimation of Miscanthus rhizomes

The continuous cooling experiment showed that the cold-acclimated Miscanthus rhizomes suffered no injury (as indicated by relative conductivity) if treated for 16 h at −5 °C compared to treatment at 0 °C. The switchgrass varieties similarly showed no evidence for injury at −5 °C, and their RC estimates were similar as for Miscanthus. In both species, however, the RC increased significantly between −5 and −10 °C. Based on results in Fig 6c, and from prior work with Miscanthus (Peixoto et al., 2015), we used a value of 20% RC as the threshold where 50% of the rhizomes are killed. The switchgrass RC corresponding to 50% mortality is unknown and thus is assumed to be similar to that of Miscanthus. With this value, we estimate that the temperature corresponding to 50% mortality was near −6 °C in both Miscanthus and lowland switchgrass varieties. The Miscanthus estimates are similar to values previously determined for continuously cooled, winter-acclimated samples from a range of genotypes growing in Canada (LT50 of −4 °C to −6 °C; Friesen et al., 2015; Peixoto et al., 2015) and are slightly more negative than lethal cold thresholds of European Miscanthus x giganteus rhizomes (LT50 of −3 °C to −5 °C; Clifton-Brown & Lewandowski, 2000). The switchgrass estimates are the first reported for any lowland varieties, but upland varieties are noted to be more cold tolerant (Lee et al., 2014). We observed in a field trial in Elora-ON that Miscanthus genotypes had no mortality after the winter of 2009–2010, while only 17% of the switchgrass Alamo and about 50% of switchgrass Kanlow resprouted the following spring from second year rhizomes (Peixoto, unpublished result). Deen et al. (2011) report that first year rhizomes of an Amuri Miscanthus genotype had 94% survivability in Elora-ON and Simcoe-ON, while a lowland switchgrass variety (Cave-in-Rock) had 78% survivability in Elora-ON and 98% survivability in Simcoe-ON. Based on our results, the difference in cold tolerance in the field is not due to physiological tolerance thresholds of the rhizomes and might instead be due to other factors, such as rhizome depth and degree of exposure to surface conditions. We recommend that future studies examine both physiological cold tolerance and actual cold exposure of rhizomes to fully understand causes of mortality in agronomic settings.

Rhizomes sampled from field populations may express cold tolerance limits that reflect conditions around the time of harvest, rather than the absolute tolerance limit the plant may express if the tissue is fully acclimated to subzero temperatures. To maximize cold acclimation, we used a staged-cooling procedure where samples were cooled at 1 °C h−1 to a treatment temperature and then held at the temperature for 24 h before cooling to the next treatment stage. The diploid M115 Miscanthus variety showed an ability to reduce its LT50 to −13 °C from the −6 °C value observed in the continuous cooling trial. By contrast, there was no LT50 shift in the triploid Illinois Miscanthus variety, indicating little subzero acclimation potential. The LT50 value for M115 in the staged-cooling study is much lower than previously reported for Miscanthus hybrids (−3 °C to −7 °C) which were not tested using a staged-cooling rate (Clifton-Brown & Lewandowski, 2000; Friesen et al., 2015; Peixoto et al., 2015). This indicates there is substantial genetic potential for increased cold tolerance in the Miscanthus gene pool. We were unable to investigate the acclimation response in the switchgrass lines, so their ability to further harden during prolonged freezing remains uncertain.

Climate data from 1950 to 1998 show that winter and early spring warming episodes are particularly frequent in southern Canada (Shabbar & Bonsal, 2003). To evaluate the impact of such an event, we treated a subset of rhizomes with a freeze–thaw cycle where the samples were thawed from −10 °C to 4 °C and maintained at this temperature for 24 h before being cooled again in stages to −10 °C. This procedure did not alter survivability or RC in the diploid M115. In the Miscanthus Illinois genotype, this freeze–thaw cycle caused a sharp increase in RC compared to before the thaw event. These results show that acclimation is not enhanced by a freeze–thaw cycle, and in M115 at least, de-acclimation was not apparent following a short warming event in winter. By contrast, the increased injury in the Illinois line following the freeze/thaw event may reduce its tolerance of actual winter conditions in cool temperate landscapes. De-acclimation can influence survivability of plants in regions where episodic winter thaw is common (Kalberer et al., 2006; Bykova & Sage, 2012).

Conclusion – the use of LT50 versus LEL50

Because procedures for thermally treating tissues affect the outcome, we recommended that researchers use, as much as possible, a standardized set of protocols that are physiologically robust and comparable between studies. Such standardized procedures are lacking in the relatively new discipline of bioenergy feedstock improvement, and thus, a principle objective of the research here has been to develop guidelines for subsequent research. While direct measurements of LT50 estimated by regression analysis are generally preferred, they can be costly and impractical due to limitations in labor, growth facilities, and availability of regenerative tissues. In such cases, LEL50 can provide a meaningful alternative for cold tolerance assessments. Electrolyte leakage tests and other proxies are advantageous over regrow because they are rapid, they can be used on
small amounts of tissue, and they do not require regenerative tissues and greenhouse space for regrowth assays. The success of an electrolyte leakage test, however, depends on knowing the LEL50, which is the RC value that corresponds to the LT50. Some authors assume the temperature at which 50% RC occurs is a suitable equivalent of regrowth (Webb et al., 1994; for oat and rye; Hannah et al., 2006; for Arabidopsis; Bykova & Sage, 2012; for Bromus spp); however, this approach overestimates cold tolerance thresholds because tissues do not recover from this level of injury (Zhang & Wilison, 1987; Teutonico et al., 1993; Maier et al., 1994; Boorse et al., 1998; Dunn et al., 1999; Waalen et al., 2011). Instead, RC values of 20% to 30% typically reflect mortality thresholds (Palta et al., 1982; Coiner, 2012; Peixoto et al., 2015). In the present study, we estimate the lethal RC of Miscanthus rhizomes is between 19% and 29%, depending on the cultivar (Fig. 6b). These values should allow for rapid assessment of LT50 in future Miscanthus studies using the electrolyte leakage method.

With the methods developed here, we provide new perspectives for cold tolerance of Miscanthus and switchgrass. In one diploid genotype of Miscanthus, rhizomes can increase acclimation upon slowly developing exposure to subzero temperatures, while the more productive triploid hybrid cannot. We report for the first exposure to subzero temperatures, while the more productive triploid hybrid cannot. We report for the first time that the LT50 of lowland switchgrass is relatively high and similar to Miscanthus. By contrast, upland switchgrass is noted for being cold tolerant and rhizomes derived from the upland Pathfinder variety are reported to be tolerant of temperatures down to −20 °C (Hope & McElroy, 1990); however, their methods differ from what our results lead us to recommend. Because lowland switchgrass is more productive and upland varieties (Lee et al., 2014), there is a strong desire to cross the cold tolerance of the upland varieties with the productivity of lowland varieties. The methods developed provide a reliable, high-throughput screening capacity to facilitate the breeding of ideal switchgrass and Miscanthus lines for cold climates.

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