



Impact of genotype and parent origin on the efficacy and optimal timing of GA_{4/7} stem injections in a lodgepole pine seed orchard

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Abstract

Seed orchards play a critical role in reforestation, but often struggle to consistently produce enough seed. A mixture of the less polar gibberellins, GA₄ and GA₇, has been shown to promote flowering in *Pinaceae* species, but the responses vary significantly based on species, genotype and provenance, application method, timing and dosage. Previous studies with lodgepole pine (*Pinus contorta* Dougl. var. *latifolia* Engelm.) suggest that the response to exogenous GA_{4/7} varied among genotypes. Gibberellins also play a critical role in seed development and germination but it remains unclear how exogenous GA_{4/7} will affect seed quality. We used 25 lodgepole pine genotypes in a commercial seed orchard in Alberta, Canada to explore the impact of genotype and geographic origin on the efficacy and optimal timing of GA_{4/7} stem injections to promote female cone bud initiation, cones and seed production. Overall, we found that GA_{4/7} stem injections promoted female cones in 40% of genotypes included in this study. For these 10 genotypes, we found that the latitude of their provenance was positively correlated with later effective application times. Of the remaining 15 genotypes, five were hypersensitive to exogenous GA_{4/7} showing needle discoloration and loss, and 10 genotypes showed no response. In general, stem-injected GA_{4/7} did not affect the number of seeds per cone while two genotypes showed a reduced seed yield and one genotype showed increased seed yield per cone. Seed weights were not affected by the stem-injected GA_{4/7}, while the July application date resulted in higher germination rates compared to control trees.

Keywords Tree improvement · Forest genetics · Seed orchards · Plant growth regulators

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Introduction

Seed orchards play a critical role in supplying high-quality seed for reforestation, but often struggle to consistently produce enough seed (Giertych 1987; Eriksson et al. 1998). Natural fluctuations, pests and diseases, as well as insufficient pollination, unfavourable environmental conditions and high ovule and conelet abortion rates can result in low numbers of mature cones, few seeds per cone and ultimately low seed yields (White et al. 1977; Yates and Ebel 1978; Katovich et al. 1989; Webber 1995; Bates et al. 2002; Owens et al. 2005; Owens and Fernando 2007). Research conducted in the late 1970s and 80s showed that in many conifers, cone bud initiation can be promoted through the application of exogenous gibberellins (GA) and other plant growth regulators (Ross and Pharis 1976; Pharis et al. 1980, 1987; Owens and Blake 1985; Bonnet-Masimbert 1987). A mixture of the less polar gibberellins, GA₄ and GA₇, has been shown to promote flowering and thereby enhance conelet production in *Pinaceae* species, but the responses can vary significantly based on species, genotype and provenance, as well as application method, timing and dosage (Eriksson et al. 1998; Zhao et al. 2011).

Wheeler et al. (1980) found that exogenous GA_{4/7}, applied as a spray or droplets, can be used to enhance female conelet production in lodgepole pine (*Pinus contorta* Dougl. var. *latifolia* Engelm.); however, the response to exogenous GA_{4/7} appeared to differ among genotypes. Likewise, in a recent study with only two genotypes in a lodgepole pine seed orchard, GA_{4/7} stem injections promoted female cone production in one of the genotypes, while the other genotype showed no response (Kong et al. 2018). Lodgepole pine is known to display high genetic diversity among populations along with a strong adaptation to local climate (Rehfeldt et al. 1999; Liepe et al. 2016). It is reasonable to assume that trees from different geographic origins will display differences in phenology, such as the timing of bud-break in the spring, when grown together in a seed orchard. It is therefore reasonable to consider that the optimal timing of any GA_{4/7} treatment will vary among seed orchard trees based on the geographic origin of the parent tree.

Gibberellins are also known to play a critical regulatory role in seed development and germination and GA's are often found in high concentrations in developing seeds (Singh et al. 2002; Hedden and Thomas 2012). It is currently unclear how the application of exogenous GA to promote cone production will affect seed development and germination rates in lodgepole pine.

In the present study we set out to explore the relationship between genotype, geographic origin of the parent and the efficacy and optimal timing of GA_{4/7} stem injections to promote female conelet development, cone and seed production. To address these questions, we selected a total of 25 genotypes in an under-producing commercial lodgepole pine seed orchard in Alberta, Canada. We then subjected individual ramets belonging to these genotypes to one of four GA_{4/7} application treatments (June, July, August, none). Specifically we wanted to test the following hypotheses: (1) GA_{4/7} stem injections will increase the number of female conelets/cones in all genotypes in a lodgepole pine seed orchard; (2) the optimal time for GA_{4/7} stem injections will vary among genotypes based on the geographic origin of the parent trees from which scions for grafting in the orchard originated from; and (3) GA_{4/7} stem injections will cause no difference in (a) the number of seeds per cone, (b) the 1000-seed weights, or (c) the germination rates.

Materials and methods

Study design

This study was conducted in a high elevation clonal lodgepole pine seed orchard, located approximately 40 km west of Grande Prairie, Alberta, Canada (55.066545°N, –119.294858°W, 690 m.a.s.l.). Establishment of the orchard began in 1994 by planting trees developed from scion material collected from 34 parent genotypes which were grafted onto 3-year-old seedling rootstock for a total of 174 trees. Additional genotypes were added to the orchard until 2013 for a total of 110 genotypes and 1550 trees. Since going into production in 2006, the orchard has been unable to reach the industry target of 5.5 million seeds per year.

In summer 2015, we selected 25 of the 110 genotypes present in the orchard based on a minimum age (12 years) and number of ramets (12). For each of these genotypes, we randomly assigned three ramets each to one of three GA_{4/7} application times (June, July and August) with the remaining three ramets serving as a control (i.e. no GA_{4/7}).

For each application time, a new batch of the GA_{4/7} mixture was prepared in the lab by mixing 17.1 g of GA_{4/7} with 213.75 ml of 95% ethanol, resulting in a GA_{4/7} concentration of 80 mg ml⁻¹. The GA component of the mixture was composed of GA₉ (0.87%), GA₇ (36.88%) and GA₄ (62.25%) (Zhejiang Shenghua Biok Biology Co. LTD, Deqing Zhejiang, China).

The mixture was then injected into holes drilled into the stem. The 6.35 mm wide holes were drilled just above the graft union at a 45° angle to a depth of approximately 2 cm and below the first whorl of branches. All holes were immediately sealed with grafting wax (Green Earth Pruning Sealer) to prevent infection. The number of holes, and with it the administered GA_{4/7} dosage, varied based on stem diameter above the graft union (see Table 1). The treatment concentration of the GA_{4/7} however, did not vary. Following the stem-injections, all treated trees were monitored visually for any sign of negative effects caused by our treatment. Findings from this monitoring effort were summarized in an internal report (Bhardwaj and Thomas 2016).

Tracking cone development

In order to track the impact of the GA_{4/7} treatments on the number of female conelets, we marked three randomly selected branches in the middle and upper part of the crown on each of the selected 300 trial trees. We then tracked the developing conelets on these marked branches, counting them at the beginning (June) and end (late August) of the 2016 and 2017 growing seasons, covering the entire 26 month reproductive cycle of lodgepole

Table 1 Stem diameter, number of holes per tree and GA_{4/7} stem injection dosages used for 25 lodgepole pine trees

Stem diameter (cm)	Holes tree ⁻¹	GA _{4/7} tree ⁻¹ (mg)	Injection volume tree ⁻¹ (ml)
5.0–7.5	1	40	0.5
7.6–10.0	2	80	1.0
10.1–15.0	3	120	1.5
> 15.0	4	160	2.0

pine (Owens 2006). At the end of each growing season (2015–2017), all cones present on our trial trees were harvested to obtain a total cone count per tree. After each cone collection, five cones were selected randomly from each ramet and seeds were extracted in 2015, 2016 and 2017.

Seed extraction and testing

Immediately after each operational cone harvest (2015–2017), we collected a random sample of five cones from each trial tree. In some cases, the sample size was smaller because the total number of cones per tree was less than five. The cone samples were stored in labelled brown paper bags and transported at room temperature back to the University of Alberta where they were spread out on the lab bench to await further processing. Prior to extracting the seeds, all samples were weighed to determine the average weight per cone. In order to extract the seeds from these serotinous cones, samples were initially submerged into an 80 °C water bath (Grant, SUB Aqua Pro, Grant Instruments Ltd.) for 1 min to dissolve the resin and then immediately transferred into labelled sealed aluminum trays and placed into a drying oven at 60 °C for 8 h to facilitate the opening of cone scales. Seeds were then extracted by hand with the help of a dissecting probe and de-winged using tweezers. The extracted seeds were subsequently weighed to determine the 1000-seed weight and scanned on a flatbed scanner (EPSON Perfection v800) using the image analysis program WinSeedle (Regent Instruments Inc., QC, Canada) to determine the number, size, shape and colour of extracted seeds. Seeds were then pooled for each genotype and treatment and stored in a fridge at 4 °C. In July 2018, we randomly selected 100 seeds from each of these composite samples, from the 2017 harvest, for germination testing. In accordance with the current seed testing standard for Alberta (Government of Alberta Agriculture and Forestry Division 2016), seeds were placed on moist tissue paper in labelled petri dishes and placed in an incubator set at 25 °C with a 12 h day/night cycle for 21 days. Petri dishes were monitored every other day and germination counts were conducted on day 7, 14 and 21. Fully germinated seeds (i.e. those with a radicle at least four times the length of the seed itself) were removed after each count.

Data analysis

We assessed the effect of GA_{4/7} stem injections on female conelet and cone numbers, seed counts per cone and 1000-seed weights using a 2-way ANOVA with GA treatment and genotype as fixed factors. The effect on germination rates was assessed using one-way ANOVA with GA treatment as a fixed factor. All analyses were executed using R (R Core Team 2013). We used Fisher's LSD test to conduct pairwise comparisons of least-square means using the *lsmeans* and *cld* commands in the *lsmeans* package (Lenth 2016).

In order to assess the relationship between parent geographic origin and the effect of GA_{4/7} application timing, we assigned a ranking factor (effective timing) to all genotypes that showed a positive response to the GA_{4/7} injection based on the respective month(s) during which the treatments were applied [i.e. effective timing (month): June=6, June/July=6.5, July=7, July/August=7.5, August=8]. We used this 'effective timing' factor in combination with the geographic origin (latitude, longitude, elevation) of the parents to build a predictive model using multiple regression. The final model was determined by stepwise selection based on the Akaike Information Criterion (AIC) using the *stepAIC* command in the *MASS* package (Venables and Ripley 2002).

Table 2 Analysis of variance including source, type III sums of squares, degrees of freedom (df), Mean square, F-value (F), significance (*p* value) and partial Eta Squared comparing the effects of GA_{4/7} treatments (trt) and genotype (clone) on initial conelet numbers in 2016

Source	Type III sum of squares	df	Mean square	F	<i>p</i> value	Partial Eta squared
Intercept	290,039	1	290,039	148.48	<0.001	0.861
trt	5613	3	1871	3.43	0.021	0.125
clone	46,881	24	1953	3.59	<0.001	0.544
trt * clone	39,224	72	544.8	1.34	0.059	0.325

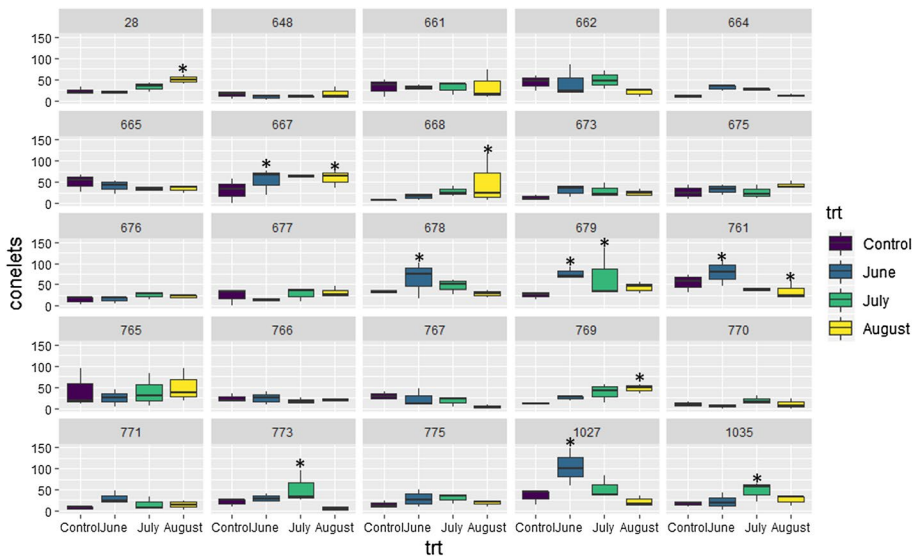


Fig. 1 Boxplot showing mean number of female conelets in June 2016 on three marked branches (sum) for all GA_{4/7} treatments applied in summer 2015. Each panel represents one of the 25 genotypes (clones) included in the study

Results

The initial (i.e. spring 2016) conelet production response to the summer 2015 GA_{4/7} stem injections varied significantly among genotypes (Table 2). Overall, 15 of 25 genotypes showed no significant increase in female conelet numbers for any of the three application times when compared to the control trees (Fig. 1). Among these 15 genotypes, five (648, 661, 677, 767, 770) appeared to be hypersensitive to the GA_{4/7} stem injections (*genotype* × *treatment*, *p* < 0.001) resulting in varying degrees of needle discoloration and loss (Fig. 2). In all five cases, hypersensitivity occurred irrespective of the time of GA_{4/7} application. For the 10 remaining genotypes that showed an increase in female conelet production in response to the exogenous GA_{4/7}, the effective application times represented the full range of treatments (i.e. June–August) (Table 3).

Stepwise multiple regression using the 10 responsive genotypes, ranked by their effective application times, and associated geographic origins (latitude, longitude and

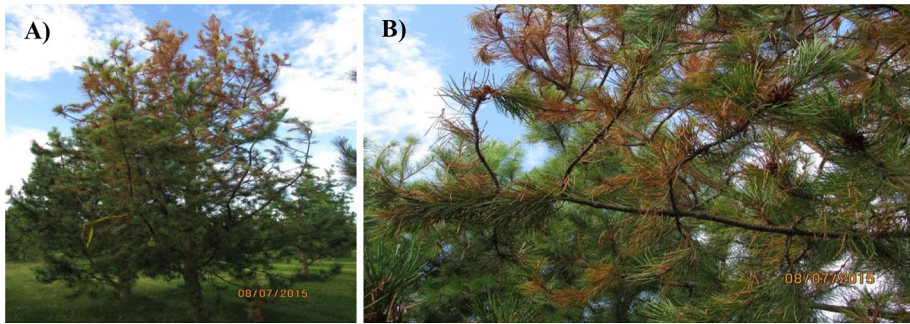


Fig. 2 **a** Severe case of needle discoloration affecting the upper crown of a lodgepole pine ramet shortly after $GA_{4/7}$ stem injections; **b** needle discoloration spreading along entire branches starting at the tips and including multiple years of needles

Table 3 Effective $GA_{4/7}$ application times for each of the 25 lodgepole pine genotypes (clones) included in the study based on pairwise comparisons of least-square means using Fisher's LSD test

Effective timing	Clones	Number of clones	% of sample population
none	648, 661, 662, 664, 665, 673, 675, 676, 677, 765, 766, 767, 770, 771, 775	15	60
June	678, 1027	2	8
June and July	679	1	4
July	773, 1035	2	8
July and August	667, 761	2	8
August	28, 668, 769	3	12

Table 4 Geographic origin of parent trees for the ten genotypes (clones) of lodgepole pine that responded to at least one of the $GA_{4/7}$ application times

Clone	Latitude (°N)	Longitude (°W)	Elevation (m)	Effective timing
1027	53.417	−117.267	1300	June
678	53.567	−117.983	1280	June
1035	53.667	−117.783	1325	July
773	53.700	−117.600	1220	July
761	53.700	−117.650	1370	July and August
769	53.717	−117.500	1200	August
679	53.733	−117.367	1140	June and July
668	53.850	−118.600	1220	August
667	53.933	−118.850	1220	July and August
28	54.400	−119.250	1310	August

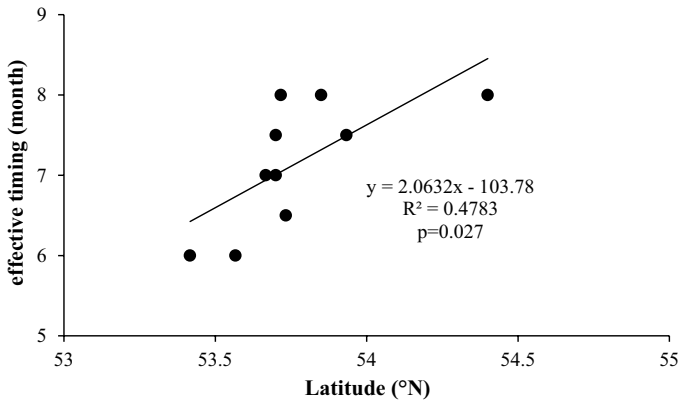


Fig. 3 Regression showing the linear relationship between latitude and the effective application time (month) of GA_{4/7} stem injections increased the number of female conelets compared to the control (10 out of 25 genotypes)

elevation) (Table 4), revealed a significant positive correlation between effective application time and northerly latitude of the parent trees ($R^2=0.48$; $p=0.027$) (Fig. 3).

Conelet abortion rates among the cohort of female conelets initiated in 2015 were high, with an overall mean of $72 \pm 24\%$ (\pm SD) over the course of the experiment. The GA_{4/7} treatments had no effect on abortion rates. There were, however, significant differences among genotypes ($p < 0.001$), with conelet abortion rates ranging from $36.6 \pm 18\%$ (genotype 664) to $99.9 \pm 0.2\%$ (genotype 668) (Fig. 4).

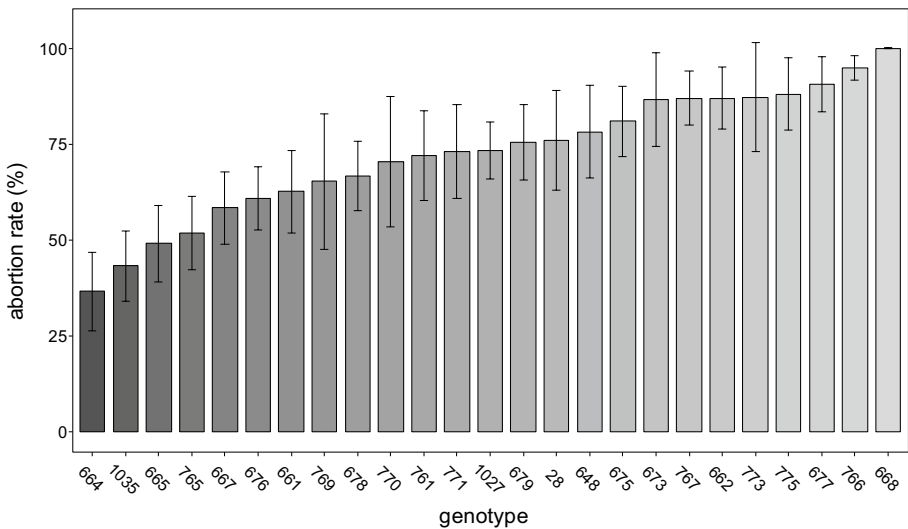


Fig. 4 Cumulative abortion rates (%) for the 2015–2017 cohort of female conelets (i.e. flower–mature cone) for each of the 25 lodgepole pine genotypes included in this study. Error bars represent 95% CI

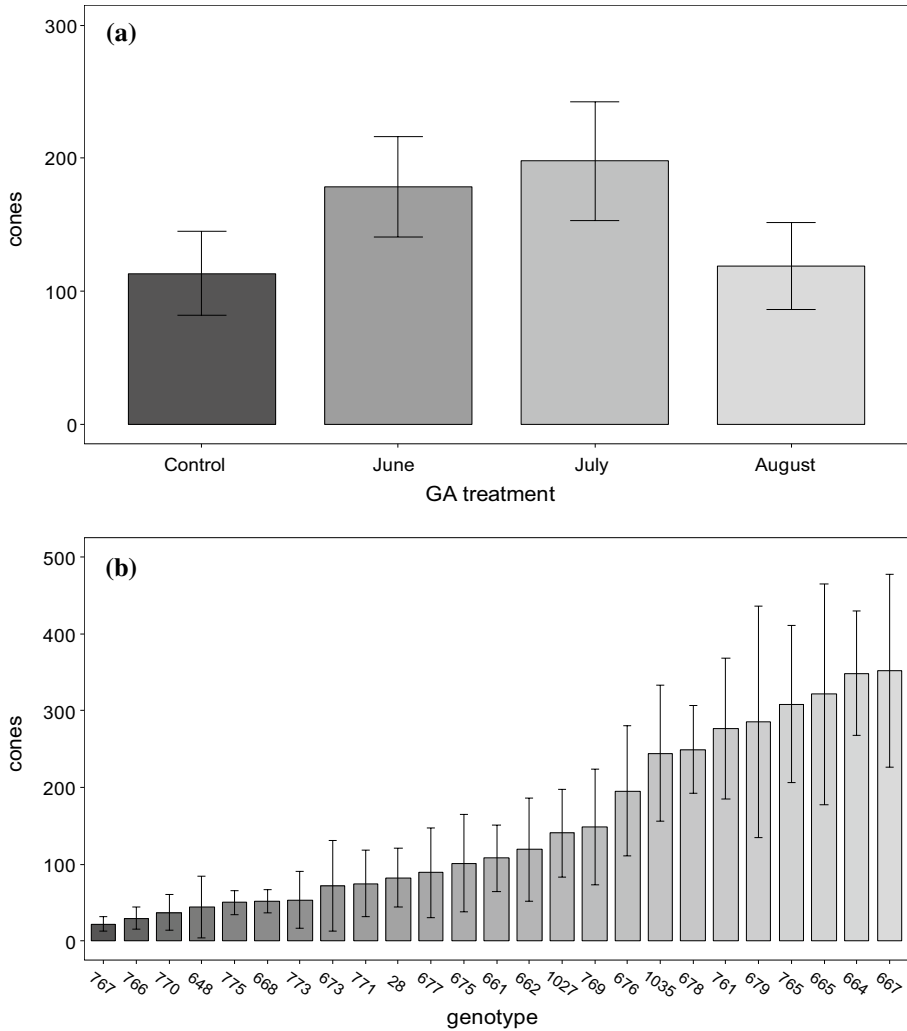


Fig. 5 Effect of **a** GA_{4/7} treatments and **b** genotype on the mean number of mature seed cones per tree (cones) in 2017. Error bars represent 95% CI

The September 2017 harvest of all cones initiated in 2015, showed that GA_{4/7} stem injections in June and July, but not August, significantly increased the total number of cones per tree compared to the control trees ($p < 0.001$) (Fig. 5a). Total cone production also differed significantly among genotypes ($p < 0.001$) (Fig. 5b). As was expected, the GA_{4/7} treatments had no effect on total cone production per tree in the two previous years of collection (2015, initiated in 2013 and 2016, initiated in 2014).

The number of seeds per cone showed a significant *treatment* × *genotype* interaction ($p < 0.001$) in 2017. For 21 of the 25 genotypes, the number of seeds per cone did not differ significantly among GA_{4/7} treatments, averaging 20.6 ± 8.7 seeds per cone. For two genotypes (662, 775) however, the August GA_{4/7} stem injections resulted in a lower

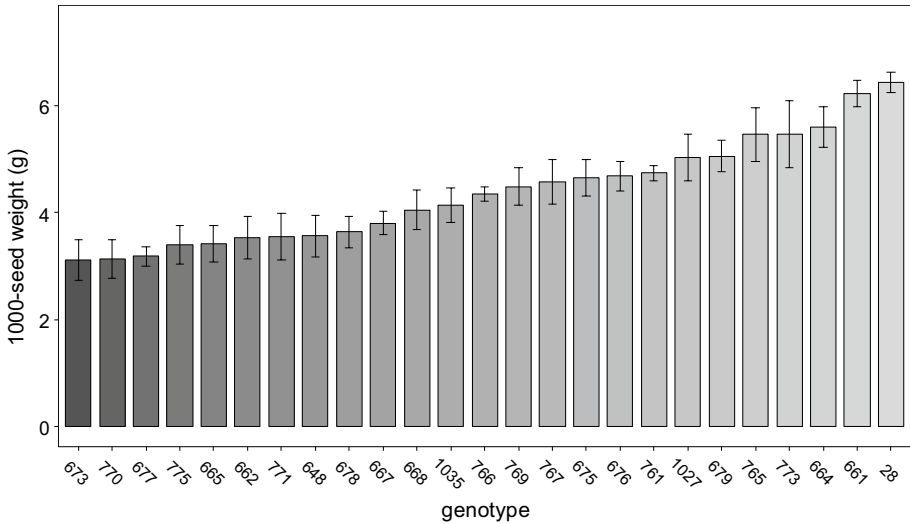


Fig. 6 Mean 1000-seed weights ($\pm 95\%$ CI) for the 25 genotypes based on seed extracted from five cones per tree in September 2017

Table 5 Impact of GA_{4/7} treatments (trt) on percent (%) germination rates (after 7, 14 and 21 days) for seeds collected at the end of the experiment (2017)

GA _{4/7} trt	Germination rate (%)		
	Day 7	Day 14	Day 21
Control	4.1 a	28 b	41 b
June	0.04 b	31 b	39 b
July	0.6 b	46 a	52 a
August	7.4 a	33 b	44 ab

Germination rates are based on 100 randomly selected seeds per genotype and treatment (n=25). Different letters indicate significant differences among treatment means

number of seeds per cone (10.3 ± 9.3 , 12.1 ± 12.4) (\pm SD) compared to the control trees (27.1 ± 3.5 , 31.7 ± 8.8), while another genotype (1035), responded with lower numbers of seeds per cone when GA_{4/7} was injected in June (21.6 ± 14.3) when compared to control trees for this genotype (38.0 ± 17.9). Only one genotype (765), showed an increase in the number of seeds per cone in response to one of the GA_{4/7} treatments, with stem injections in July resulting in 32.9 ± 14.3 seeds per cone, compared to 19.1 ± 7.7 seeds per cone for control trees of the same genotype. For comparison, each cone contained on average 29.0 ± 10.5 seeds in 2015 and 29.9 ± 10.5 in 2016, irrespective of which GA_{4/7} treatment trees had received.

In 2017, we collected a sample of five cones from each trial tree and extracted all viable seeds. Our analysis of seed weights showed no differences in the 1000-seed weights among the GA_{4/7} treatments ($p=0.12$), while there were strong differences among genotypes ($p < 0.001$) (Fig. 6).

Seeds from control trees and seeds from trees that received their GA_{4/7} in August showed significantly higher germination rates, than seeds from trees that received either the June or July application ($p < 0.001$) on day 7 of the germination trial (Table 5). After

14 days, seeds from trees that received their GA_{4/7} in July showed higher germination rates (46%) than all other treatments (31%). At the end of the trial (day 21) the July application still resulted in a higher germination rate compared to the control trees, as well as those that received GA_{4/7} in June.

Discussion

We hypothesized that at least one of our GA_{4/7} stem injection treatments would increase the number of female conelets, and therefore cones for all genotypes included in our study. Instead, we found that only 40% of the sample population responded with increased female conelet production (spring 2016) to at least one of our GA_{4/7} treatments at the given dosage. Moreover, we found that all three application times (June, July and August) were effective in promoting female conelet production, although which timing proved effective varied strongly among genotypes.

Among the 60% of genotypes that did not respond positively to any of the GA_{4/7} stem injection treatments, one-third appeared to be hypersensitive to the dosage of exogenous GA applied. These genotypes showed varying degrees of needle loss and discoloration within a few weeks after the stem injections. It may still be possible to promote flowering in these genotypes, however, finding an appropriate dosage for such genotypes may not be feasible as it risks significant damage to valuable orchard trees. Instead, it may be advisable to test all genotypes for hypersensitivity to exogenous GA_{4/7} prior to operational stem injections and to simply exclude those showing a negative reaction. A simple and inexpensive test consists of spraying a GA solution onto an individual branch early in the spring and observing the needles for several weeks (Kong and von Aderkas, personal communication) to determine which genotypes may be hypersensitive. Conversely, the remaining two-thirds of ‘non-responsive’ genotypes may need a higher dose of exogenous GA to enhance female conelet production. Further testing would be required to identify if there is an effective dosage for these genotypes.

Our findings thus showed that the initial conelet induction response to GA_{4/7} stem injections in lodgepole pine was genotype specific (clonal). Kong et al. (2018) likewise found clonal differences in the efficacy of GA_{4/7} stem injections among two lodgepole pine genotypes. At present, it remains unclear what causes the clonal differences in conelet induction response to the injection of exogenous GA_{4/7}. However, it has been suggested that clonal differences in endogenous hormone levels, as well as the ability to metabolize exogenous GA_{4/7} may play a critical role (Kong et al. 2018).

Contrary to our expectations, the results from the 2017 cone harvest did not match the findings from the initial conelet numbers observed in spring 2016. Rather than showing a strong genotypic response, the total cone counts suggested a main treatment effect, with GA_{4/7} stem injections in June and July leading to an overall higher number of cones per tree compared to the control trees. The apparent contradiction between these two findings may be rooted in differences in the underlying methodology (i.e. 3 branches vs. whole tree) and the high level of conelet abortion among certain genotypes. In our opinion, the initial response in female conelet induction is a more accurate representation of the actual effect of the GA_{4/7} treatments, as the initial number of female conelets was not influenced by the high rates of conelet abortion.

We also hypothesized that the optimal timings for GA_{4/7} stem injections will vary among genotypes based on the geographic origin of the parent tree from which scion material

was collected. Our findings appear to support this hypothesis, as we found a relationship between optimal timing of GA_{4/7} stem injections, leading to an increase in conelet production and the geographic origin, specifically, latitude of the parent trees, where more northerly origins were positively correlated with the later application of GA_{4/7} stem injections.

Lodgepole pine is one of the most wide-ranging and genetically diverse conifer species in western North America, displaying a high degree of differentiation and local adaptation among and even within populations (Rehfeldt et al. 1999; Wu and Ying 2004; Liepe et al. 2016).

Several studies have highlighted the role of climatic differences in driving genetic diversity among lodgepole pine populations (Chuine et al. 2001; Wang et al. 2010; Liepe et al. 2016).

The formation of reproductive organs represents a significant carbon cost and as a consequence plants adapt to their local climate in order to avoid losing reproductive organs during unfavourable weather conditions such as late frosts or during drought stress (Wheeler et al. 1980).

Genotypes from more northerly geographic origins are likely adapted to a cooler climate with slower spring warming and an increased risk of late frosts and may therefore show a delay in their reproductive cycle when compared to more southerly provenances (Chuine et al. 2001, 2006; Howe et al. 2003).

In order to promote the development of female conelets, the application of exogenous GA_{4/7} has to occur at the proper stage of cone bud initiation in late spring to early summer. Ho and Hak (1994) found that early (May–July) application of GA_{4/7} led to higher seed cone numbers in small potted lodgepole pine seedlings, whereas late (July–August) application resulted in increased pollen cone numbers.

The exact timing of these developmental stages, however, may vary based on both genotype and geographic origin, in particular the northern latitude, of the parent trees (O'Reilly and Owens 1987, 1988). Kong et al. (2016) found that exogenous GA_{4/7} applied to developing buds of lodgepole pine genotypes in a seed orchard in late spring was metabolized quickly with concentrations dropping drastically 2–4 weeks after application.

Given that the intent of treating commercial orchard trees with exogenous GA_{4/7} is typically to increase seed production, it would be counterproductive if the treatments negatively affected the number of seeds per cone, seed weights or germination rates. In our study, we were able to show that in a relatively small fraction of genotypes (12.5%) GA_{4/7} stem injections did in fact cause changes in the number of seeds per cone. In most cases at least one of the application times caused a reduction in seeds per cone when compared to the control trees. In one genotype however, one of the treatments resulted in a higher number of seeds per cone.

In our study, none of the GA_{4/7} treatments caused any change in the 1000-seed weight. In contrast, Eriksson et al. (1998) found a reduction in 1000-seed weights when studying the effects of GA_{4/7} stem injections on seed and pollen production in a Scots pine (*Pinus sylvestris* L.) seed orchard in Sweden. Any reduction in seed size is undesirable as larger seeds are typically associated with better viability and increased seedling vigour (Wahid and Bounoua 2013).

Although we did not find any differences in seed weights, we did find that the GA_{4/7} treatments had an effect on germination rates, with the July application resulting in a higher germination rate than seeds from control trees after 21 days. Gibberellins are known to play a role in seed germination and GA treatments have been used to promote germination when seed availability may be severely limited or when seeds have been stored under less than ideal conditions (Singh et al. 2002; Hedden and Thomas 2012;

Vishal and Kumar 2018). Further research would be needed to determine if this positive effect carries over into increased seedling vigour after outplanting.

In summary, our study showed that GA_{4/7} stem injections can be used to stimulate female conelet production in lodgepole pine, however, the response to such a treatment is complex and strongly influenced by genotype and parent origin.

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Author contributions BT, SB: study conception and design. SB, BT: acquisition of data. SB, BT: analysis and interpretation of data. SB: drafting of manuscript. BT, SB: critical revision. BT, SB: final approval of the version to be published.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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