

Impact of waterlogging and temperature on autumn growth, hardening and freezing tolerance of timothy (*Phleum pratense*)

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Abstract

Precipitation has generally increased in Norway during the last century, and climate projections indicate a further increase. The growing season has also become longer with higher temperatures, particularly in autumn. Previous studies have shown negative effects of high temperatures and, depending upon temperature conditions, contrasting effects of waterlogging on hardening capacity of timothy. We studied effects of waterlogging on seedlings of timothy (*Phleum pratense*, cv. Noreng) under three pre-acclimation temperatures: 3°C, 7°C, 12°C, and in autumn natural light in a phytotron at Holt, Tromsø (69°N). After temperature treatments, all plants were cold acclimated at 2°C for three weeks under continued waterlogging treatments. Freezing tolerance was determined by intact plants being frozen in pots at incremental temperature decreases in a programmable freezer. Waterlogging resulted in a higher probability of death after freezing, and a significantly reduced regrowth after three weeks at 18°C, 24 hrs light in a greenhouse. Increasing pre-acclimation temperatures also had a clear negative effect on freezing tolerance, but there was no interaction between temperatures and waterlogging. The results indicate that waterlogging may have negative implications for hardening of timothy and may contribute to reduced winter survival under the projected increase in autumn temperatures and precipitation.

KEYWORDS

autumn temperatures, climate change, flooding, winter acclimation, winter hardiness

1 | INTRODUCTION

Winter acclimation or hardening is imperative for winter survival of perennial grasses in regions with cold winters, and especially at high latitudes where winters are long and cold. Temperatures have increased during the last century in northern latitudes (Hanssen-Bauer et al., 2015; Mikkonen et al., 2015). This increase has occurred more during late autumn, winter and spring than in summer (Uleberg, Hanssen-Bauer, Oort, & Dalmansdottir, 2014). Precipitation has

also increased in the same period (Hanssen-Bauer et al., 2015; Vikhamar-Schuler et al., 2016). Temperature variability and number of winter warming events leading to rain, snowmelt and ground ice formation have also increased and are projected to further increase in northern Scandinavia, thus forming stressful winter conditions for overwintering plants (Vikhamar-Schuler et al., 2016). Increased autumn temperatures and heavy precipitation could adversely affect winter acclimation and winter survival in grassland and thereby limit forage production in regions with cold winter conditions. Climate

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projections for the end of this century indicate that temperature, average precipitation and heavy precipitation events will continue to rise in the future in the northern hemisphere, especially in winter (Hov et al., 2013; Lehtonen, Ruosteenoja, & Jylhä, 2014).

Freezing tolerance is often used as a measure of winter hardiness (Rapp & Junttila, 2000). The freezing tolerance capacity of plants is determined by their genetic makeup and the level of the tolerance by environmental cues such as temperature and light conditions (Harrison, Tonkinson, Eagles, & Foyer, 1997). Hardening in grasses is mainly triggered by exposure to low temperatures (Palva et al., 2002). Higher autumn temperatures before winter acclimation conditions give poorer hardening and lower freezing tolerance, compared with lower autumn temperatures both in timothy (*Phleum pratense* L.) and in perennial ryegrass (*Lolium perenne*) (Dalmannsdottir et al., 2017, 2016).

Due to the slow diffusion of oxygen in water, the exchange of soil air with the atmosphere is strongly reduced in flooded or waterlogged soils, and the respiration of microorganisms and plant roots can lead to hypoxic or near-anoxic conditions in the soil. Anoxic plants shift from oxidative phosphorylation to anaerobic glycolysis, which results in severe energy deficits of the plants and production of toxic ethanol or lactate as end products (Jackson & Drew, 1984; Vartapetian & Jackson, 1997). Photosynthesis may be reduced in flooding-sensitive plants due to, for example flooding-induced stomatal closure and thus reduced CO₂ fixation. In more tolerant species, photosynthetic rates may increase to prevent photoinhibition (Caudle & Maricle, 2012). Waterlogging is less damaging than total submergence, as shoots above water potentially can capture oxygen and transport it to oxygen deficient organs through aerenchyma (Loreti, Van Veen, & Perata, 2016; Yamauchi, Shimamura, Nakazono, & Mochizuki, 2013). The risk of hypoxic or anoxic conditions in the soil increases under higher temperatures as increased metabolic activity requires a greater supply of oxygen to both plant roots and competing soil microorganisms (Drew, 1992).

It is not entirely clear how the interaction of waterlogging with autumn temperature affects plant growth and winter acclimation, and responses differ between species and among cultivars within species (Jurczyk, Krepski, Kosmala, & Rapacz, 2013; Pomeroy & Andrews, 1979). Flooding at higher temperatures in grasses is more damaging than flooding at low temperatures (Beard & Martin, 1970). Low-temperature waterlogging causes lower freezing tolerance in both winter wheat and winter barley compared to non-flooding, but winter wheat treated with low-temperature waterlogging has increased survival of ice encasement (Andrews & Pomeroy, 1981, 1989). Jurczyk et al. (2013) found that flooding increased the freezing tolerance of some, but not all genotypes of meadow fescue (*Festuca pratensis*). In another study with low-temperature flooding, no effects or reduced freezing tolerance in meadow fescue and in perennial ryegrass was found (Jurczyk, Rapacz, & Krepski, 2015). Waterlogging led to increased freezing tolerance in timothy and red clover (*Trifolium pratense*) in a location with low autumn temperatures, but no effects on freezing tolerance were found in a location with higher average autumn temperature (Dalmannsdottir, Østrem, & Larsen, 2012).

Climate change projections indicate increased risk of waterlogged soils and higher temperatures during autumn in the Nordic regions. This may affect winter acclimation and survival of perennial forage grasses. Timothy is the most important grass species used for forage production in the Nordic countries. It is winter-hardy and quite tolerant of hypoxic/anoxic conditions resulting from flooding or ice encasement (Banach et al., 2009; Höglind, Bakken, Jørgensen, & Østrem, 2010). In the current study, we investigated the effect of waterlogging in combination with different temperatures before winter acclimation on hardening, determined as freezing tolerance and winter survival of timothy. The cultivar used in this study is a northern type, well adapted to winter conditions. We hypothesized that waterlogging would have negative effects on hardening, thereby resulting in lower freezing tolerance and poorer winter survival of timothy. Furthermore, we assume that these negative effects would be more pronounced at higher than at lower pre-acclimation temperatures.

2 | MATERIALS AND METHODS

2.1 | Plant material, temperature and waterlogging treatment

Timothy seedlings (cv. Noreng, winter-hardy cultivar bred for high latitudes) were sown in mid-July 2015 in a greenhouse at Holt, Tromsø (69°39'7"N, 18°57'12"E), Norway. The seedlings were transplanted into pots (12 cm diameter, 8 cm height), five plants per pot, in mid-August (Figure 1). The pots were filled with fertilized sphagnum and perlite (3:1). In late August, the plants were transferred to a phytotron, all in one chamber with a temperature of 12°C, relative humidity (RH) 62% and natural light conditions. After 17 days, in mid-September, the plants were placed in three different chambers in the phytotron at 3°C (RH 40%), 7°C (RH 42%) or 12°C (RH 62%) and natural daylight, giving three different pre-acclimation regimes. Half of the pots in each chamber were waterlogged by placing pots in 25 cm × 55 cm trays that were filled with water up to ca. 1 cm below the pot rim. As control, we placed the same number of pots in trays and watered with tap water only when required. The plants were tillering at the start of the treatments and had 3–4 fully expanded leaves per tiller. In mid-October, after 32 days of pre-acclimation under the different temperature treatments, all plants were cold acclimated for three weeks at 2°C under natural daylight conditions until early November, while continuing waterlogging treatments.

2.2 | Plant measurements

In mid-September before treatments commenced, plant height was measured on a subset of five pots with five plants per pot. In the same plants, chlorophyll content as mg chlorophyll/m² was recorded with a CCM-300 chlorophyll meter (Opti-Sciences, Hudson, NH, USA) on the newest fully expanded leaf in each plant

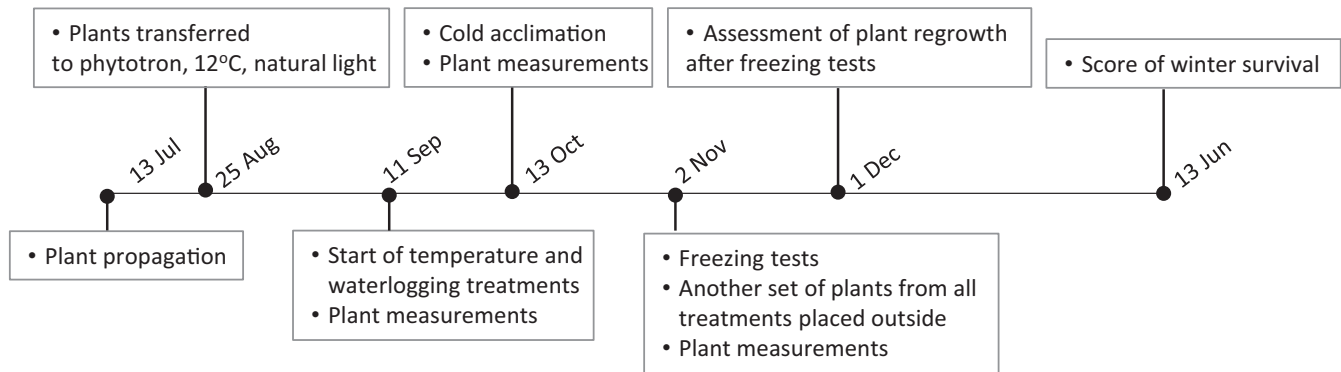


FIGURE 1 Timeline of treatments

per pot. Photosynthesis was measured using an EGM-4 CO₂ analyser (PP Systems, Amesbury, MA, USA) connected to a custom-made transparent (20 cm × 20 cm × 20 cm) respiration chamber. Then, shoots were cut at the soil base and roots cleaned carefully under tap water. We weighed shoots fresh and recorded shoot and root dry matter (DM) after drying at 60°C, 48 hrs. In mid-October, after one month of temperature and waterlogging treatments, photosynthesis was measured again on another subset of five pots per treatment and chlorophyll; number of tillers and number of leaves per tiller were recorded. In early November, after three weeks cold acclimation, chlorophyll content, plant height and biomass of roots and shoots were recorded on these plants in the same way as in September.

2.3 | Freezing tests

After cold acclimation, plants were cut 3.5 cm above soil. Freezing tests were performed as described by Höglind et al. (2010) with modifications since we placed individual pots with plants in two programmable freezers. Before freezing tests started, the temperature was lowered from 2°C to -3°C by 1°C h⁻¹ and kept at -3°C for approximately 12 hrs to avoid supercooling of the plants. Thereafter, we froze plants to five pre-determined temperatures ranging from -11°C to -26°C, two pots with five plants per pot per freezing temperature, treatment and freezer, that is 20 pots per treatment for the freezing tests. The cooling rate was 1°C h⁻¹ until -10°C was reached, and from then, the freezing progressed at 3°C h⁻¹. A subset of four pots from each treatment was placed at 2°C and in darkness for comparison. After freezing, all plants were thawed at 2°C and darkness for 24 hrs and then transferred to a greenhouse with 18°C, 24 hrs light for three weeks to record survival and measure regrowth. Light was provided with cool white fluorescent lamps—Philips TLD 58W 840, with 150 μmol/m² s⁻¹ irradiance at plant level, measured with a LI-COR LI-190 Quantum sensor (LI-COR, NE, USA), within the range 400–700 nm. The number of surviving plants per pot was counted, and the regrowth of the surviving plants was measured as dry matter (DM) after cutting at 3.5 cm above soil surface and drying at 60°C for 48 hrs.

We estimated the probability of death of the plants based on the scores of the number of surviving plants.

2.4 | Winter survival

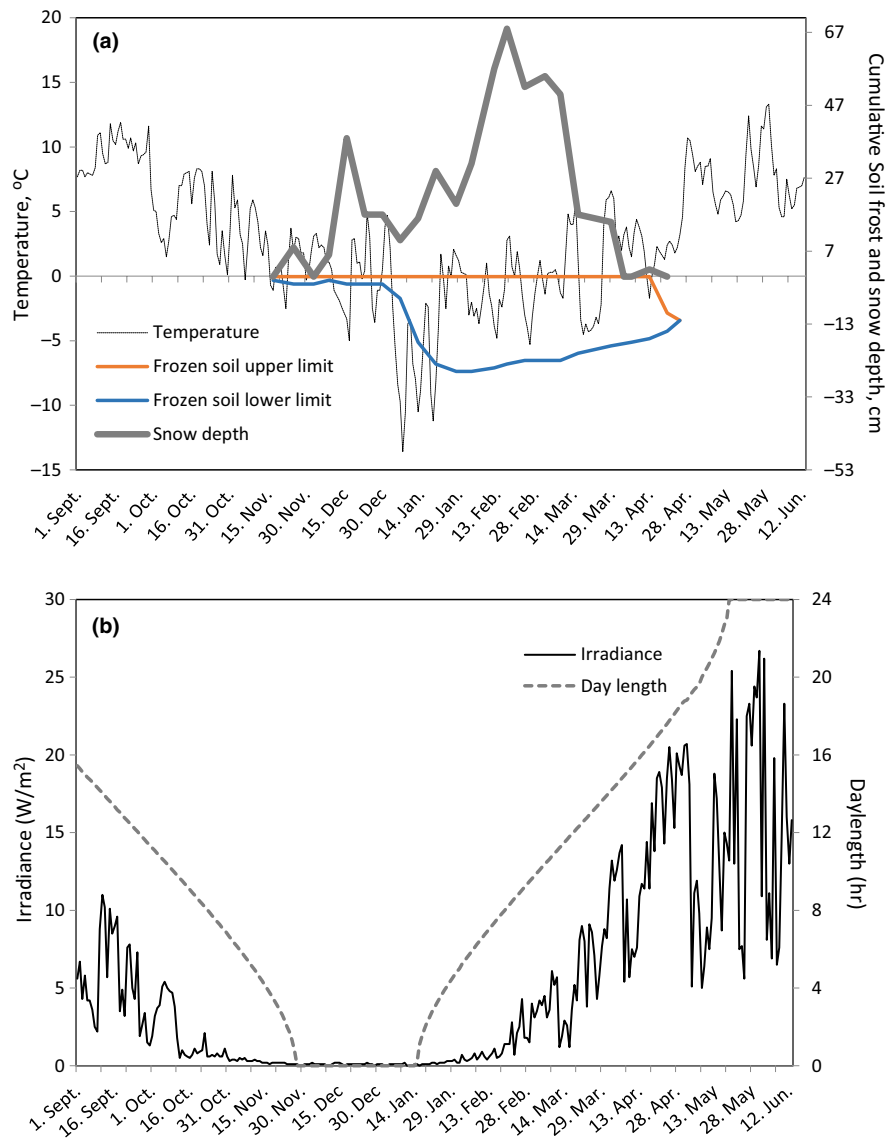
Another set of plants from each temperature and waterlogging treatment was placed outside in the beginning of November under ambient winter conditions. There were 5–7 pots per temperature and waterlogging/no-waterlogging treatment. In addition, 10 pots with plants not treated with the different temperature or waterlogging were placed outside from mid-September. In mid-June the following year, we counted the number of surviving plants and tillers and measured DM regrowth by cutting leaves at soil surface and drying at 60°C for 48 hrs.

The winter weather in 2015–16 was reasonably stable. The accumulated snow depth was measured manually about once a week at a single point with a graded rod. Soil frost depth was measured at the same time using a graded soil frost tube containing a solution of methylene blue dye. When frozen, the solution turns colourless (Rickard & Brown, 1972). The melting of soil frost in spring was measured both at the soil surface (upper limit) and at the lower limit of the frost. A continuous snow cover from the beginning of December lasted until early April (Figure 2a). The air temperatures during the winter/spring were higher than normal in almost all winter months, except January. Precipitation was higher than normal during the autumn months, but lower than normal from January on. May was warmer than normal (7.9°C versus 5.1°C), while June was slightly colder (8.8°C versus 9.3°C). Figure 2b shows the irradiance and day length during the experimental period. As Tromsø is located above the Arctic Circle, day length decreases rapidly during autumn. Between 27 November and 15 January the sun never rises, while it never sets between 20 May and 22 July.

2.5 | Statistical analysis

The experiment was designed with waterlogged and non-waterlogged (control) treatments in trays, 8 pots per tray. The trays were

FIGURE 2 A: Daily average air temperature, 2 m height (°C) and cumulative depth (cm) of frozen soil and snow. The melting of soil frost in spring was measured both from soil surface (upper limit) and from the lower limit of the frost. B: irradiance (Wm⁻²) and day length (h) during the experimental period (September 2015–13 June 2016) at Holt, Tromsø. [Colour figure can be viewed at wileyonlinelibrary.com] Source: Agrometeorology Norway (LMT)



randomized within each of the three phytotron chambers (temperature treatments of 3°C, 7°C or 12°C, respectively). For most plant measurements, we selected five pots randomly from each waterlogging and temperature treatment, and these were considered parallels in the statistical analyses. For freezing tests, we randomly selected 20 pots per treatment. The pots were placed in two freezers, ten pots per treatment in each freezer; thus, there were two pots per freezing temperature and treatment in each freezer.

The effect of temperature and waterlogging on death rate after freezing was analysed using the following mixed logistic model.

$$\pi_{ijklt} = P(y_{ijkl}(t) = 1 | F_k) = P(y = 1) = \frac{e^{\mu + \alpha_i + \beta_j + \gamma \cdot t + F_k}}{1 + e^{\mu + \alpha_i + \beta_j + \gamma \cdot t + F_k}}$$

The effect of temperature and waterlogging on leaf regrowth after freezing was analysed using the following mixed linear model.

$$z_{ijkl}(t) = \mu + \alpha_i + \beta_j + \gamma \cdot t + (\alpha\gamma)_i \cdot t + F_k + \epsilon_{ijkl} \quad (2)$$

In (1) and (2), μ is an unknown constant, α_i is the main effect of temperature i ($i = 3, 7, 12^\circ\text{C}$), β_j is the main effect of waterlogging j ($j = \text{wl}, \text{c}$), γ and $(\alpha\gamma)_i$ are coefficients that estimate the effect of the actual freezing temperature (t) on the response variable (death rate or leaf regrowth), in (2) depending on temperature (i), F_k is the random effect of freezer k ($k = 1, 2, 3, 4$), and ϵ_{ijkl} is an error term. $y_{ijkl}(t)$ in (1) is the observation [dead (1)/ alive (0)] made on a plant in pot l (5 plants per pot), at temperature i , waterlogging j in freezer k exposed to the actual freezing temperature t . $z_{ijkl}(t)$ in (2) is the measured biomass in pot l , at temperature i , waterlogging j in freezer k exposed to freezing temperature t . Before settling on the models in (1) and (2), models including all main effects, second- and third-order interactions were estimated, and we decided to omit clearly non-significant terms.

The effect of temperature and waterlogging on plant morphological characteristics (plant height, leaf numbers, chlorophyll content), net CO₂ assimilation and on biomass, dry matter content in shoots and root: shoot ratio was analysed using the following linear model.

$$z_{ijk} = \mu + \alpha_i + \beta_j + \epsilon_{ijk} \quad (3)$$

In (3), z_{ijk} is the measured response variable in pot k ($k = 6, 7, \dots, 35$) at temperature i ($i = 3, 7, 12^\circ\text{C}$) and waterlogging j ($j = \text{wl}, \text{c}$). μ is an unknown constant, α_i is the main effect of temperature i , and β_j is the main effect of waterlogging j . The interaction effects $(\alpha\beta)_{ij}$ are omitted because they were clearly not significant for any of the response variables. ε_{ijk} is an error term.

The Glimmix procedure in SAS[®] was used to estimate all models.

3 | RESULTS

3.1 | Plant morphology, growth and photosynthesis

We measured photosynthesis and recorded plant characteristics before temperature and waterlogging treatments started (Mid-September, starting point) and after one month of treatments. Plant height increased from start to Mid-October, and both height and number of leaves per plant were significantly affected by pre-acclimation temperature treatments with taller plants and more leaves per plant at 12°C than at 7°C or 3°C (Table 1). The temperature also affected chlorophyll content, with higher content in plants pre-acclimated at 12°C than at 3°C . There were no significant effects of waterlogging treatments on these plant characteristics. There were no significant effects of waterlogging or temperature on photosynthesis.

The biomass of the plants was measured in mid-September before treatments started and in the beginning of November after waterlogging and temperature treatments and three weeks of cold acclimation (Table 2). Waterlogging treatments affected shoot growth significantly ($p = .041$), with more shoot biomass in waterlogged plants than in control plants, but waterlogging did not affect root biomass. The temperatures affected the root: shoot ratio significantly with a higher root: shoot ratio at 7°C than at 3°C . The DM content in leaves was also significantly affected by pre-acclimation temperatures, with the lowest DM percentage at 12°C . There were no effects of waterlogging on root: shoot ratio or DM content, nor any interactions between temperature and waterlogging treatments on any of these plant characteristics.

3.2 | Freezing tolerance

We recorded regrowth and survival in a subset of plants at 2°C and darkness while other subsets were subjected to programmed freezing to detect freezing tolerance. All plants kept at 2°C survived, and the regrowth after three weeks at 18°C was significantly ($p = .0003$) higher in plants pre-acclimated at 3°C than at 7°C or 12°C (Figure 3).

Freezing tolerance with LT_{50} values for the different waterlogging and pre-acclimation temperatures is shown in Figure 4. Waterlogging affected freezing tolerance negatively and plants that

TABLE 1 Effect of four weeks of pre-acclimation temperature and waterlogging treatments (C—watered when required, WL—waterlogged) measured mid-October on plant height (cm), numbers of tillers and leaves per plant, chlorophyll content (mg/m^2) and on net CO_2 assimilation (net ppm CO_2 assimilated per minute per pot). Results are Lsmeans (15 pots \times 5 plants for waterlogging treatments and 10 pots \times 5 plants per temperature treatment) followed by standard error of the means. "Starting point" refers to plants ($n = 5$ pots \times 5 plants) measured mid-September before temperature and waterlogging treatments started

Treatment	Plant height, cm		No of tillers per plant		No of leaves per plant		Chlorophyll, mg/m^2		ppm CO_2 $\text{pot}^{-1} \text{min}^{-1}$	
		SEM		SEM		SEM		SEM		SEM
Starting point	24.6	0.24	–	–	–	–	250	6.3	16.55	4.38
Waterlogging										
C	28.1		3.37		12.7		249		12.33	
WL	28.9		3.43		13.2		257		15.58	
Pre-acclimation temperature										
3°C	27.6b		3.34		11.1b		238b		11.76	
7°C	27.6b		3.26		12.5b		251ab		13.12	
12°C	30.3a		3.60		15.2a		270a		16.98	
Significance										
Waterlogging	ns	0.56	ns	0.12	ns	0.65	ns	5.56	ns	1.24
Temperature	*	0.69	ns	0.15	***	0.53	*	7.19	ns	1.52

Note: Lsmeans in the same column and the same treatment (waterlogging, temperature level) followed by different letters are significantly different according to Tukey's test ($p \leq .05$)

ns $0.05 < P$

*** $p \leq .001$

** $0.001 < p \leq .01$

* $0.01 < p \leq .05$

TABLE 2 Effects of waterlogging (C—watered when required, WL—waterlogged) and pre-acclimation temperature on biomass (mg dry matter (DM) pot⁻¹), DM content and root: shoot ratio in plants measured on 2 November after one month of water and temperature treatments, and three weeks cold acclimation at 2°C. Results are lsmeans (15 pots á 5 plants for waterlogging treatments and 10 pots á 5 plants per temperature treatment). The “Starting point” are means followed by standard error of plants ($n = 5$ pots á 5 plants) measured on 11 September before temperature and waterlogging treatments started

Treatment	Shoot DM, mg pot ⁻¹		Root DM, mg pot ⁻¹		Total plant DM, mg pot ⁻¹		% DM in shoots		Root: shoot ratio	
		SEM		SEM		SEM		SEM		SEM
Starting point	569	65	300	61.6	869	125	15.0	0.66	0.50	0.06
Waterlogging										
C	1,151b		1,195		2,346		21.7		1.04	
WL	1,377a		1,321		2,698		21.9		0.99	
Pre-acclimation temperature										
3°C	1,302		1,128		2,431		22.4a		0.88b	
7°C	1,088		1,201		2,288		22.6a		1.12a	
12°C	1,402		1,445		2,847		20.4b		1.05ab	
Significance and S.E.M										
Waterlogging	*	74	ns	78	ns	139	ns	0.23	ns	0.05
Temp.	ns	91	ns	96	ns	170	***	0.34	*	0.06

Note: Lsmeans in the same column and the same treatment (waterlogging, temperature level) followed by different letters are significantly different according to Tukey's test ($p \leq .05$).

ns $0.05 < P$

*** $p \leq .001$

** $0.001 < p \leq .01$

* $0.01 < p \leq .05$

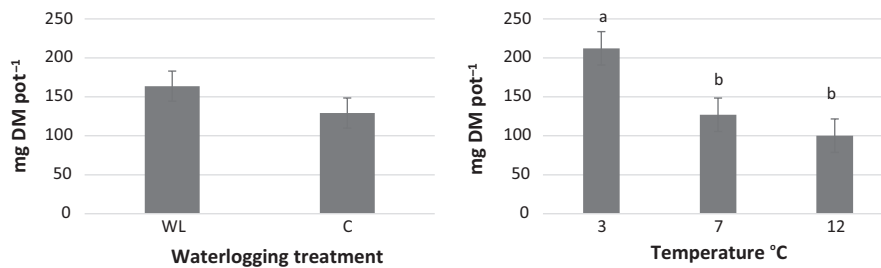


FIGURE 3 Regrowth of waterlogged plants (WL) or control (C, watered when required) measured on 2 November after one month of water and temperatures treatments, and three weeks cold acclimation at 2°C. The plants were then cut at 5 cm stubble height and placed for 48 hr at 2°C in darkness. Regrowth was measured as lsmeans mg DM pot⁻¹ ± standard error ($n = 4$ pots, 5 plants per pot) after three weeks at 18°C and 24 hr light

were waterlogged had a significantly ($p < .0001$) higher probability of death than control plants. Pre-acclimation temperature also affected freezing tolerance, and plants treated at 12°C before cold acclimation had significantly higher probability of death ($p < .0001$) after freezing than after treatments of 7°C or 3°C.

Regrowth capacity after the freezing treatment was affected negatively by waterlogging ($p = .0001$) with less regrowth in waterlogged plants than in control plants (Figure 5). There was a clear overall negative effect of increasing pre-acclimation temperature on regrowth ($p < .0001$). These differences were significant until a temperature -24°C, at which regrowth of plants treated with 3°C and 7°C was similar. There were no interactions between pre-acclimation temperature and waterlogging treatment.

3.3 | Overwintering

The waterlogging and temperature treatments had no significant effects on regrowth in spring or on the number of tillers and surviving plants. There were also no differences between pre-acclimation treatments and waterlogging versus. control in plants that had overwintered under ambient winter conditions (Figure 6).

4 | DISCUSSION

The results of our study on a northern cv. of timothy show that both waterlogging and increasing pre-acclimation temperatures

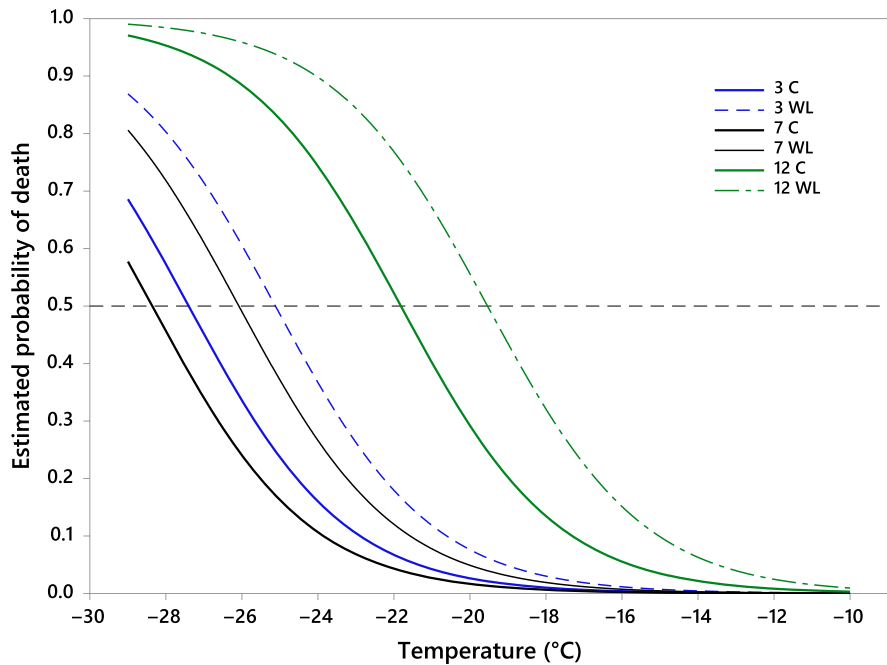


FIGURE 4 Estimated probability of death and LT_{50} after freezing tests of waterlogged plants (WL) or control (C, watered when required) measured on 2 November after one month of water and temperature treatments (3°C, 7°C or 12°C), and three weeks cold acclimation at 2°C. LT_{50} is estimated as the temperature (°C) corresponding to where the dotted line crosses the actual curve [Colour figure can be viewed at wileyonlinelibrary.com]

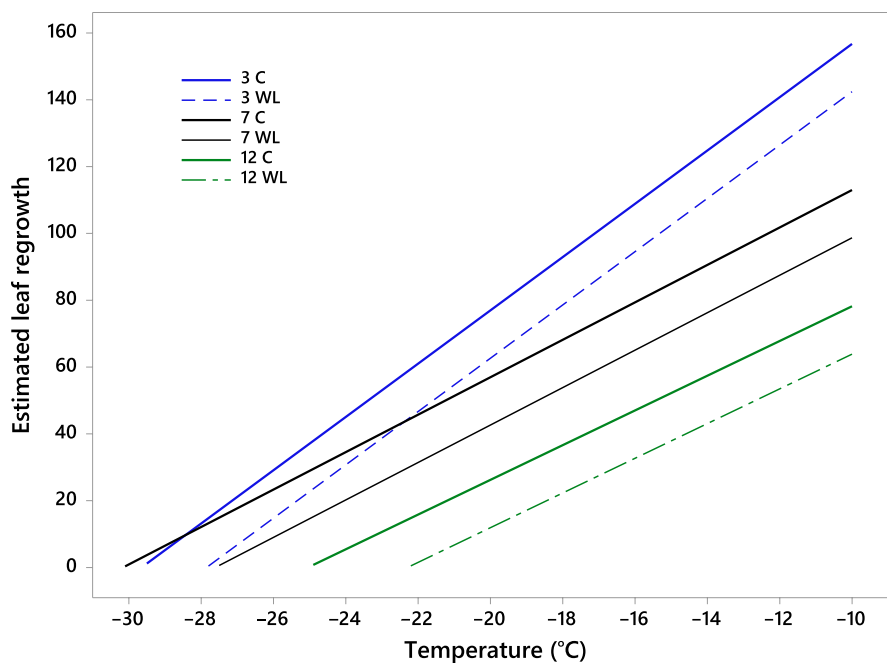


FIGURE 5 Estimated leaf regrowth (mg DM pot^{-1}) after freezing tests on waterlogged plants (WL) or control (C, watered when required) that were kept for one month at three different pre-acclimation temperatures (3°C, 7°C or 12°C) and three weeks cold acclimation at 2°C. The regrowth was recorded when plants had grown for three weeks at 18°C after freezing tests [Colour figure can be viewed at wileyonlinelibrary.com]

have negative effects on hardening, with lower freezing tolerance and less regrowth after freezing. The present study is to our knowledge the first to combine waterlogging with different temperature treatments. However, we found no interaction between pre-acclimation temperatures and waterlogging treatments, contrary to our hypothesis.

The adverse effect of increasing pre-acclimation temperature on hardening was more pronounced than the negative effect of waterlogging. A pre-acclimation temperature of 12°C reduced the leaf regrowth by about 69% compared to the 3°C treatment, as an average over all freezing and pre-acclimation temperatures.

This agrees with Dalmannsdottir et al. (2016, 2017), who also found that a rise in pre-acclimation temperatures resulted in lower freezing tolerance and reduced photoacclimation compared with lower pre-acclimation temperatures. The negative effect of rising autumn temperatures on freezing tolerance may contribute to poorer overwintering of timothy in northern regions, as temperatures continue to rise, and winter conditions generally become more stressful (Hanssen-Bauer et al., 2015; Mikkonen et al., 2015; Vikhamar-Schuler et al., 2016).

Waterlogging caused higher death rates after freezing compared to the control treatments. Regrowth after freezing was 24% lower

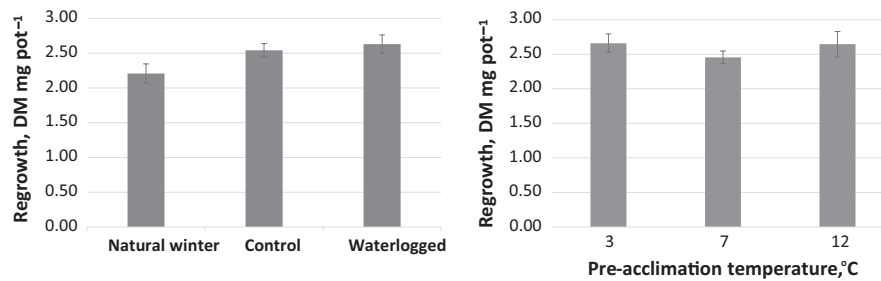


FIGURE 6 Regrowth in spring 13. June of plants treated with one month on waterlogging treatments (A) and three different pre-acclimation temperatures (B) in autumn. The plants were placed outside under natural winter conditions after three weeks cold acclimation at 2°C. The treatment “natural winter” was not subjected to waterlogging or temperature treatments but overwintered from autumn under ambient winter conditions. Results are lsmeans mg DM pot⁻¹ ± standard error

in waterlogged plants than in control plants averaged over all freezing temperatures and pre-acclimation temperatures. Jurczyk et al. (2013) and Jurczyk et al. (2015) reported conflicting results with both reduced and improved freezing tolerance in meadow fescue and perennial ryegrass after waterlogging. They related these result to differences in temperature conditions under waterlogging in the two studies, that is higher temperature under waterlogging led to improved freezing tolerance. Dalmanndottir et al. (2012) found, on the other hand, increased freezing tolerance in timothy treated with waterlogging in a location with low average autumn temperatures (average of 3°C), while there were no differences in freezing tolerance between control and waterlogged treatments in a location with higher autumn temperatures. This contrasts with our findings that waterlogging reduced freezing tolerance at all tested pre-acclimation temperatures.

Bertrand et al. (2001) suggested that timothy reduced its metabolic rate as a response to anaerobic conditions. Bertrand et al. (2003) found an increase in total non-structural carbohydrates (TNC), particularly of sucrose, in timothy when grown under oxygen deficient conditions. They suggested this increase in sucrose in timothy was a reallocation of storage carbohydrates from stubbles and roots to leaves. This could partly explain the greater reduction of regrowth we found in waterlogged plants after freezing compared to control plants. The effect of flooding or waterlogging on freezing tolerance also varies between both species and genotypes. Timothy is considered a very winter-hardy and flooding-tolerant grass species that can sustain long periods of hypoxic/anaerobic conditions caused either by flooding or ice encasement (Banach et al., 2009; Gudleifsson, 2010). However, there are variations in tolerance between cultivars in response to anoxic conditions (Höglind et al., 2010). Therefore, in a southern type of timothy responses to waterlogging could be more severe than the effects we found in this northern winter-hardy cv.

The waterlogged plants were larger, with significantly more shoot biomass than control plants. Waterlogging may have disturbed growth cessation, which is considered a pre-condition of successful cold acclimation, thereby contributing to the lower freezing tolerance in waterlogged timothy (Østrem, Rapacz, Larsen, Dalmanndottir, & Jørgensen, 2014). Although flooding generally impairs growth of herbaceous plants, waterlogging is found to be less stressful than total submergence (Jackson & Drew, 1984; Liu & Jiang, 2015), and in

some tolerant species, waterlogging even stimulated shoot growth (Zong et al., 2015).

Andrews and Pomeroy (1981) found that low-temperature flooding reduced freezing tolerance in winter barley and winter wheat when plants were totally submerged for two weeks, while waterlogging to 2 cm below soil level did not affect freezing tolerance. In our study, the water level in the pots was approximately 1 cm below soil level in waterlogged plants, which is less harmful than total submergence, but the plants were waterlogged for a long time. Due to the increase in precipitation observed during the last decades in northern regions, scenarios of this kind of prolonged soil waterlogging are quite realistic (Hanssen-Bauer et al., 2015; Hartmann et al., 2013).

There were no significant differences in photosynthetic rate between waterlogging treatments. Flooding is generally considered to impair photosynthesis, but Caudle and Maricle (2012) found an increased photosynthetic rate in tolerant plants as a response to flooding compared to more sensitive plants where photosynthetic rates decreased during flooding.

The pre-acclimation temperatures affected plant morphology, with taller plants and more leaves at 12°C than at 7°C and 3°C, but there were no differences in plant biomass between the temperature treatments. Solar radiation diminishes rapidly during the autumn months in Sub-arctic and Arctic regions, and light conditions may restrict growth more than temperature. Similar trends were observed by Dalmanndottir et al. (2017), who at the same latitude found less differences in biomass production between high (12°C) and low (6°C) pre-acclimation temperatures in autumn months with less daylight (September–October) than in August–September. Plants pre-acclimated at 7°C had a higher root: shoot ratio than plants at 12°C and 3°C. Davidson (1969) suggested that the root:shoot ratio was smallest at the optimum growth temperature. However, this may not apply to plants under cold acclimation, where the ability to store photosynthates in storage organs such as stubbles and roots is important for winter survival. Dry matter content was lowest in plants grown at 12°C. Andrews and Pomeroy (1981) found a strong negative correlation between water content and freezing tolerance in crown tissue of winter cereals. Dehydration of cells is an important strategy to increase the osmotic potential in cell sap and thereby minimize the risk of intracellular freezing (Kacperska-Palacz, 1978).

The difference in photosynthetic rate per pot was not significant between temperature treatments although the plants at 12°C had a significantly higher chlorophyll content than plants grown at 3°C. The acclimation of the photosynthetic apparatus to cold involves increased photosynthetic activity (photochemical quenching), or dissipation of surplus energy as heat (non-photochemical quenching). Reducing the physical size of the light-harvesting complex may be another strategy to protect the photosystem. All these mechanisms are involved in protection against photoinhibition caused by cold (Hüner et al., 1993). Dalmannsdottir et al. (2017) found that photochemical quenching increased at lower temperatures. This mechanism is more often used by plants of northern origin as a response to high light intensity/low temperature to reduce photoinhibition. The reduction of chlorophyll content may be a way of reducing the risk of photoinhibition; thus, the plants grown at 3°C may have both reduced the chlorophyll content and increased photosynthetic activity to avoid photoinhibition (Dalmannsdottir et al., 2017; Hüner et al., 2012). This could also explain why the difference in photosynthetic rate per pot was not significant between plants grown at 12°C and 3°C, even though plants grown at the lower temperature had both less leaf biomass and lower chlorophyll content than plants grown at 12°C. The plants pre-acclimated at 12°C may have allocated more carbohydrates from photosynthesis to growth, while plants at 7°C and 3°C may have allocated more to storage (Thorsteinsson, Harrison, & Chatterton, 2002).

The waterlogging and temperature treatments had no negative effects on survival or growth in spring, after overwintering outside. The winter conditions were favourable that year with a relatively stable snow cover insulating the plants from extreme frosts, and no problems with ice encasement (Figure 1). This could explain the good winter survival of the plants. However, plants are dependent on cold acclimation for adequate winter hardiness against biotic and abiotic winter stresses, and the level of freezing tolerance is highly correlated with winter survival (Larsen, 1994). In timothy for instance, freezing tolerance correlates with the ability to tolerate ice encasement (Höglind et al., 2010). Therefore, in a winter with adverse conditions, the lower freezing tolerance caused by waterlogging or increasing pre-acclimation temperatures could have resulted in poorer winter survival of timothy.

5 | CONCLUSIONS

Increasing pre-acclimation temperatures and waterlogging reduced freezing tolerance leading to an increase in the probability of death and a reduction in regrowth after freezing, compared to treatments without waterlogging and with lower pre-acclimation temperatures. The results suggest that in years with stressful winter conditions, waterlogged soils and higher autumn temperatures could increase the risk of poor winter survival.

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