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JOURNAL TITLE: Environmental and experimental botany

USER JOURNAL TITLE: Environmental and experimental botany.

ARTICLE TITLE: Photoperiod, [CO₂] and soil moisture interactively affect phenology in trembling aspen: Implications to climate change-induced migration

ARTICLE AUTHOR: Inoue, Sahari

VOLUME: 180

ISSUE:

MONTH:

YEAR: 2020

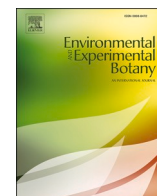
PAGES: 104269-

ISSN: 0098-8472

OCLC #: 38907803

Processed by RapidX: 3/17/2021 12:19:46 PM

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Photoperiod, [CO₂] and soil moisture interactively affect phenology in trembling aspen: Implications to climate change-induced migration

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ARTICLE INFO

Keywords:

[CO₂]
Phenology
Photoperiod
Populus tremuloides Michx.
Soil moisture
Tree migration

ABSTRACT

Trees may migrate northward in response to climate change, which would expose them to different environmental conditions, such as [CO₂], photoperiod, soil moisture. Changes in these environmental conditions will likely influence tree phenology and growth. We grew *Populus tremuloides* Michx. from seeds for two growing seasons under two [CO₂] (400 vs 1000 μmol mol⁻¹), four photoperiod regimes (corresponding to 48 °N (seed origin), 52 °N, 55 °N, and 58 °N latitude), two soil moistures (60 %–75 % vs 13 %–20 %) and examined phenological responses. We found that longer photoperiods extended growing season by delaying autumnal phenology and increased growth. Low moisture also delayed autumnal phenology but reduced growth, and the effects were reduced by elevated [CO₂]. Surprisingly, low moisture reduced cold hardiness of aspen. Elevated [CO₂] delayed bud break in the spring and the effects were magnified by the low moisture. The interactive effects of photoperiod, CO₂, and soil moisture on phenology, cold hardiness, growth, and growing season indicate the complex nature of northward migration that has been predicated under the scenario of climate change. Our results suggest that these interactions should be considered when planning latitudinal seed transfers and predicting the migration of boreal trees in response to climate change.

1. Introduction

Rising atmospheric CO₂ concentration ([CO₂]) will continue to cause changes in the climate conditions of the earth, such as increase global temperature and changes in the spatial and temporal patterns of precipitation (Pachauri et al., 2014). The climate change can cause the northward shift of the present-day climate niches for 130 North American tree species by as much as 700 km but it is unknown to what extent populations of trees will actually track climate shifts through migration (McKenney et al., 2007). Migrating to higher latitudes, either naturally or by human assistance, however, will expose them to different photoperiod regimes than what they are adapted to, e.g., longer photoperiods during the growing season and faster rates of changes in photoperiod during seasonal transitions (Thomas and Vince-Prue, 1997). These will likely alter the timing of phenological events, which are major determinants of tree growth and species distribution (Chuine, 2010; Chuine and Beaubien, 2001; Way and Montgomery, 2015). In addition to the potential effects of photoperiod change, the expected increases in [CO₂] and the associated changes in water availability (Pachauri et al.,

2014) may also affect the tree phenological responses (Estiarte and Peñuelas, 2015; Piao et al., 2019). Past studies, however, have primarily focused on the effects of individual factors (e.g., Leuzinger et al., 2005; Taylor et al., 2008). While a good understanding of individual effects is essential, changes in latitudes and climate variables will more likely interact with each other, and the interactive effects may be different from the summation of individual effects.

Photoperiod is one of the critical environmental factors regulating seasonal changes in the phenology, physiology, and growth of temperate and boreal plants (Inoue et al., 2019, 2020; Jackson, 2009; Junttila, 1980; Li et al., 2015; Newaz et al., 2017a, 2016; Newaz et al., 2017b; Tedla et al., 2019, 2020a; Weiser, 1970). Growth cessation, bud set, and cold acclimation in the fall are induced mainly by longer nights and lower temperatures (Jackson, 2009; Soolanayakanahally et al., 2013; Weiser, 1970). These phenological events are generally related to the adaptation of the species to the local environment, for example, northern populations set bud earlier and acquire greater cold hardiness than southern populations (Campbell and Sorensen, 1973; Junttila, 1982; Kozłowski and Pallardy, 2002). While many studies have demonstrated

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the importance of temperature to the regulation of leaf senescence, the influence of photoperiod on leaf senescence has only been reported for few tree species, e.g., *Populus tremula* (Fracheboud et al., 2009), *Populus tremula* (Keskitalo et al., 2005). Since trees will be exposed to new photoperiod regimes when migrating northward, their responses to the change in photoperiod can influence the success of the migration, for instance, the change in photoperiod may lead to a loss of synchronization between their phenology and the environment at the new site. Longer photoperiods can delay growth cessation and increase the risk of frost damage in the autumn (Howe et al., 1995). Furthermore, the faster rate of photoperiod change during the summer-autumn transition at higher latitudes may jeopardize the proper development of cold hardiness and buds (Bigras et al., 2001; Newaz et al., 2017a; Tedla et al., 2020b, 2019). Trees without an adequate level of cold hardiness may not survive cold winters (Li et al., 2003, 2015; Welling et al., 1997) and frost-damaged buds may not be able to flush in the following spring (Ostry et al., 1989; Tedla et al., 2020b). Delays in senescence may reduce nutrient resorption and reduce nutrient reserves. On the other hand, delayed senescence can extend the period of photosynthetic carbohydrate production (Hoch et al., 2001) and increase the storage of carbohydrates, which may benefit bud break next spring (Kaelke and Dawson, 2005; Regier et al., 2010). Although the effect of photoperiod on bud break is largely unclear, some studies have found that longer photoperiods can promote dormancy release and lead to earlier bud break in some species (Laube et al., 2014; Newaz et al., 2016). A good understanding of how photoperiod regimes at higher latitudes may affect tree growth, phenology, dormancy induction, and cold hardiness development may be critical for predicting the extent of northward migration and the potential performance of trees at new locations in the context of climate change-induced northward migration.

[CO₂] and soil moisture can also affect tree phenology and cold hardiness, and the effects can vary with species and other environmental factors (Gunderson et al., 1993; Piao et al., 2019). 3Free Air CO₂ Enrichment (FACE) experiments on *Populus* spp. (Dickson et al., 2000; Karnosky et al., 2003) have found delays in the timing of leaf senescence under elevated [CO₂], suggesting a possible extension of the growing season in the future (Taylor et al., 2008), whereas the opposite effect of elevated [CO₂] has been found in some other species (Jach and Ceulemans, 1999). Elevated [CO₂] may also expedite bud burst in the spring (Jach and Ceulemans, 1999). Soil moisture is a potentially important cue to both autumn and spring bud phenology. Low water availability may delay bud burst in the spring but advance bud formation and leaf senescence in the fall (Estiarte and Peñuelas, 2015; Myers et al., 1998). Interactions among different factors can complicate the prediction of trees' responses and performance. For instance, the effects of low soil moisture on the phenology of trees in the fall (Holland et al., 2016; Winner and Pell, 2012) and cold hardiness development (Kreyling et al., 2012) can be modified by changes in photoperiod and elevated [CO₂]. However, the interactive effects of multiple factors are generally not well understood.

Trembling aspen (*Populus tremuloides* Michx.) is the most abundant deciduous tree species in Canada's boreal forest and has important ecological and commercial values. The phenological events of *Populus* species are known to be strongly adapted to local photoperiod regimes (Luquez et al., 2008) and strong genetic differentiation and clinal variation along the latitudinal gradient have been found in common garden studies (Soolanayakanahally et al., 2013). Generally, shorter photoperiods are a significant trigger for the initiation of autumnal phenological events (Keskitalo et al., 2005; Pauley and Perry, 1954). Thus, changes in photoperiod associated with northward migration may have a strong impact on their phenology. Furthermore, trembling aspen is sensitive to water stress (Dang et al., 1997; Peterson and Peterson, 1992). Recent severe droughts, in combination with other stress factors such as insects and pathogens, have resulted in declines and die-backs in large areas of trembling aspen in west-central Canada (Hogg et al., 2008; Marchetti et al., 2011; Worrall et al., 2013), raising serious concerns over the

future of deciduous boreal forests as rising temperatures will likely lead to more frequent and more severe droughts in the future (Hogg and Hurdle, 1995; Hogg and Bernier, 2005). Since aspen shows a considerable genotypic variation in growth performance, hydraulic traits, water use efficiency, and drought tolerance (DesRochers et al., 2007; Fichot et al., 2009; Monclus et al., 2006; Morrison et al., 2000; Silim et al., 2009), climate change will likely cause shifts in the distribution of its populations. Controlled environment studies are necessary for projecting the potential scope of the shift.

The main objective of this study was to investigate the phenotypic plasticity of trembling aspen in response to the interactions of [CO₂], photoperiod, and soil moisture regimes in the context of climate change-induced northward migration. We hypothesize that the changes in photoperiod regime associated with northward migration and elevated [CO₂] would delay the autumn phenological events of trembling aspen, resulting in a longer growing season and more growth, and that low soil moisture would partially offset the delay in the development of dormancy and cold hardiness. We also hypothesize that photoperiod regimes at latitudes north of seed origin in combination with elevated [CO₂] would expedite bud break in the spring.

2. Materials and methods

2.1. Plant materials

Trembling aspen (*Populus tremuloides* Michx.) catkins were collected on 9 June 2016 from 10 free-pollinated trees in Thunder Bay, Ontario, Canada (48.38 °N, 89.25 °W). The catkins were air-dried for five days at room temperature, and seeds were extracted according to Moench (1999). The seeds from different trees were mixed and stored in a sealed glass bottle at -4 °C.

Seeds were sown in germination trays filled with 1:1 (v/v) mixture of peat moss and vermiculite in a greenhouse at Lakehead University Thunder Bay campus. The day/night temperatures in the greenhouse were maintained at 24/14 (±2) °C, and the natural photoperiod was extended to 16 h by using high-pressure sodium lamps (P.L. Systems, Grimsby, ON, Canada) which gave about 600 μmol m⁻² s⁻¹ PAR at the canopy level. All trays were well-watered but not fertilized during the germination phase. The seedlings were individually transplanted into pots (12 cm in height, and 12 cm and 9.5 cm in the top and bottom diameter, respectively) filled with a mixture of peat moss and vermiculite (7:3 v/v) 18 days after the completion of germination.

2.2. Experimental design

The experiment was carried out in four greenhouses at Lakehead University, Thunder Bay campus. The treatments consisted of two levels of [CO₂] (400 μmol mol⁻¹ (AC-ambient [CO₂]) and 1000 μmol mol⁻¹ (EC-elevated [CO₂])), four photoperiod regimes corresponding to the photoperiod of 48.38 °N (seed origin) (48 °N), 51.68 °N (52 °N), 54.98 °N (55 °N), and 58.28 °N (58 °N) latitude, and two soil moisture regimes (high soil moisture - HSM and low soil moisture - LSM). The elevated [CO₂] treatment level (1000 μmol mol⁻¹) is consistent with a representative concentration pathway (RCP) 8.5 scenario adopted by Intergovernmental Panel on Climate Change (IPCC) (Pachauri et al., 2014), where the current high rate of CO₂ emission into the atmosphere is predicted to continue and the atmospheric [CO₂] will reach >1000 μmol mol⁻¹ in the year 2100. The experiment design was a split-split plot with the [CO₂] as the main plot, photoperiod as sub-plot, and soil moisture as sub-sub-plot. Each of the two [CO₂] treatments were randomly assigned to two of the four greenhouses (two replicates for each [CO₂]). The photoperiod treatment was nested within the [CO₂] treatment, and soil moisture was nested within the photoperiod.

2.3. Treatments and greenhouse environment controls

The environmental conditions in each greenhouse were independently monitored and controlled using the Argus Titan Environment Control System (Argus Controls Systems Ltd., Surrey, BC, Canada). The EC was achieved with the model GEN2E gas CO₂ generators made by Custom Automated Products Inc. (Riverside, California, USA). The photoperiod in each greenhouse was set to the longest photoperiod of the four treatments: the natural light level was supplemented (on cloudy days, early mornings, and late evenings) and natural day length was lengthened by using high-pressure sodium lamps (Model LR48877, P.L. Systems, Grimsby, ON, Canada). The shorter photoperiods were achieved by shading. A wooden frame was installed for each photoperiod treatment, and neutral density shading cloth was used to block light in the shorter photoperiod treatments. To avoid temperature modification inside the wooden frame due to the shading, we used a double-layered blackout film (white outside and black inside) which is specifically designed for greenhouse applications. The white side is an ultra-white layer to reflect all incident light and the black inside layer stops the transmission of longwave radiation. Furthermore, the benches were raised from the floor with welded mesh and fans were installed to ensure proper ventilation and air mixing. The photoperiod setting was adjusted weekly according to the calculated photoperiod for each of the four latitudes. The soil volumetric water content was monitored with Delta-T ML2x probe and HH2 moisture meter (Delta-T Devices, Cambridge, UK) and maintained between 60 % and 75 % of the field capacity for HSM and between 13 % and 20 % of the field capacity for LSM.

The experiment was run for two growing seasons, 1 November 2016 to 16 April 2017 (emulating the natural environmental conditions of 7 June to 20 November) and 1 November 2017 to 15 May 2018 (emulating the natural environmental conditions of 26 April to 7 November). The emulated natural dates were used in the subsequent presentations of the paper. The temperature and photoperiod were adjusted weekly based on the weekly averages of the past ten years for each of the four latitudes (Environment Canada weather records, ON). The daily temperature in each greenhouse was ramped at 6-h set points (averages of the past ten years). We fertilized seedlings in three phases, during which the fertilizer solution contained 33 mg N L⁻¹, 73 mg P L⁻¹ and 55 mg K L⁻¹ in the establishment/early season phase (7–21 June in the first growing season (first) and 26 April – 25 May in the second growing season (second)); 100 mg N L⁻¹, 60 mg P L⁻¹, 150 mg K L⁻¹, 80 mg L⁻¹, 40 mg L⁻¹ and 60 mg L⁻¹ in the rapid growth phase (22 June to 30 August in the first growing season 26 May to 30 August in the second growing season); and mg N L⁻¹, 44 mg P L⁻¹, and 83 mg K L⁻¹ in the hardening phase (1–25 September). After the completion of bud set and cold hardening in the first growing season, the seedlings were stored in walk-in cold storage at -4 °C. The seedlings were covered with plastic sheets to avoid freeze-desiccation during the storage. The seedlings were transferred into bigger pots (18 cm in height, and 16 cm and 14 cm in top/bottom diameter) in the second growing season.

2.4. Measurements and observations

2.4.1. Growth and growth cessation

Three seedlings were randomly selected from per treatment combination (two levels of [CO₂], 4 levels of photoperiods, and two levels of soil moisture) and two replications per combination (3 × 2 × 4 × 2 = 96 seedlings in total). The weekly measurement of stem elongation started on Julian day 225 (i.e., August 13th) and continued until the completion of the elongation (i.e., measured for 6 weeks). The height growth measurements were used to develop the height growth curve for each sample tree, which was used to determine the timing of growth cessation. According to MacLachlan et al. (2017), the date of growth cessation was defined as the date when 95 % of annual height increment was achieved, and the percentage height growth was calculated as follows:

$$\text{Height growth(\%)} = \frac{\text{Cumulative height increment (cm)}}{\text{Total height increment (cm)}} \times 100$$

The height growth curve was fitted using a 3-parameter sigmoidal model in SigmaPlot 12.5 (Systat Software Inc., San Jose, CA, USA). Due to time constraints, we only obtained height growth curve data in the first growing season.

2.4.2. Autumn bud phenology

The autumn bud phenology was observed at two-day intervals from August (emulated dates) to the completion of bud set in the first and second growing season. The development of terminal buds was monitored and recorded using four scales: stage 0 = no bud formation (Fig. 1A), stage 1 = appearance of terminal bud (Fig. 1B), stage 2 = terminal bud in light brown color (Fig. 1C), and stage 3 = terminal bud fully developed and covered by dark brown scales (referred to as bud set hereafter) (Fig. 1D).

2.4.3. Leaf senescence

Leaf senescence was observed at four-day intervals starting from August until all leaves turned yellow in both growing seasons. Leaf color change and the number of leaves that turned yellow were assessed visually. Leaves were determined as senescence when either more than 75 % of the leaf area turned yellow or the leaf fell off (Fu et al., 2018; Vitasse et al., 2009). The dates of 10 % (initiation) and 100 % (completion) of the leaves on the individual seedlings turned yellow were determined from the data.

2.4.4. Electrolyte leakage test

The electrolyte leakage method was used to determine the cold hardiness after the seedlings were stored at -4 °C for a month at the end of each of the two growing seasons (corresponding to mid-December of the natural season). The same seedlings were used for the phenological observations and cold hardiness determination. The tip of the terminal shoot from each seedling was cut into four 2-cm long sections and washed with distilled water (DW). Each section was put into a separate 50 mL falcon tube and stored at 4 °C for 24 h. Then, the samples were exposed to -5 °C, -15 °C, -30 °C and -45 °C using a programmable freezer (Model: 45-6.8, Scientemp Corporation, Adrian, MI, USA). The freezer temperature was lowered at a constant rate of 5 °C h⁻¹ (starting at 5 °C) and held for 1 h after each 5 °C change. The samples were maintained at each target temperature for 1 h before they were taken out of the freezer, placed in falcon tubes with 20 mL of DW, and incubated for 24 h at room temperature (21 °C). The samples were well shaken before the initial electrical conductivity of the solution was measured using an Accumet AR 20 electrical conductivity meter (Fisher Scientific, Ottawa, Canada). The falcon tubes were then heated in a dry oven at 80 °C for 2 h, and the second measurement of electrical conductivity was taken after 24 h at room temperature. The relative electrolyte leakage (REL) was calculated as follows (Zhang & Willison 1987);

$$\text{REL(\%)} = \left(\frac{\text{Initial conductivity}}{\text{Second conductivity}} \right) \times 100\%$$

LT₅₀ (the temperature causing 50 % REL) was estimated from a linear regression of REL with the target freezing temperatures (Arora, Wisniewski & Scorza 1992; Boorse, Bosma, Ewers & Davis 1998; Rapacz 2002; Morin et al. 2007). For the ease of graphic presentation, the absolute value of LT₅₀ was used in figure presentations

2.4.5. Spring bud phenology

The observation of spring phenology was conducted in the spring of the second growing season. Three seedlings from each treatment combination and replication were monitored at two-day intervals using a five-stage scale: stage 0 = dormant (Fig. 2A), stage 1 = bud open with a visible green tip (Fig. 2B) (referred to as bud break hereafter), stage 2 =

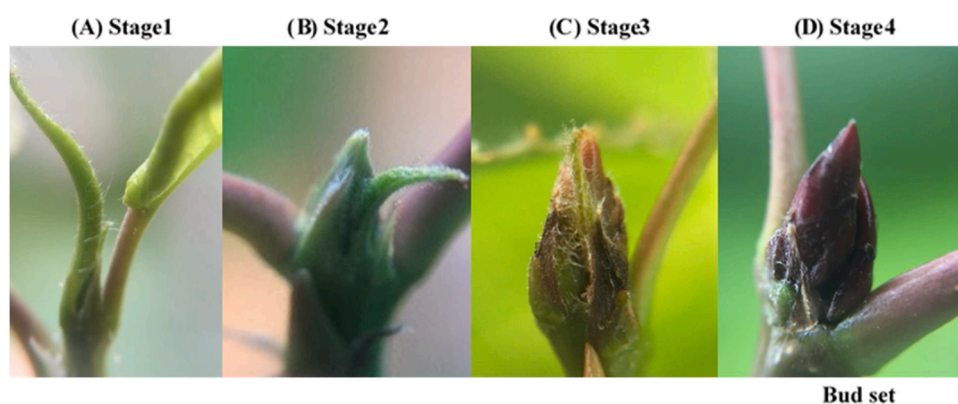


Fig. 1. A four-stage scale was used for monitoring autumn bud development. Picture (A) stage 0; no bud set (active growth phase), (B) stage 1; initiation of a terminal bud, (C) stage 2; terminal bud becoming light brown, and (D) stage 3; terminal bud fully developed and covered by dark brown scales (bud set).

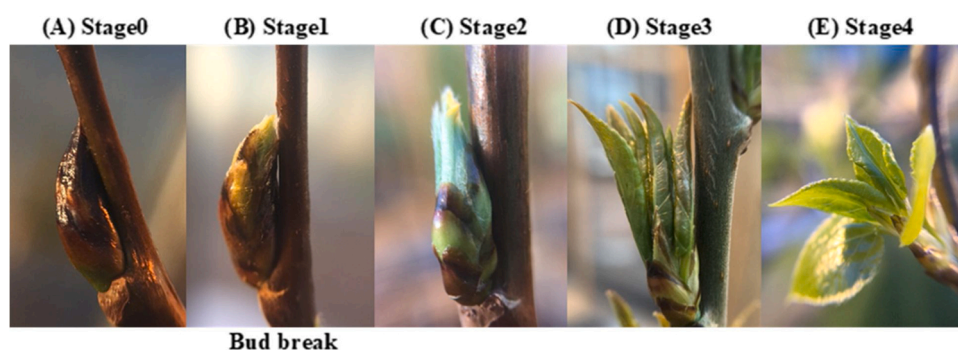


Fig. 2. A five-stage scale was used for monitoring spring bud phenology. Picture (A) stage 0; dormant bud, (B) stage 1; bud open with visible green tip (bud break), (C) stage 2; the large visible green tip is shown, (D) stage 3; green leaf emerged from the bud with leaf bases hidden, and (E) stage 4; leaf unfolding with very small leaves and visible petiole (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 1

ANOVA results (*p* values) for the effects of [CO₂] (C), photoperiod (P), soil moisture (SM) and their interactions on height increment, growth cessation, final height growth, autumn bud phenology, leaf senescence, spring bud phenology, and cold hardiness. The seedlings were exposed to two levels of [CO₂] (400 and 1000 μmol mol⁻¹), four photoperiod regimes (at 48 °N (seed origin), 52 °N, 55 °N, and 58 °N), and two soil moisture regimes (high (60 %–75 %v of field capacity) and low (13 %–20 %v of field capacity)). The numbers in bold are significant *p* values at <0.05.

Variable	Year	C (DF = 1)	P (DF = 3)	SM (DF = 1)	C × P (DF = 3)	C × SM (DF = 1)	P × SM (DF = 3)	C × P × SM (DF = 3)
<i>Growth cessation</i>	2017	0.672	0.047	0.012	0.808	0.777	0.309	0.544
<i>Final height</i>	2017	0.202	0.035	0.002	0.718	0.247	0.558	0.661
<i>Autumn bud phenology</i>								
<i>Bud set</i>	2017	0.984	0.137	0.818	0.017	0.251	0.648	0.930
	2018	0.444	0.028	<0.001	0.171	0.911	0.538	0.897
<i>Leaf senescence</i>								
<i>Initiation</i>	2017	0.324	0.054	0.084	0.181	0.107	0.575	0.748
	2018	0.010	0.235	<0.001	0.814	0.008	0.051	0.424
<i>Duration</i>	2017	0.356	0.217	0.229	0.622	0.573	0.215	0.494
	2018	0.036	0.574	0.001	0.772	0.009	0.075	0.456
<i>Completion</i>	2017	0.926	0.056	0.153	0.127	0.070	0.318	0.281
	2018	0.159	0.116	0.001	0.892	0.477	0.666	0.879
<i>Cold Hardiness</i>								
<i>LT50</i>	2017	0.535	0.003	0.397	0.042	0.501	0.992	0.876
	2018	0.343	0.316	<0.001	0.875	0.455	0.946	0.740
<i>Spring bud phenology</i>								
<i>Bud break</i>	2018	0.038	0.611	0.055	0.622	0.024	0.459	0.264
<i>Leaf unfolding</i>	2018	0.067	0.275	0.797	0.112	0.009	0.168	0.980

*DF = degree of freedom.

large visible green tip shown (Fig. 2C), stage 3 = green leaf emerged from bud scales with leaf bases hidden (Fig. 4.2D) and stage 4 = leaf unfolding with very small leaves and visible petiole (Fig. 2E). The monitoring started when seedlings were moved back into the greenhouses and ended when all seedlings reached the leaf unfolding stage. Leaf unfolding time was calculated as the duration days from stage 1 to stage 4.

2.5. Data analysis

The data were examined graphically for the normality of distribution (probability plots of residuals) and homogeneity of variance (scatter plots) using the R software (Version 3.5.0, R Development Core Team 2018) before being subjected to the analysis of variance (ANOVA). An effect was considered significant at $p \leq 0.05$. When the ANOVA showed a significant ($p \leq 0.05$) photoperiod effect or significant interaction, Fisher's Least Significant Difference post hoc test was used to compare treatment means.

3. Results

3.1. Growth cessation and height growth

Growth cessation of seedlings grown in longer photoperiods corresponding to 55 and 58 °N latitude was delayed by 5 and 6 days, respectively, as compared to 48 °N photoperiod (Table 1; Fig. 3A). Low soil moisture (LSM) delayed growth cessation by 2 days (Table 1;

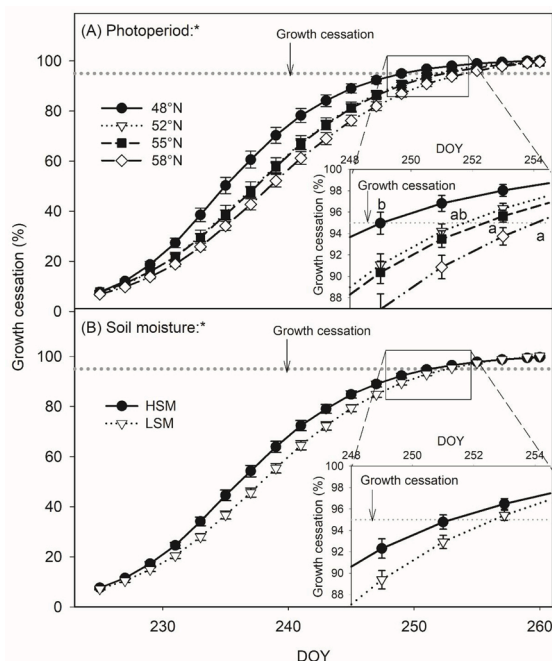


Fig. 3. Height growth curve fitted with a 3-parameter sigmoidal model indicating the day of the year for growth cessation. (A) photoperiod effect ($n = 24$: three seedlings with two replications per treatment, two levels of $[CO_2]$ and two levels of soil moisture, $3 \times 2 \times 2 \times 2 = 24$) and (B) represents the effect of soil moisture ($n = 48$: three seedlings with two replications per treatment, two levels of $[CO_2]$ and four levels of photoperiod, $3 \times 2 \times 2 \times 4 = 48$). The dashed line represents growth cessation. Growth cessation was defined as the date when 95 % of height growth increment was achieved. The data were fitted with a 3-parameter sigmoidal model by using SigmaPlot 12.5 (Systat Software Inc., San Jose, CA, USA). The inserted figures are the magnified section of the curves where growth cessation occurred and the different letters in the insert of Fig. A indicate significant differences between treatments in the timing of growth cessation ($p < 0.05$) as determined by Fisher's Least Significant Difference post hoc test. The level of significance is shown (* $p < 0.05$).

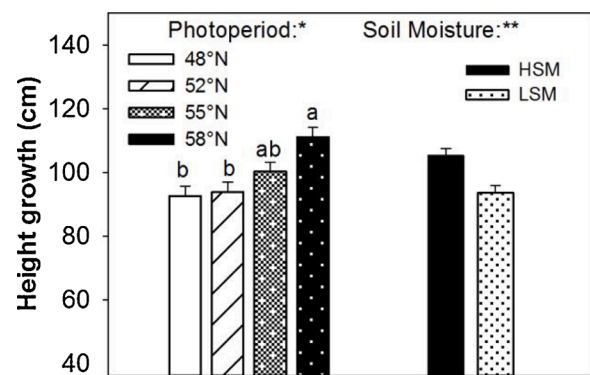


Fig. 4. Total height by four photoperiods (at 48 (seed origin), 52, 55, and 58 °N) (left) and two soil moisture levels (high and low) (right) in trembling aspen seedlings. Data are pooled across $[CO_2]$ and soil moisture for (left) ($n = 24$ (see Fig. 3 for explanations), $[CO_2]$ and photoperiod for (right) ($n = 48$, (see Fig. 3 for explanations). Different letters above the bars indicate significant differences under Fisher's Least Significant Difference post hoc test ($p < 0.05$). The level of significance is shown (* $p < 0.05$; ** $p < 0.01$).

Fig. 3B). Total height growth showed a general increasing trend with increasing photoperiod, but not all the differences between adjacent treatments were statistically significant (Fig. 4). LSM reduced height growth by about 12 cm (Table 1; Fig. 4).

3.2. Autumn bud phenology

In the first growing season, bud set was delayed in the combined treatments of EC with longer photoperiods (55 °N and 58 °N) by ~ 16 and 13 days, respectively compared to that of seed origin (Fig. 5A). In the second growing season, bud set was delayed by ~ nine and six days, respectively in the longer photoperiods of 55 and 58 °N (Fig. 5B) and by ~20 days in LSM (Fig. 5C).

3.3. Leaf senescence

The onset of leaf senescence in the first growing season was delayed by ~9 days and ~14 days by longer photoperiods of 55 °N and 58 °N latitude, respectively ($p = 0.054$) as compared to the seed origin (Table 1, Fig. 6A). However, the delaying effect on the completion of leaf senescence was much smaller ($p = 0.056$, Fig. 6A), leading to a faster rate and shorter duration of leaf senescence at longer photoperiods or higher latitudes, particularly at 58 °N (duration about 10 days shorter than at the seed origin). The photoperiod effects, however, disappeared in the second growing season (Table 1). $[CO_2]$, soil moisture, and their interaction became significant on leaf senescence initiation and duration (Table 1). LSM significantly delayed the initiation but shortened the duration of leaf senescence, but the soil moisture effect was substantially reduced by EC (Table 1; Fig. 6B). However, the completion of leaf senescence was only delayed by ~4 days in LSM.

3.4. Electrolyte leakage test

In the first growing season, LT_{50} generally became less negative with increases in photoperiod, and the EC significantly increased LT_{50} in photoperiod at 48 °N and 52 °N (Table 1; Fig. 7A). In the second growing season, however, the photoperiod effect on LT_{50} became statistically insignificant (Table 1). However, LSM substantially reduced the cold hardness of the seedlings in the second growing season, as indicated by the much less negative LT_{50} (Table 1; Fig. 7B).

3.5. Spring bud phenology

EC substantially delayed bud break in the spring, and the magnitude

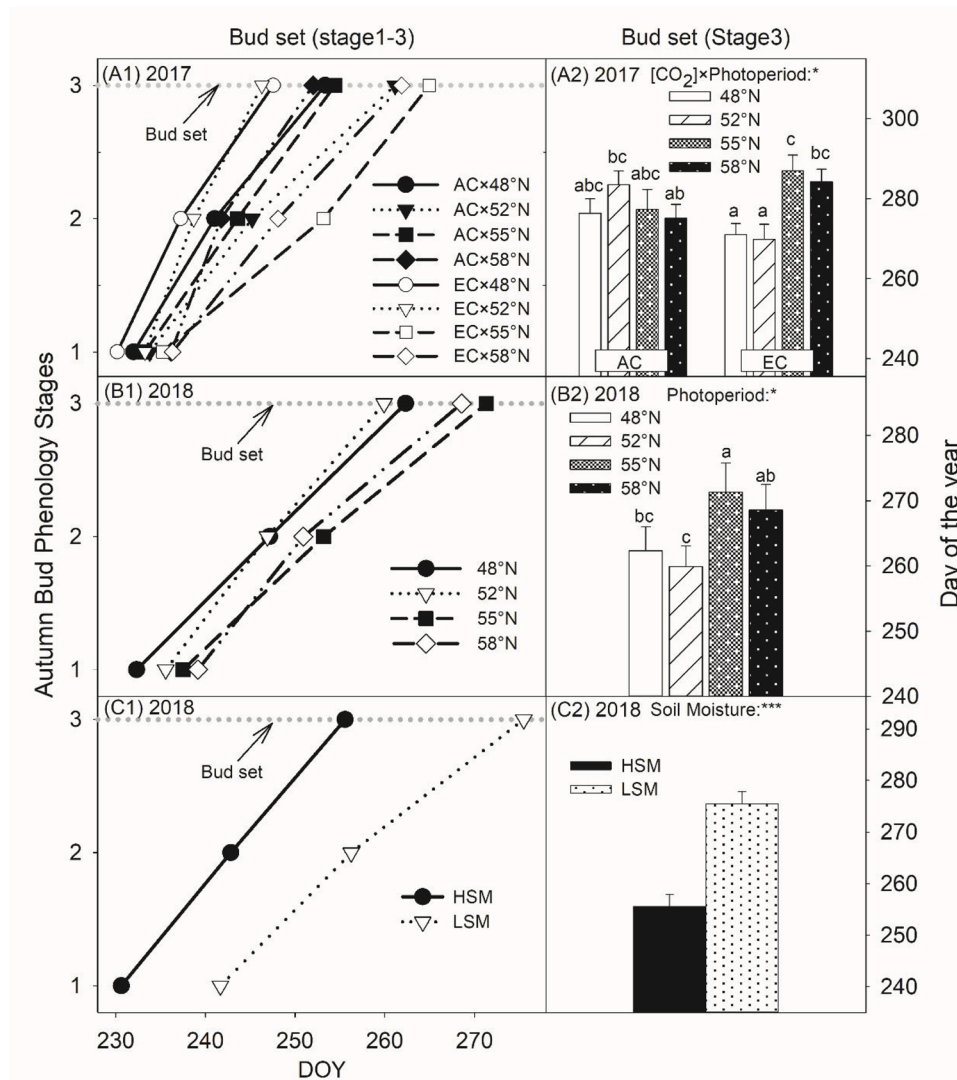


Fig. 5. Mean day of the year at each autumn bud set stage in 2017 (A1) and 2018 (B1) and (C1) and mean (+SE) of the day of the year at stage3 (A2) 2017, and (B2) and (C2) 2018. (A1) and (A2) represent the interactive effect of $[CO_2]$ and photoperiod effect ($n = 12$: three seedlings with two replications per treatment, two levels of soil moisture, $3 \times 2 \times 2 = 12$). (B1) and (B2) represents the effect of photoperiod ($n = 24$ (see Fig. 4.3 for explanations)). (C1) and (C2) represent the soil moisture effect ($n = 24$ (see Fig. 3 for explanations)). Different letters above the lines of (A2) and (B2) indicate significant differences under Fisher's Least Significant Difference post hoc test ($p < 0.05$). The level of significance is shown (* $p < 0.05$; *** $p < 0.001$).

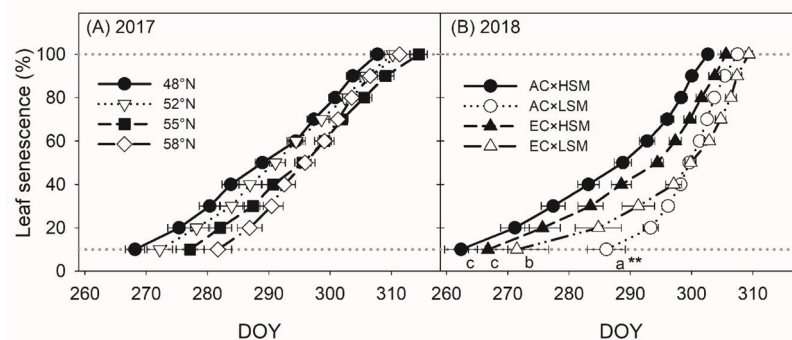


Fig. 6. Mean (+SE) day of the year of leaf senescence (%) in 2017(A), and 2018 (B). Data are pooled across $[CO_2]$ and soil moisture (A) ($n = 24$ (see Fig. 3 for explanations)), and photoperiod for (B) ($n = 24$: three seedlings with two replications per treatment, four levels of photoperiod, $3 \times 2 \times 4 = 24$). Different letters at the bottom of (B) indicate significant differences under Fisher's Least Significant Difference post hoc test ($p < 0.05$). The level of significance is shown (** $p < 0.01$).

of the delay was greater in LSM (delayed by ~13 days in HSM, ~18 days in LSM) (Table 1; Fig. 8A). LSM delayed bud break only under EC (Fig. 8A). LSM slowed down leaf unfolding under AC but not under EC (Table 1; Fig. 8B). However, EC expedited leaf unfolding in both moisture treatments and all photoperiod treatments (Table 1; Fig. 8B).

4. Discussion

Our results support the hypothesis that longer photoperiods increase growth but delay autumnal phenological events/processes. The two longest photoperiods in this study delayed growth cessation in the fall by 5–6 days and bud set by 6–9 days, leading to an extended growing

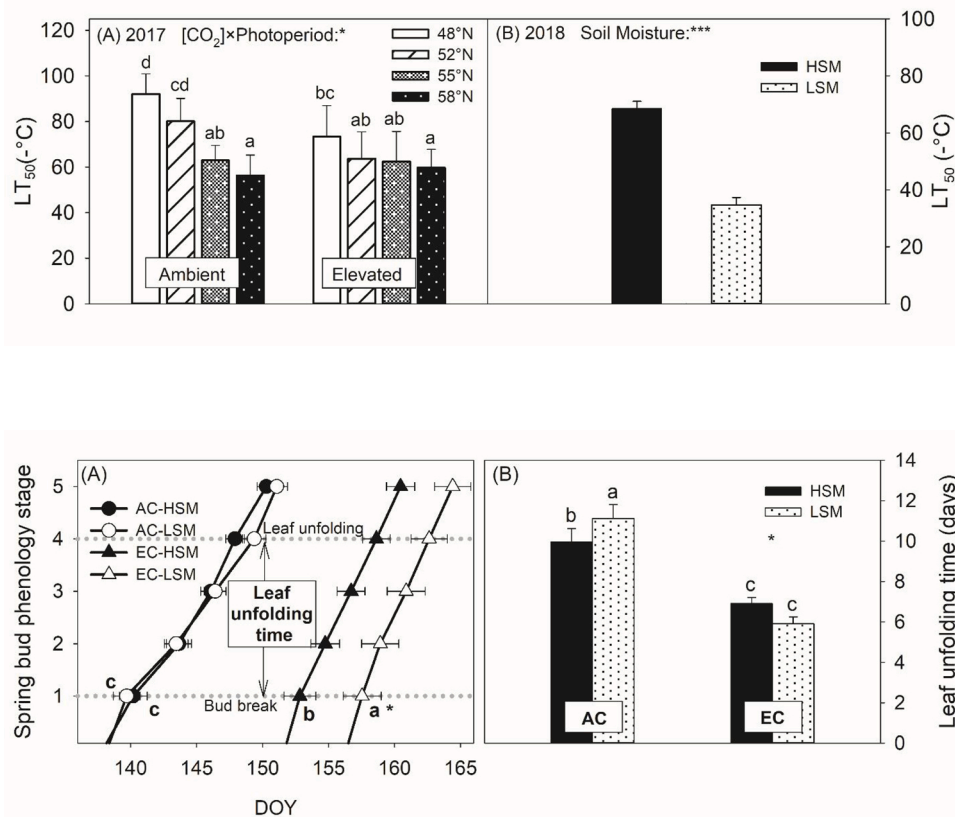


Fig. 7. Mean (+SE) LT₅₀ by an interactive effect of photoperiods (at 48 (seed origin), 52, 55, and 58 °N) and [CO₂] (ambient and elevated) in 2017 (A) and the main effect of soil moisture (HSM and LSM) in 2018 (B) of trembling aspen seedlings. Data are pooled across [CO₂] and soil moisture for (A) ($n = 12$, (see Fig. 5 for explanations)), [CO₂] and photoperiod for (B) ($n = 48$, (see Fig. 3 for explanations)). Different letters above the bars represent significantly different means under Fisher's Least Significant Difference post hoc test ($p < 0.05$). The level of significance is shown (* $p < 0.05$; *** $p < 0.001$) on the top.

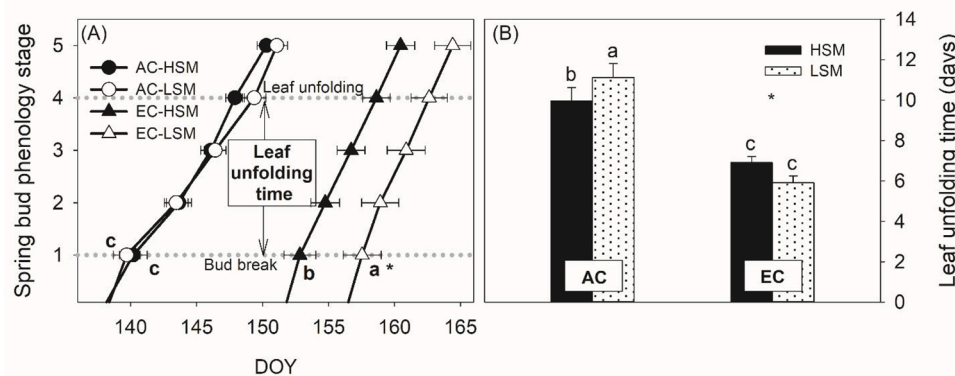


Fig. 8. Mean (+SE) day of the year at each spring bud phenology stage of trembling aspen seedlings in 2018 (A). Figure (B) represents the interactive effect of [CO₂] and soil moisture on leaf unfolding time. Data are pooled across photoperiod and soil moisture for (A) (B) ($n = 24$, (see Fig. 6 for explanations)) because of the lack of significant effect ($p > 0.05$). Different letters in the bottom (A) and above the bars (B) represent significantly different means as determined by the Fisher's Least Significant Difference post hoc test ($p < 0.05$). The level of significance is indicated by * ($p < 0.05$).

season and greater growth. These results suggest that the fall phenology of trembling aspen is presumably triggered by a critical photoperiod and that the critical photoperiod is probably coded genetically which will not change when a population migrates or is moved to a new location with a different photoperiod regime. This conclusion agrees with the findings of Brissette and Barnes (1984); Frewen et al. (2000), and Howe et al. (1995). Other researchers have also concluded that photoperiod is the key environmental factor determining the initiation of the growth cessation and bud set in *Populus* spp. (Barr et al., 2004; Pauley and Perry, 1954). *Salix pentandra* (Junttila and Kaurin, 1990) and *Betula pedula* (Viherä-Aarnio et al., 2005) also show delayed growth cessation in the fall when grown at higher latitudes than seed origin. A 10-years field study has demonstrated that trembling aspen grows faster when it is grown at 7° north of seed origin (Schreiber et al., 2013). It is interesting to note that in the present study, the delay in bud set under photoperiods at higher latitudes (55 and 58 °N) only occurred under elevated [CO₂] in the first growing season. This interactive effect may reflect the effect of elevated [CO₂] on the production and accumulation of carbohydrates (Ainsworth and Long, 2005) and the effect of carbohydrate concentration on bud set (Ruttink et al., 2007). While the delay in autumn phenology and subsequent extension of the growing season promotes growth when trees migrate to higher latitudes (Thomas and Vince-Prue, 1997), such migration may also increase the risk of frost injuries if it also delays or jeopardizes the proper development of cold hardiness (Howe et al., 2000). Indeed, longer photoperiods reduced cold hardiness in this study. Additionally, frost-damaged buds and reduced leaf nitrogen resorption due to delays in leaf senescence may prevent bud break in the following spring and make trees more susceptible to pest attacks (Ostry et al., 1989). Reoccurring damages will reduce the vigor and growth of trees (Zalasky, 1976).

At the two longest photoperiods, leaf senescence was delayed by 9–14 days than that of seed origin. In the few studies on leaf senescence and related processes (e.g., Friedman et al., 2011), a clear causal

relationship among these processes or the environmental cues that regulate these processes is not well established. However, leaf senescence tends to occur near the end of the growth cessation and bud set (Estiarte and Peñuelas, 2015). Hence, the onset of leaf senescence, to a certain extent, can be assumed to be under photoperiodic control, like other autumnal phenophases. *P. tremula* (Fracheboud et al., 2009) and *P. tremuloides* (Barr et al., 2004) have shown a consistent date for leaf senescence, as demonstrated by the consistency across multiple years, suggesting a strong photoperiodic regulation on the onset of leaf senescence. Also, we should note that we only carried out the experiment for two growing seasons and longer-term and/or field studies are warranted for further insights.

Interestingly, low soil moisture delayed the onset of autumnal phenology and reduced cold hardiness in trembling aspen. Low soil moisture delayed both growth cessation and bud set, leading to a longer growing season in trembling aspen. However, despite the lengthening of growing season, the seedling height growth decreased in the low moisture treatment. While it has been reported that water stress induces growth cessation and accelerates bud set (McIntyre, 1987; Timmis and Tanaka, 1976) likely due to increased ABA accumulation under water stress (Ding et al., 2016; Mwange et al., 2005), our result showed the opposite effect on the timing of growth cessation and bud set. Similarly, the delay in the initiation of leaf senescence in response to the low soil moisture is in contradiction to our hypothesis and the results of some previous studies. For instance, Holland et al. (2016) have found that water stress treatment advances the onset of leaf senescence in *Quercus pubescens* by about one month. Water deficit has also resulted in earlier leaf senescence in *Betula pendula* (Juknys et al., 2012). However, our findings that the timing of bud set and leaf senescence were significantly delayed under low soil moisture appear to be supported by several other studies (Arend et al., 2016; Čehulić et al., 2019; Vander Mijnsbrugge et al., 2016) but the physiological mechanisms responsible for the delay are still unclear.

The result that low soil moisture reduced the cold hardiness is also contrary to our hypothesis and other studies on the effect of water stress on cold hardiness. For instance, Kreyling et al. (2012) found increases in cold hardiness in *Pinus nigra* following summer drought. The decrease of cold hardiness in low soil moisture was possibly associated with the delay of phenological events by low soil moisture as discussed earlier. The effect of low soil moisture increased in the second growing season. It can be reasonably assumed that water stress was more severe in the second growing season since the effects of water stress increase with tree size (Koch and Fredeen, 2005). The lower cold hardiness (LT₅₀ below −37 °C) due to low soil moisture could increase freezing damage and tree mortality in extremely cold winter.

The cold hardiness of aspen seedlings showed a decreasing trend with increasing photoperiod, and the photoperiod effect was reduced by elevated [CO₂]. The reduction in cold hardiness was accompanied by delays in leaf senescence, growth cessation, and bud set. The cold hardening process is initiated by short days or a combination of short days and cooler temperatures (Bigras et al., 2001), but the development of cold hardiness is complex and influenced by many factors, including the speed of acclimation after the first stage of cold hardiness occurs (Greer et al., 1989). Despite the changes in cold hardiness, aspen seedlings in all photoperiods and [CO₂] levels were sufficiently hardy (LT₅₀ below −50 °C) to withstand the cold winter in the Canadian boreal forest region (Black et al., 2005; Gusta et al., 2009). The influence of photoperiod on cold hardiness, however, disappeared in the second year measurement. The difference in the effect of photoperiod in each year may be in part due to the different age of trees (Lim et al., 2014). Especially the seedling stage is the most vulnerable to cold injury (Howe et al., 2003).

Surprisingly, elevated [CO₂] delayed bud break but expedited leaf unfolding in the spring, and the effect was magnified by the low soil moisture treatment. The results of CO₂ effects on tree phenology in the literature vary with tree species and studies. Elevated [CO₂] does not affect the bud break of *Populus* spp in some studies (e.g., *Populus trichocarpa* (Sigurdsson, 2001) and *Populus* × *euramericana* (Calfapietra et al., 2003)) but delayed spring bud break of *Populus* clones in others (Ceulemans et al., 1995). A possible explanation for the delay in bud break under elevated [CO₂] in our study may have to do with a reduction of nitrogen concentration (N) under elevated [CO₂]. The timing of bud break is known to be related to the induction of enzymes and plant hormones such as cytokinins that are important for growth resumption in the spring (Liu and Sherif, 2019; Yordanov et al., 2014), which could be influenced by N concentration in plants (Thitithanakul et al., 2012). It has been reported that trembling aspen grown under elevated [CO₂] tends to reduce tissue N concentration (Zak et al., 2000), possibly leading to a delay of spring bud break (Bigras et al., 2001; Lumme and Smolander, 1996). The delay we found, however, was quite significant compared to the other studies. Delays in leafing out in the spring can have a negative impact on the competitiveness of pioneer tree species such as trembling aspen (Lechowicz, 1984).

In conclusion, photoperiod regimes associated with northward migration significantly affected autumnal phenological events/processes in trembling aspen, such as delays in growth cessation, bud set, and leaf senescence that led to the longer growing season. The consequent increases in height growth may provide trembling aspen competitive advantages with other species. Height growth is recognized as an important trait for survival and frequently used as a measure of fitness (Ying and Yanchuk, 2006), especially in the juvenile stage when a greater height will be advantageous in competition for light (Burns et al., 1990). Having a faster rate of height growth is crucial for the survival of pioneer species as trembling aspen (Ying and Yanchuk, 2006). However, the delays in growth cessation and bud set can lead to delays in the development of dormancy and cold hardiness and expose trees to greater risks of frost injury. Moreover, other factors may constrain or offset the positive effect of longer photoperiod on growth. For instance, elevated [CO₂] delayed bud break in the spring and had the effect of shortening

the growing season. If northward migration is accompanied by reduced soil moisture as predicted for many parts of the boreal forest (Pachauri et al., 2014), the interactions between elevated [CO₂], longer photoperiod, the faster rate of its change and drier soil moisture can have detrimental effects on trees, particularly on frost resistance and frost damages. Therefore, changes in photoperiod, [CO₂] and soil moisture should be considered when planning latitudinal seed transfer and predicting the migration of boreal trees in response to climate change.

Author contributions

S Inoue had the main responsibility in all phases of the study. The co-authors were QL Dang, R Man, and B Tedla. Both QL Dang and R Man contributed to manuscript writing and discussion of ideas. B Tedla contributed to discussions of experimental design and ideas, as well as performing the experiment.

Declaration of Competing Interest

The authors declare that there is no conflict of interests for the publication of this manuscript for any of the authors.

Acknowledgment

We acknowledge Ms. Joan Lee, former Greenhouse Manager, and Ms. Keri Pidgen, the current Greenhouse Manager of Lakehead University for their logistic support and other operational assistance during the experiments. The study was supported by an NSERC Discovery grant to QL Dang (Project No. 203198-2013-RGPIN), an Ontario Trillium Scholarship to S Inoue, and Lakehead University Graduate Assistantships to S Inoue.

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