Effectiveness of winter temperatures for satisfying chilling requirements for reproductive budburst of red alder (*Alnus rubra)*

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Background. Experiencing an adequate amount of cold temperatures over winter is necessary for many temperate tree species to break dormancy and flower in spring. Thus, changes in winter and spring temperatures associated with climate change may influence when trees break dormancy and flower in the future. There have been several experimental studies that have quantified the effectiveness of cold temperatures for chilling requirements for vegetative budburst of temperate trees, however, there are few experimental studies addressing the chilling requirements for reproductive budburst of trees, as it is difficult to place reproductively mature trees in temperature-controlled environments.

***Methods*.** To identify how changing temperatures associated with climate change may impact reproductive phenology, we completed a temperature-controlled growth chamber experiment using cuttings of reproductive branches of red alder (*Alnus rubra),* one of the most widespread hardwood tree species of the Pacific Northwest, USA. The purpose of this study was to examine how colder (4 °C) and warmer (9 °C) winter temperature regimes influenced the timing of reproductive budburst of red alder cuttings in spring.

Results. We found that cuttings flowered earlier after pretreatment with a 4 °C winter temperature regime than after a 9 °C winter temperature regime. We used our experimental data to estimate a "possibility-line" showing the accumulated chilling and forcing temperatures necessary prior to reproductive budburst of red alder.

***Discussion**.* This study provides a preliminary indication that warmer winters with climate change may not be as effective for satisfying chilling temperature requirements of Northwest hardwood tree species.

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Keywords: Climate change, flowering, phenology

Abstract

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Introduction

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Tree phenology is strongly controlled by temperature, and as climate change alters seasonal temperatures, tree phenology may shift in unexpected ways. Trees in temperate regions have evolved to time spring phenological events so that they occur after the risk of frost has passed, and thus many tree species, or genotypes within species, require a certain period of cold (chilling) temperatures to break dormancy prior to flowering or leaf-out (Perry, 1971; Körner, 2007). One of the most noticeable phenological changes over the recent past has been earlier leaf-out and flowering of tree species in temperate ecosystems (Fitter & Fitter, 2002; Parmesan & Yohe, 2003). However, continued warming, especially over winter, may result in a lack of chilling temperatures required for initiation of spring phenological events (Luedeling, Zhang & Girvetz, 2009), thus leading to a possible delay in spring phenology (Cook, Wolkovich & Parmesan, 2012). Additional research is needed on the specific chilling requirements of temperate tree species to enable prediction of how tree phenology, and associated changes in important ecosystem services, will change with climate change (Chuine et al., 2016).

There is a large body of literature that relies on observational data to estimate the chilling and forcing requirements for budburst of tree species (e.g. Hannerz, 1999; Chuine, 2000; Luedeling et al., 2009; Prevéy, Harrington & St. Clair, 2018). For some species photoperiod may also be important in the timing of spring budburst (Laube et al., 2014), however, temperature and photoperiod are often highly correlated in spring. Thus, observational studies may not be able to parse out the influence of the two environmental cues, and may not accurately identify temperature cues for budburst (Basler & Körner, 2012). To separate the effects of temperature from other environmental cues, there have been a number of experimental studies that have examined the influence of simulated winter temperature regimes on vegetative budburst of small potted trees or twigs (e.g. Harrington, Gould & St.Clair, 2010; Basler & Körner, 2012; Nanninga et al., 2017). However, there have been few experimental studies that examine environmental cues that are important for reproductive budburst of trees (but see Viti & Monteleone, 1995), since it is difficult to place reproductively-mature trees in experimental treatments, such as growth chambers or greenhouses, as they are usually quite large. One method to overcome this obstacle is to take cuttings (cut twigs) of reproductively mature trees, and place these cuttings in simulated temperature environments (Basler & Körner, 2012; Vitasse & Basler, 2014; Nanninga et al., 2017).

From previous experimental studies, several patterns have emerged. Multiple studies indicate that exposure to increased chilling temperatures reduces the amount of forcing temperatures needed for reproductive budburst in spring (Harrington, Gould & St.Clair, 2010;

Nanninga et al., 2017). Thus, a "possibility-line" can be modeled that predicts the amount of forcing needed for reproductive budburst based on the amount of chilling a tree has received (Harrington, Gould & St.Clair, 2010; Prevéy, Harrington & St. Clair, 2018). One previous study indicated that subfreezing temperatures may accelerate vegetative budburst of trees (Rinne et al., 1997). Another study found that simulated high winter temperatures led to reduced fruit production and necrosis in apricot cultivars (Viti & Monteleone, 1995).

Here, we focus on the influence of winter temperature on flowering dates of red alder (*Alnus rubra*), the most common hardwood tree species of the Pacific Northwest (Harrington, 2006). Historically, red alder has received less research attention than some of the widespread conifer species of the region. However, more recently, the values of red alder as an important component of ecosystems and as a timber crop are being recognized, and thus more attention is being paid to this species (Deal & Harrington, 2006; Harrington, 2006). Red alder plays an important role in northwestern ecosystems by stabilizing streambanks, fixing nitrogen in soil, and providing food and cover for animals (Harrington, 2006; Harrington et al. 2008). Additionally, it has become a valuable timber species, and interest in the effects of management practices on tree growth, as well as flowering, has grown (Harrington & Debell, 1995; Ahrens & Bluhm, 2017). However, to date, there has been relatively little research on the environmental cues that are important for the reproductive phenology of red alder.

In the current study, we examine how experimental winter temperature regimes influence the date of reproductive budburst of cuttings of red alder. We created a range of experimental conditions in temperature-controlled growth chambers and greenhouses to address two questions: (**1**) How effective are colder (4 °C) and warmer (9 °C) winter temperature regimes for chilling prior to reproductive budburst of red alder? Based on previous research, we hypothesized that temperatures at or below 5 °C would be more effective (or more quickly satisfy chilling requirements) than temperatures above 5 °C, so cuttings in treatments experiencing colder temperatures over winter would flower earlier than those experiencing warmer winters when exposed to forcing temperatures in spring. (**2**) How does short-term exposure to sub-freezing events influence the date of reproductive budburst? We hypothesized that short-term exposure to sub-freezing temperatures during dormancy would be effective for satisfying chilling requirements, and cuttings exposed to sub-freezing periods would flower earlier than those that did not experience sub-freezing periods when exposed to forcing conditions in spring (Rinne et al 1997). Finally, we tested the utility of the equations developed for the reproductive phenology model in Prevéy, Harrington & St. Clair (2018) for creating a “possibility-line” for reproductive budburst of red alder.

Methods

Sample collection and treatment

On 11/1/2016, we collected cuttings of red alder (*Alnus rubra*) from a riparian corridor along the edge of Webster Nursery, south of Olympia, WA (46°57'05.8"N, 122°57'50.8"W). All sampled trees were flagged so we could compare phenology of cuttings to phenology on intact trees in spring. We collected 5 reproductive twigs from each of ten individual trees. We placed the cut ends of twigs in water, and transported the twigs immediately to the USFS Olympia Forestry Sciences Laboratory, in Olympia WA, where the experiment was conducted. The collection site is owned by the Washington State Department of Natural Resources, and they granted us permission to take plant samples from their property. Flowering usually occurs from mid-winter through early spring, with seed ripening from late August to October (Harrington et al. 2008)

Cutting preparation

Prior to being placed in experimental treatments, all cuttings were recut to similar lengths (30-40 cm) and then the lower portions were submerged into a disinfectant sodium hypochlorite solution (200 ppm active chlorine) for ten seconds. They were then recut underwater and placed in containers filled with 400 ml water. The sides of all containers were covered in aluminum foil to block sunlight and reduce algal growth. Every seven days over the course of the experiment we changed the water in containers, recut the stems underwater, and randomized the location of containers in experimental treatments. We also recorded “survival” of cuttings each week. A cutting was considered dead if the cut stem was no longer green, or if the cutting had shed its reproductive buds. Portions of these methods were adapted from Basler & Körner (2012).

Experimental treatments

We placed one cutting from each sampled tree (ten cuttings per treatment in total) in one of four different experimental treatments. The four treatments were: **4 °C** - a 4 °C temperature regime in a growth chamber, **4 °C/freeze** - a 4 °C temperature regime in a growth chamber with one 0 °C freezing event on 1/6/2017 (freezing events were accomplished by placing cuttings in a 0 °C freezer overnight), **9 °C** – a 9 °C temperature regime in a growth chamber, **9 °C/freeze -** a 9 °C temperature regime in a growth chamber with one 0 ° C freezing event on 1/6/2017, and

ambient/greenhouse - ambient temperatures in a lathhouse (Table 1). All cuttings were placed in treatments from 11/2/2016 through 1/31/2017, and then moved to a greenhouse with a variable temperature that averaged 16 °C to simulate forcing conditions. Temperature regimes for treatments were accomplished with a combination of growth chambers, ambient conditions in a lathhouse, and forcing conditions in a greenhouse. The growth chambers were Percival growth chambers (Model PGC - 105X). Photoperiods in growth chambers were set to match ambient photoperiods. Growth chambers were lit with a combination of 25-W incandescent and 160-W florescent bulbs (Phillips F27T12/CW/VHO).

We also had an additional **ambient** treatment where cuttings remained in a lathhouse over winter and spring. We then compared the dates of reproductive budburst on these cuttings to the reproductive phenology on intact trees at Webster Nursery to examine how phenology of cut branches from trees may differ from whole-tree phenology. Finally, we collected an additional set of cuttings from seven of the ten originally sampled red alder trees at Webster Nursery on 1/12/2017 and placed them in the greenhouse to increase the range of temperature conditions for modelling the possibility line of chilling and forcing conditions necessary to flower (**Webster/greenhouse** treatment, Table 1).

After 1/31/2017, we began to check for reproductive budburst on the cuttings twice weekly. We defined the day of year (DOY) of reproductive budburst as the first day we observed open male (staminate) catkins that were shedding pollen, or female (pistillate) catkins with bracts that had opened enough to allow for pollination. We also monitored the sampled trees from Webster Nursery for reproductive budburst from 2/1/2017 through 3/20/2017.

Statistical analyses

We used linear mixed-effects models to compare the day of year (DOY) of female versus male reproductive budburst between all treatments to observe if there were differences in the timing of male and female budburst, and if the different treatments influenced those differences. We employed a linear mixed-effects model with the DOY of budburst as the response variable, and treatment and sex as predictor variables. For all linear mixed-effects models, the sampled tree ID was included as a random effect to reduce the influence of variation between individual trees on final results. To examine if relatively colder temperatures or freezing events over the dormant season led to earlier dates of reproductive budburst than warmer temperatures, we compared the

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dates of reproductive budburst of red alder cuttings that experienced different dormant season temperatures, but then experienced the same forcing temperatures when moved to the greenhouse on January 31st (Table 1). We statistically compared dates of budburst between the different treatments using a linear mixed-effects model with day of year (DOY) of budburst as the response variable and treatment as the predictor variable, and individual tree ID as a random variable.

To examine whether phenology of cuttings differed from phenology of the branches still attached to trees, we compared the DOY of reproductive budburst of cuttings in the lathhouse, which received ambient temperature conditions, to the originally sampled trees at Webster Nursery. We used a linear mixed-effects models with the DOY of reproductive budburst as the response variable, treatment (Ambient or Webster) as the response variable, and tree ID as a random variable. All models were conducted using the lmer function in the lmertest package (Kuznetsova, Brockhoff & Christensen, 2017) in the statistical program R (R Core Team, 2017). Code and data for the analyses and are included in the Supplemental files 2-4.

Testing the reproductive phenology model

We used the reproductive phenology model described in Prevéy, Harrington & St. Clair (2018) to calculate the chilling and forcing hour accumulations by the date of reproductive budburst of cuttings in all treatments. This reproductive phenology model was originally parameterized using a large database of Douglas-fir flowering data, which allowed for the determination of a “possibility-line” for flowering of Douglas-fir (Prevéy, Harrington & St. Clair, 2018). Here, we test whether the equations developed to estimate the effectiveness of chilling and forcing temperatures for flowering of Douglas-fir (Fig. S1.) could be used to estimate a “possibility-line” for reproductive budburst of red alder. We calculated hourly chilling and forcing units, and summed unit accumulations by the date of reproductive budburst, for cuttings from all experimental treatments as well as for reproductive budburst dates from trees at Webster Nursery. We then fit the possibility-line with a hierarchical linear model to account for variation in the date of reproductive budburst between different treatments and individual cuttings. We fit both linear and logarithmic models and compared fit statistics to identify the best-fit possibility-line for reproductive budburst of red alder.

Results

Temperature conditions averaged 4 °C in the colder growth chamber, and 9 °C in the warmer growth chamber (Fig. 1). Ambient conditions in the lathhouse and at Webster Nursery averaged 4.4 °C, and 4.3 °C, respectively, from 11/2/2016-1/31/3017, and there were multiple freezing events (Fig. 1).

Survival of red alder cuttings that experienced a simulated freezing event in the 4 °C treatment was low (2 cuttings), and no cuttings survived in the 9 °C /freeze treatment (Fig. 2). Thus, we removed those treatments from further analyses. Across the other treatments, an average of 63% of red alder cuttings survived to reproductive budburst (Fig. 2). The reproductive buds on one of the ten originally sampled trees at Webster Nursery stopped developing in mid-winter and did not flower in spring (Fig. 2).

All red alder twigs had both male and female catkins, however, after male reproductive budburst, many of the female catkins did not develop further (Table 2). There were no significant differences in the dates of reproductive budburst of male and female catkins for most treatments. However, there was earlier reproductive budburst for male versus female red alder cuttings harvested on 1/11/2017 in the Webster/greenhouse treatment, and on trees at Webster Nursery (Table 2).

Red alder cuttings in the 4 °C treatment had earlier male and female reproductive budburst than red alder cuttings in the 9 °C or ambient/greenhouse treatments ($t > 2.2$, $p < 0.04$, Figs. 3). Cuttings in the warm treatment took the longest to reach 100% reproductive budburst (Fig. 4).

Reproductive phenology models

The equations for the reproductive phenology model in Prevéy, Harrington & St.Clair (2018) were used to define a possibility-line for reproductive budburst of red alder (Fig. 5). We used only male reproductive budburst data to model the possibility-line, as we had more observations of male reproductive budburst than female reproductive budburst. A natural log relationship between chilling and forcing unit accumulation fit the data better than a linear relationship ($R^2 = 0.69$ for the natural log model versus $R^2 = 0.49$ for the linear model, Fig. 5).

Discussion

We found that pretreatment with colder (4 °C) winter temperatures accelerated the reproductive budburst of red alder more than warmer (9 °C) winter temperatures after cuttings were placed in forcing conditions in a greenhouse. These results provide preliminary evidence that relatively colder temperatures may be more effective for satisfying chilling requirements for reproductive budburst of temperate tree species that flower prior to leaf-out in spring. The importance of cold temperatures for flowering also indicates that warmer winter temperatures in the future may not be as effective for satisfying chilling requirements for flowering trees, and this could eventually lead to a delay in reproductive budburst if winter temperatures increase appreciably, especially along the southern portion of species ranges (Luedeling, Zhang & Girvetz, 2009; Luedeling, 2012).

Although colder temperatures led to earlier reproductive budburst, cuttings from all temperature treatments in this experiment did experience male reproductive budburst, indicating that a fairly wide range of winter temperatures (4 to 9 °C) can contribute to chilling requirements. A wide range of temperatures was similarly found to be effective for chilling prior to vegetative budburst of Douglas-fir (Harrington, Gould & St.Clair, 2010). Additionally we found that increased exposure to chilling temperatures led to less forcing temperatures required prior to reproductive budburst, similar to other studies of vegetative and reproductive phenology (Harrington, Gould & St.Clair, 2010; Nanninga et al., 2017; Prevéy, Harrington & St. Clair, 2018). Thus, even if winter temperatures become warmer in the future, increasing temperatures in spring may still result in advancing budburst dates.

The equations used to calculate chilling and forcing unit accumulations for the reproductive phenology model of Douglas-fir (Prevéy, Harrington & St. Clair, 2018) worked well to describe a possibility-line for reproductive budburst of red alder. The best fit model for the Douglas-fir possibility-line was linear, whereas a log model was a better fit for the experimental red alder flowering data, which covered a wider range of winter temperatures than the observational data used to create the Douglas-fir model (Prevéy, Harrington & St. Clair, 2018). Examining how phenology is altered under a wide range of experimental temperatures is important, as it can be difficult to predict the effects of novel climates from observational field data if the field data doesn't include the range of temperature conditions that may occur in the future (Harrington, Gould & St.Clair, 2010).

Our test to observe whether the phenology of cuttings in the ambient treatment was a good proxy for phenology on whole trees was met with mixed results. On one hand, the timing of male reproductive budburst in the ambient treatment was very similar to the timing of male reproductive budburst outside on trees. This indicates that the reproductive phenology of cuttings can match that of branches on intact trees, and can be a useful way to expose reproductive buds to experimental conditions (Vitasse & Basler, 2014). On the other hand, the development of all female reproductive buds stopped prior to budburst on the cuttings in the ambient treatment, whereas most female reproductive buds on trees continued to develop. So, buds on cuttings may not develop in the same way as trees, especially if they are removed from trees for long time periods. Perhaps shortening the length of time cuttings are kept in growth chambers, or adding nutrients to the water that cutting are kept in, may result in more female reproductive budburst of cuttings.

We observed much earlier reproductive budburst in our experimental treatments than was observed for red alder in outside conditions. While we did not specifically alter photoperiod in this study, our results indicate that the influence of temperature alone can accelerate reproductive budburst much earlier than has happened historically, indicating that photoperiod may not constrain the advancement of early-season phenology of trees in the Pacific Northwest. However, future research using reproductive cuttings should include treatment combinations that alter both photoperiod and temperature, as there may be interactive effects between temperature and photoperiod (Heide, 1993; Basler & Körner, 2012) that may influence phenological responses to climate change (Way & Montgomery, 2015).

Conclusion

This experiment provides evidence that warmer winters with climate change may not be as effective for satisfying chilling requirements of reproductive budburst of red alder. However, multiple different combinations of chilling and forcing temperatures can result in reproductive budburst of red alder, similar to vegetative budburst of other Pacific Northwest tree species (Harrington & Gould, 2015). These results provide information on the effectiveness of different temperatures for chilling requirements prior to red alder reproductive budburst. This study also provides an example of how cuttings can be used in place of whole trees to expose reproductive buds to different simulated winter temperature regimes, and to model the combinations of chilling and forcing temperatures that can result in reproductive budburst. This information can then be used to predict how the timing of reproductive budburst may change in the future.

Acknowledgements

We thank Teresa Vail, Yianna Bekris and Leslie Brodie for their assistance with this project, William Carlson for his thoughts on an earlier version of this manuscript, and the Washington State Department of Natural Resources for permission to take plant samples and access to their lands.

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Table 1(on next page)

The locations and average temperatures (°C) for all experimental treatment combinations, and at Webster Nursery, over the course of the experiment: 11/2/2016 – 3/30/2017.

Table 1. The locations and average temperatures (°C) for all experimental treatment combinations, and at Webster Nursery over the course of the experiment: 11/2/2016 – 3/30/2017.

Treatment	November	December	January	February through budburst
4 °C	Constant 4 °C			Greenhouse (ave. 16 °C)
4 °C /freeze	Constant 4 °C w/ freeze event (0 °C)			Greenhouse (ave. 16 °C)
9 °C	Constant 9 °C			Greenhouse (ave. 16 °C)
9 °C /freeze	Constant 9 °C w/ freeze event (0 °C)			Greenhouse (ave. 16 °C)
Ambient	Variable temp. (ave. 5.7 °C)			
Ambient/greenhouse	Variable temp. (ave. 4.4 °C)			Greenhouse (ave. 16 °C)
Webster/greenhouse	Variable temp. (ave. 4.3 °C)		Greenhouse (ave. 14.4 °C)	
Webster Nursery	Variable temp. (ave. 4.3 °C)			

Figure 1

Daily mean temperatures for all experimental treatments, and from Webster Nursery, over the course of the experiment: 11/2/2016 – 3/21/2017. The dashed line denotes 0 °C.

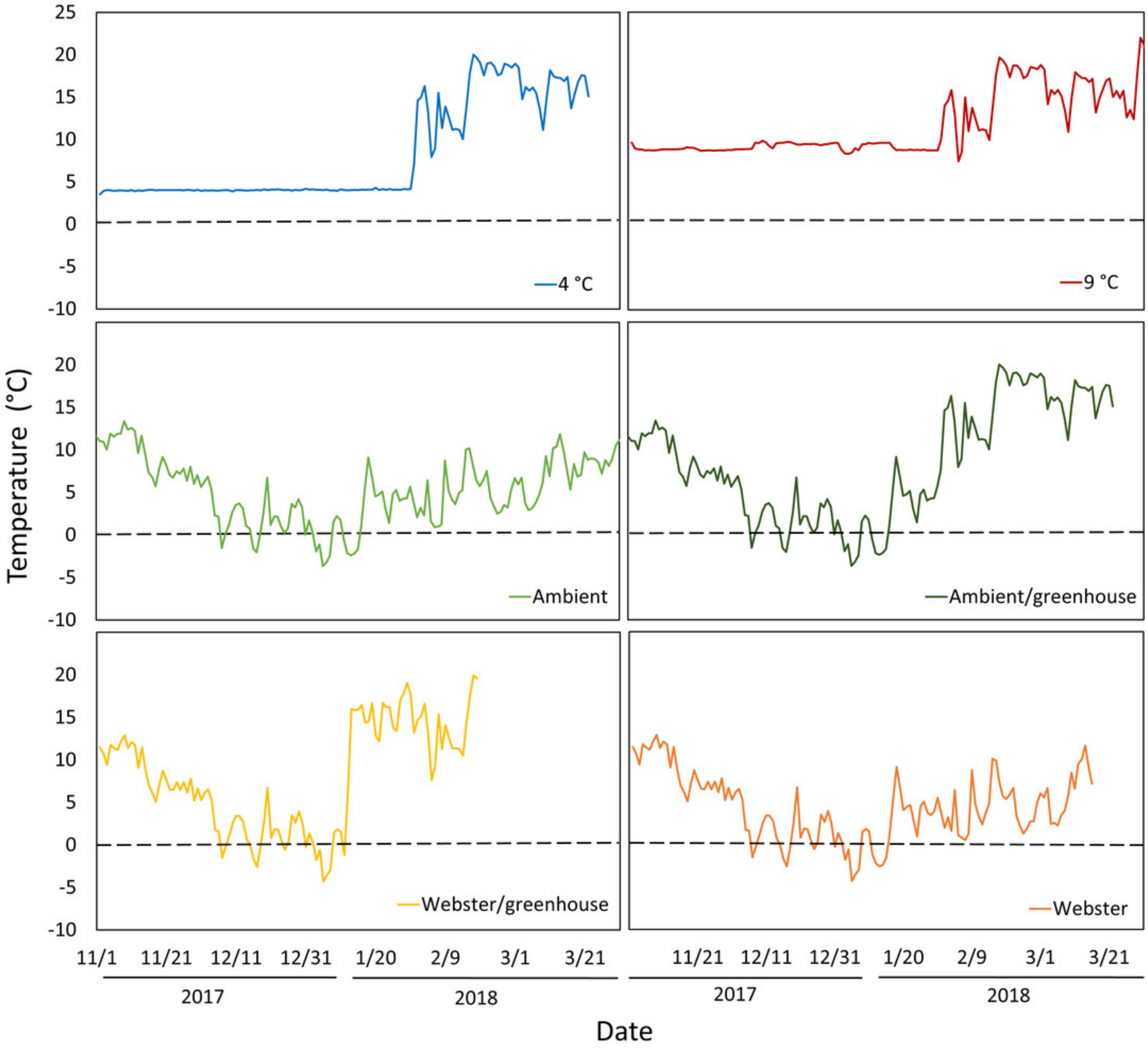

Figure 2

Percentage of cuttings that survived to reproductive budburst in each experimental treatment, and percentage of trees with reproductive buds at Webster Nursery that flowered in spring.

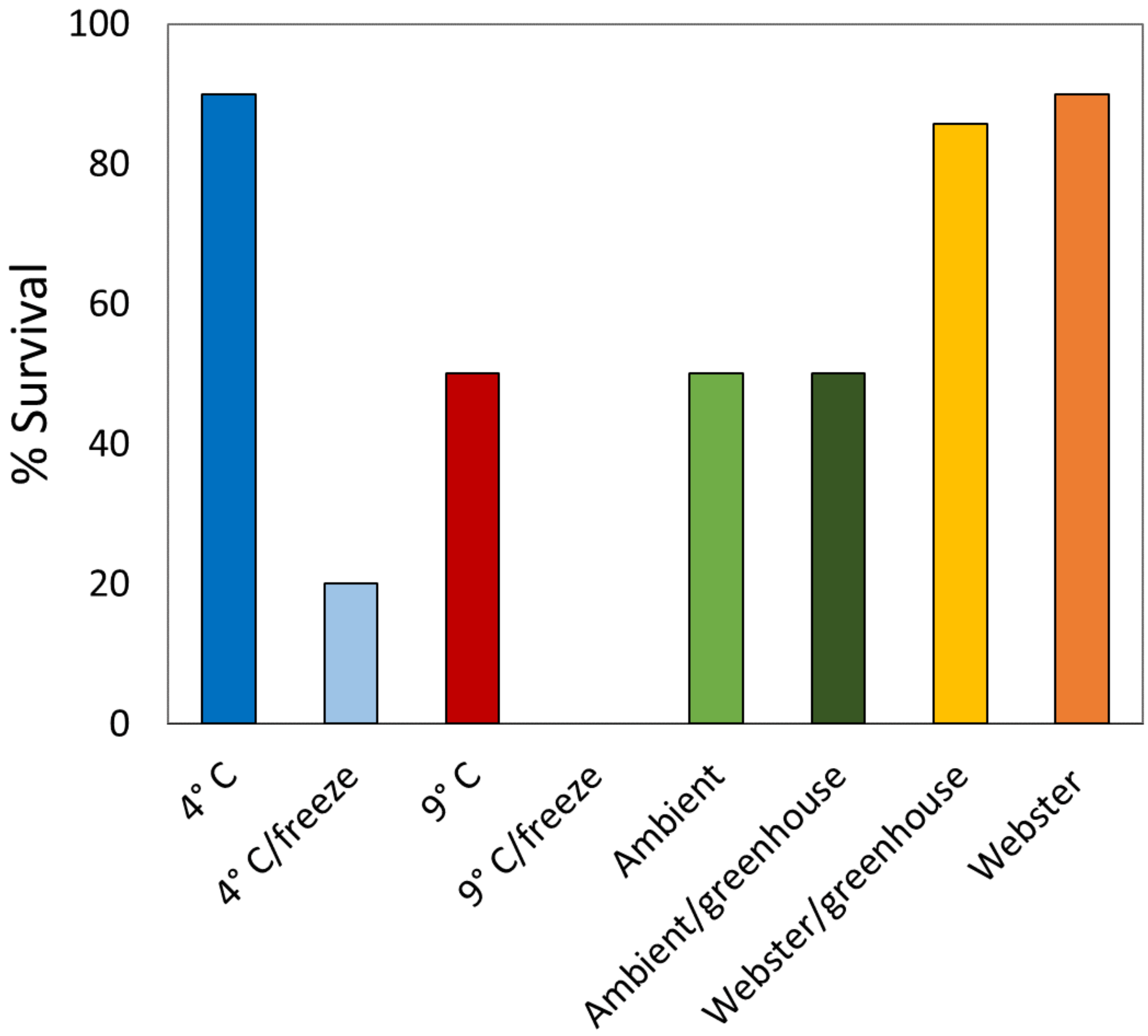

Figure 3

Average DOY of (A) male and (B) female reproductive budburst for red alder in all experimental treatments with survival greater than 40%.

The 4 °C, 9 °C, and ambient/greenhouse treatments all received the same forcing temperatures in the greenhouse from 1/31/2017 onward. No female catkins burst bud on cuttings in the ambient treatment. Different letters above bars denote significant differences at the $p < 0.05$ level.

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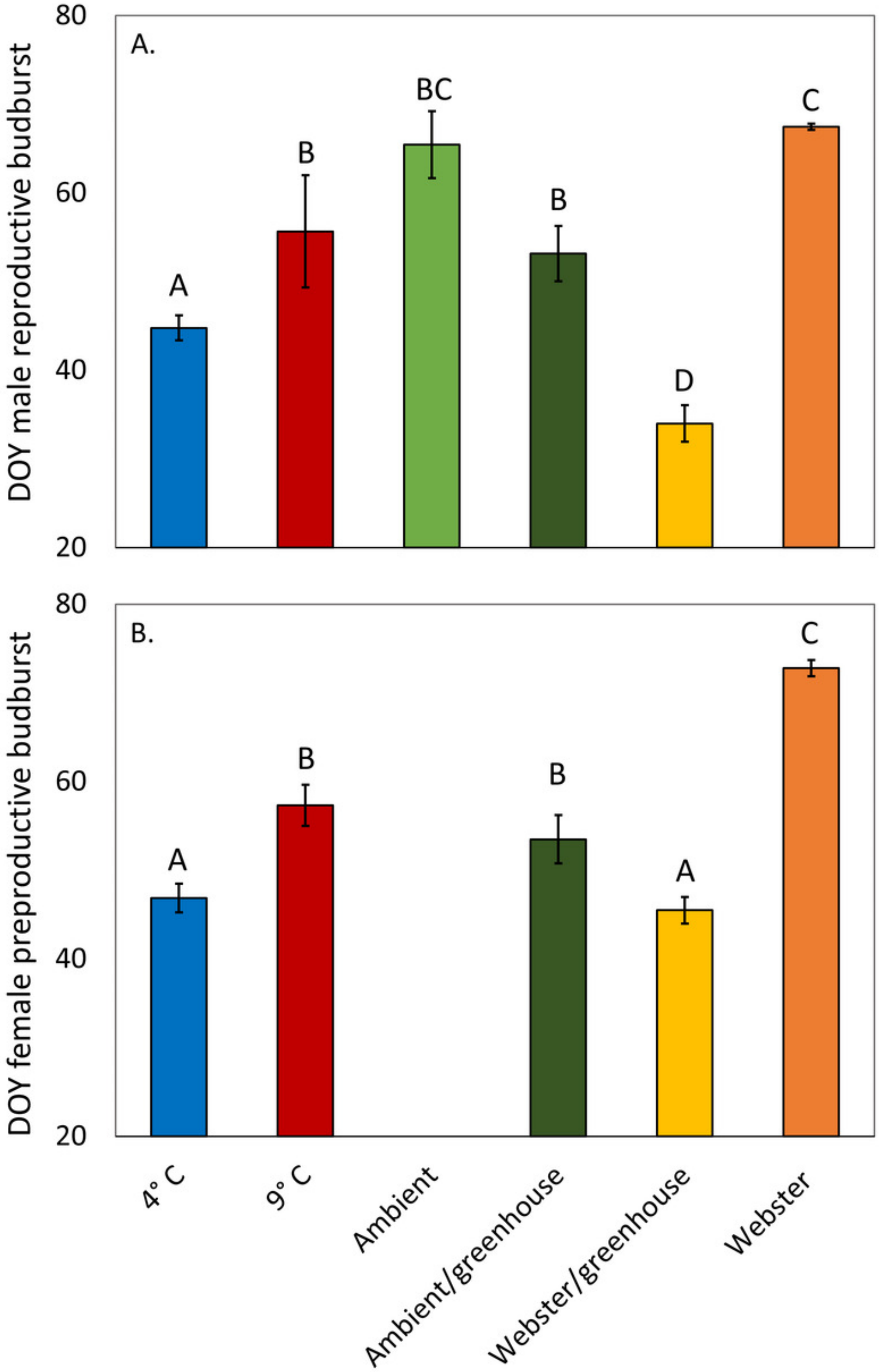

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Table 2(on next page)

Number of cuttings with male or female reproductive buds that survived to budburst, and the average DOY of reproductive budburst for each sex in experimental treatments and at Webster Nursery.

Bolded values indicate significant ($p < 0.05$) differences between the day of year (DOY) of male and female budburst for treatment.

Table 2. Number of cuttings with male or female reproductive buds that survived to budburst, and the average DOY of reproductive budburst for each sex in experimental treatments and at Webster Nursery over the course of the experiment (11/2/2016 – 3/21/2017). Bolded values indicate significant ($p < 0.05$) differences between the day of year (DOY) of male and female budburst for treatment.

Treatment	Sex	# of cuttings with reproductive buds	DOY flowering +/-SE
4 °C	M	9	44.8 ± 1.4
	F	7	46.9 ± 1.6
9 °C	M	4	55.7 ± 2.3
	F	4	57.3 ± 6.3
Ambient	M	5	65.5
	F	0	N/A
Ambient/greenhouse	M	4	53.2 ± 2.7
	F	4	53.5 ± 3.1
Webster	**M**	**9**	**67.5 ± 0.4 ***
	F	**9**	**71.1 ± 0.9**
Webster/greenhouse	**M**	**6**	**34 ± 2.0 ***
	F	**4**	**45.5 ± 5.0**

Figure 4

Percentage of red alder cuttings reaching (A) male and (B) female reproductive budburst over time in treatments receiving the same forcing temperature conditions from 1/31/2017 onward.

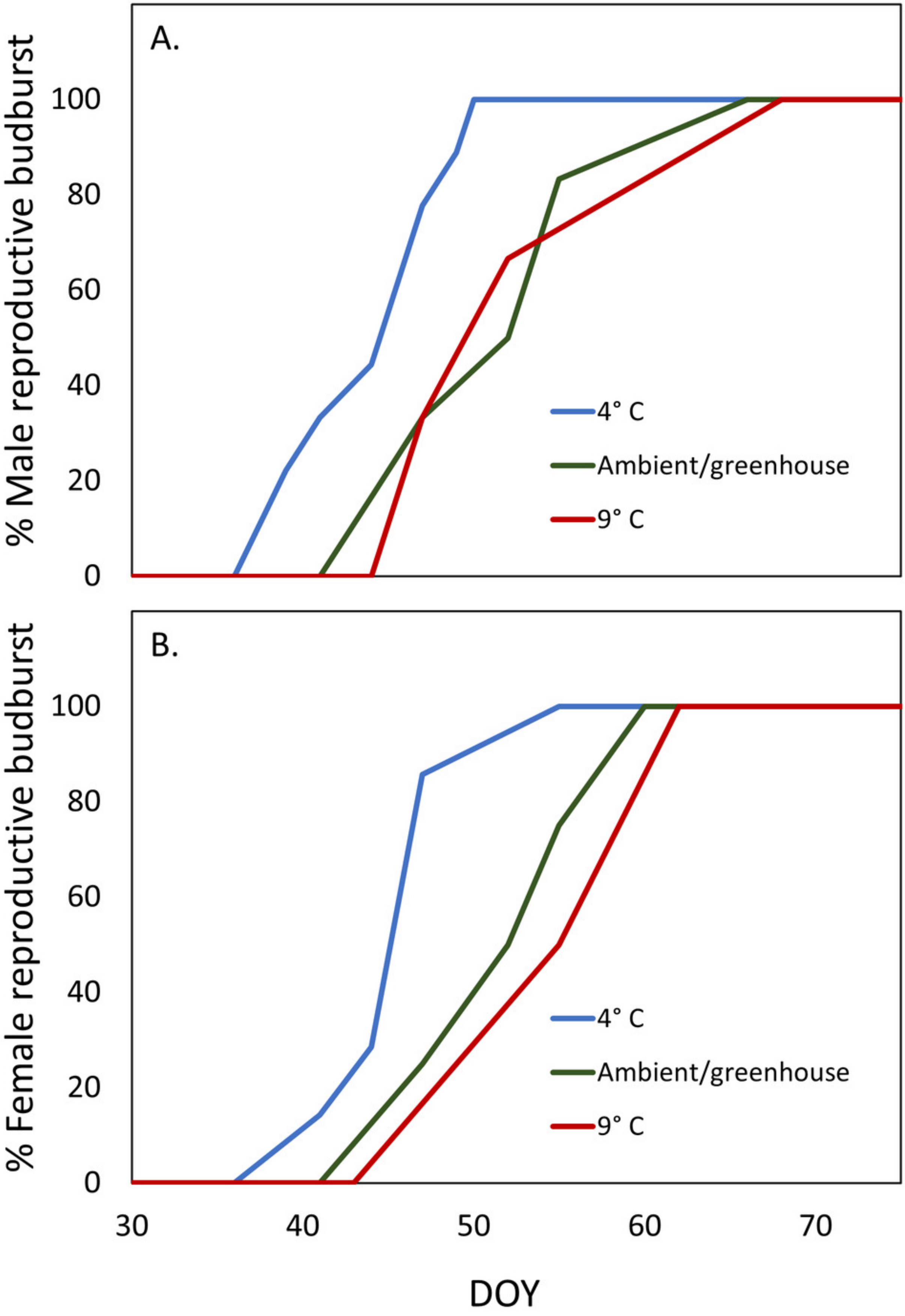
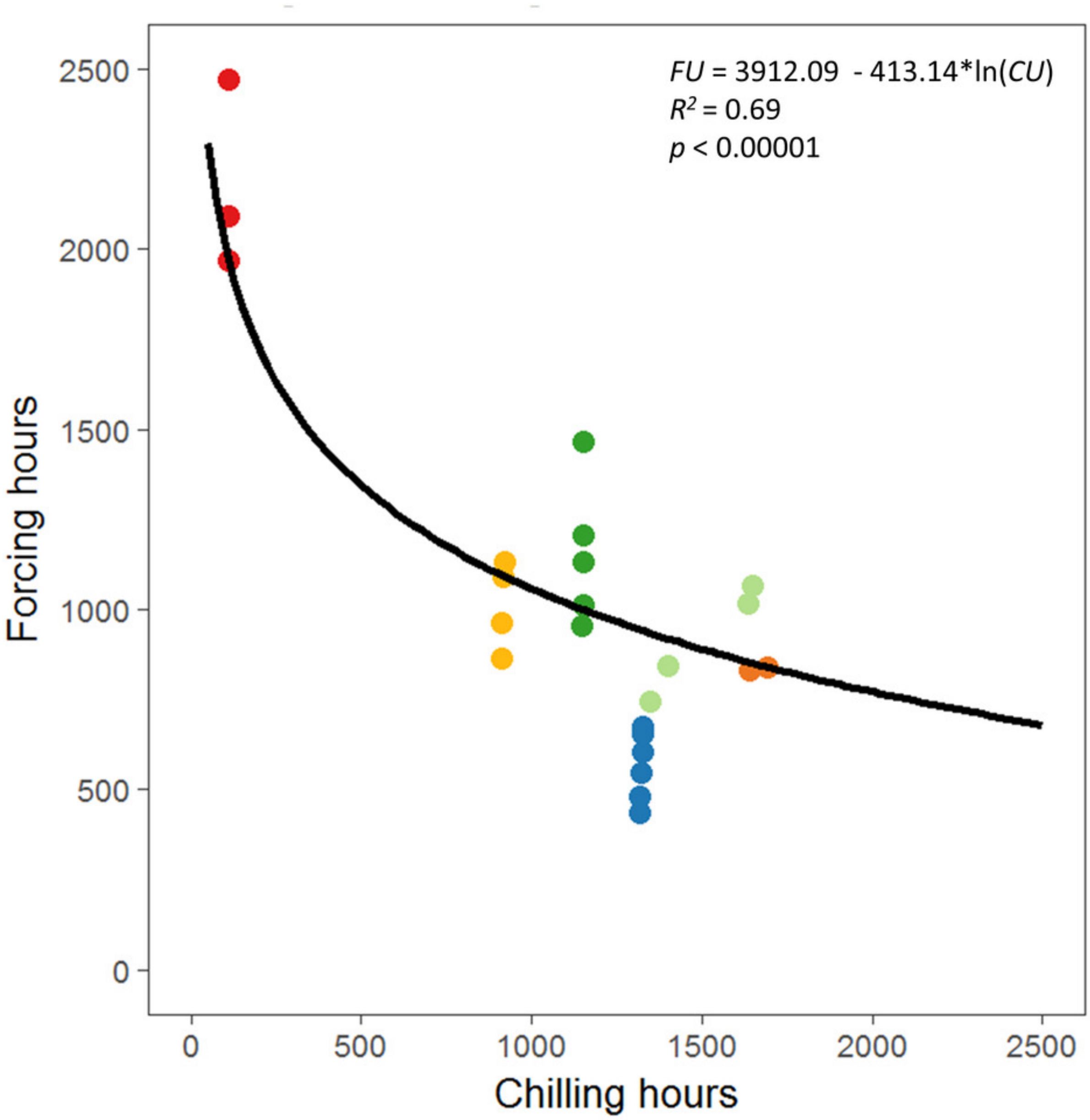

A.
B.
% Male reproductive budburst
% Female reproductive budburst
0
20
40
60
80
100
4° C
Ambient/greenhouse
9° C
30
40
50
60
70
DOY

Figure 5

Possibility line for reproductive budburst of red alder.

The possibility line is the slope of the natural log relationship between chilling units accumulated by reproductive budburst and forcing units accumulated from 11/2/2017 through reproductive budburst for red alder cuttings in the different experimental treatments. Points above the line indicate combinations of chilling and forcing where reproductive budburst is likely, and points below the line indicate combinations where budburst is less likely to occur.


$FU = 3912.09 - 413.14*\ln(CU)$
$R^2 = 0.69$
$p < 0.00001$
Forcing hours
2500
2000
1500
1000
500
0
Chilling hours
0
500
1000
1500
2000
2500