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RESEARCH ARTICLE

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Effect of giberellic acid ($GA_{4/7}$) and partial stem girdling on induction of reproductive structures in *Pinus patula*

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Abstract

Aim of study: Seed production in forest tree species commonly takes a long time due to the length of the juvenile stage. Even though several treatments have been used to induce early flowering in conifer species, experience on their use in subtropical *Pinus* species is limited. This study aimed to evaluate the effect of $GA_{4/7}$ dose (0, 1.27 and 2.54 mg cm^{-2}) and application time (July-October), alone or in combination with partial stem girdling, on male and female strobili production in young *Pinus patula* Schiede *ex* Schltdl. *et* Cham. clones.

Area of study: Nine clones with different flowering background of high-elevation *Pinus patula* growing in a six-year old seed orchard established in Central México (Aquixtla, Puebla) at 2,800 m elevation.

Material and methods: Two independent flowering trials (FT_1 and FT_2) were carried out in the seed orchard during the 2009 and 2010 flowering cycles; similar factors were evaluated at both trials but time of application, clones tested, and experimental design used varied for each of them. Partial stem girdling was done at the base of the trunk and the $GA_{4/7}$ solution was injected into the xylem above the point of girdling. The following spring, the percentage of trees with strobili and the number of strobili per tree were determined for both male and female structures.

Main results: Significant differences ($p \leq 0.05$) among clones in flowering capacity were found at both trials. None of the treatments applied in FT_1 resulted in an increase of strobili formation, most probably because they were applied too late in the growing season. In FT_2, however, application of $GA_{4/7}$ combined with partial stem girdling increased the percentage of trees with strobili and the number of strobili of both sexes, particularly when applied in early July. Partial stem girdling was more effective on promoting male strobili than female ones in gibberellin-treated grafts.

Research highlights: Timing of $GA_{4/7}$ application and stem girdling was important, with a significant interaction with clones tested in FT_2 on production of male strobili but not on female strobili. Application of 1.27 mg cm^{-2} $GA_{4/7}$ and stem girdling in July promoted the highest percentage of trees with strobili, and increased 25-fold the number of female strobili and 5-fold the number of male strobili per tree as compared to the control treatment. Thus, operational use of this induction treatment would be valuable to increase and accelerate seed production in *Pinus patula* seed orchards in the region.

Keywords: early flowering; gibberellins; juvenile stage; seed orchard; seed production.

Abbreviations: GA: Gibberellic acid; **GA_4:** Gibberellic acid 4; **GA_7:** Gibberellic acid 7; **$GA_{4/7}$:** Mix of gibberellic acids 4 and 7; **FT_1:** Flowering trial carried out in the 2009 season; **FT_2:** Flowering trial carried out in the 2010 season.

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Introduction

Pinus patula Schiede ex Schltdl. *et* Cham is one of the most economically important tree species among the subtropical pines natives to Mexico and Central America (Perry, 1991). Despite its restricted natural distribution range in the Sierra Madre Oriental, in eastern Mexico, the species has a fast growth rate and adaptive capacity to different soil and climate conditions, so it has been highly successful in forest plantations established in subtropical regions (Wormald, 1975; Velázquez *et al.*, 2004), including southern and eastern Africa, South America and Australia (Perry, 1991; Dvorak *et al.*, 2000); in South Africa, *P. patula* occupies about 50% of all the planted land (Malabadi & van Staden, 2005).

Due to the productive potential and wood quality of *P. patula*, there is a growing interest in Mexico in the genetic improvement of the species, with several progeny trials and seed orchards with phenotypically selected trees being established in the last decade in central Mexico (Salaya-Domínguez *et al.*, 2012; Morales-Gonzalez *et al.*, 2013). However, in most forest trees, particularly conifers, a long time is required before reaching sexual maturity (von Aderkas *et al.* 2004). Even when sexually mature scions from selected trees are grafted into seedlings, abundant production of genetically improved seed might take up to 10-15 years after grafting (Sirikul & Luukkanen, 1987; Cherry *et al.*, 2007). In addition, variation in maturity and fertility among genotypes in the orchard, particularly during the early development of grafts, commonly leads to an irregular seed production and uneven clonal representation in the seed crop, negatively affecting genetic diversity and genetic gain in the seedling population (Harrison & Slee, 1991; Codesido & Merlo, 2007). An option to increase seed production and homogenize parental contribution in the seed crop of young grafts is to promote differentiation of reproductive structures in them. Several cultural practices and treatments have been successfully used to promote flowering in forest tree species, such as root pruning, water and high temperature stress, mineral nutrition, stem girdling and application of plant hormones (Philipson, 1990; Meilan, 1997; Owens *et al.*, 2001).

Most treatments for flower induction are generally effective, but the highest production of reproductive structures is commonly obtained when plant hormones are applied, alone or combined with stem girdling (Pharis *et al.*, 1987; Kong & von Aderkas, 2004; Wakushima, 2004). Gibberellic acid (GA) is the phytohormone most used to promote early flowering in forest trees, particularly the combination of GA_4 and GA_7, which has been very effective in several conifers (Philipson, 1990; Kong & von Aderkas, 2004), including various pine species (Wheeler & Bramlett, 1991; von Aderkas *et al.*, 2004; Peng *et al.*, 2011).

The success of $GA_{4/7}$ application for flower induction depends on age of trees, timing and method of application, dosage, and combined effect of other cultural practices (Ross, 1991, 1992; Philipson, 1992, 1996). Since gibberellins affect cell division and differentiation of reproductive structures (Ho & Schnekenburger, 1992; Kende & Zeevaart, 1997), as well as growth and development of female strobili (Owens, 1985), time of application is critical to get both male and female flower induction in these species (Ho & Eng, 1995; Siregar & Sweet, 1996; Wakushima *et al.*, 1997). Thus, $GA_{4/7}$ must be applied during the period of shoot elongation, weeks before differentiation of reproductive structures would occur, in order to be effective (Ho & Eng, 1995; Siregar & Sweet, 1996; Muñoz-Gutiérrez *et al.*, 2010).

In temperate pine species having only one shoot growing cycle, differentiation of pollen and seed cones occurs in late spring or early summer, before the rainy season, so $GA_{4/7}$ is usually applied in early spring (Ho & Eng, 1995). However, tropical and subtropical pine species have a more complex seasonal pattern of shoot growth (Sirikul & Luukkanen, 1987; Dick, 1995; Owens, 1995), with several overlapped shoot growing cycles per year (Gómez-Cárdenas *et al.*, 1998; Isaza, 2008), so knowing the exact time for differentiation of reproductive buds is difficult, and the effective application of GA becomes more complicated (Sirikul & Luukkanen, 1987; Harrison & Slee, 1991; Codesido & Merlo, 2007). For these species, we need first to determine the appropriate timing of $GA_{4/7}$ application in order to develop effective treatments for early flowering.

In 2003, a clonal seed orchard of *P. patula* was established in central Mexico (municipality of Aquixtla, Puebla) as a part of a regional tree improvement program. The purpose of the orchard is to produce seed from phenotypically selected trees for commercial plantations within the natural range of the species in the region. This study was done to contribute in developing a cultural practice to accelerate and increase the production of genetically improved seed in orchards of the species; the specific objectives of the study were (1) to evaluate the effect of $GA_{4/7}$ injection and stem girdling at different times during the growing season on induction of male and female strobili on young *P. patula* grafts, and (2) to compare the effectiveness of strobili induction among clones with different cone production history.

Materials and methods

Location of flower induction trials

The flower induction trials were done in the *P. patula* clonal seed orchard established at the "Conjunto Predial Forestal", in Aquixtla, Puebla, Mexico (19° 43´ 13´´ N and 97° 59´ 20´´ W; 2,800 m). The orchard was established in 2003; it included 95 clones (a total of 1,230 grafts) obtained from phenotypically selected superior trees throughout the natural stands in the region. Two independent flower induction trials were carried out in successive reproductive cycles, in 2009 (FT_1) and 2010 (FT_2), when grafts were six- and seven-years old.

Trials design and application of treatments

In FT_1 10 treatments were applied across six clones, with a graft from each clone randomly assigned to each treatment (i.e. 10 grafts per clone were used). The treatments were generated from the combination of two levels of partial stem girdling (with and without partial girdling), two $GA_{4/7}$ doses (1.27 and 2.54 mg $GA_{4/7}$ cm^{-2} cross-sectional area of stem, equivalent to 100 and 200 mg de $GA_{4/7}$ in a tree with 10 cm in diameter over bark), and two application dates (September 19 and October 17, 2009), plus two control treatments without $GA_{4/7}$ application, one of them without girdling and the other girdled on September 19. The six clones included in this trial varied in amount of cone production the two previous years, from high (average number of cones per ramet >30.0, clones 19 and 116) to low (average number of cones per ramet <1.5, clones 12, 14, and 46) or no production (clone 33). Thus, for the purpose of statistical analysis, the clones were grouped in two production classes: "high" production (clones 19 and 116) and "low" production (clones 12, 14, 33 and 46).

In FT_2 seven treatments were applied across four clones, with three grafts from each clone randomly assigned to each treatment (21 grafts per clone were used). Treatments resulted from the combination of three application dates (July 3, August 3, and September 4, 2010) of 1.27 mg $GA_{4/7}$ cm^{-2} and two partial girdling levels (with and without girdling), plus a control (no $GA_{4/7}$, no girdling) included on July 3. The four clones (clones 27, 32, 33 y 34) included in this trial varied from a mean of 0 to 10 cones produced per ramet in previous years.

Before starting the trials, stem diameter over bark was measured at breast height for all trees in order to calculate cross-sectional area and determine the volume of $GA_{4/7}$ solution to be applied, according to the treatment assigned to each of them. Stem was partially girdled by making two double-overlapping, half-circumferential cuts with a small hacksaw at the base of the tree, above the grafting point; the first cut was made at a height of 50 cm from the ground, and the second at 1.5 times the stem diameter above the first (Muñoz-Gutierrez *et al.*, 2010). The depth of the incisions just reached the outermost xylem layers. The amount of $GA_{4/7}$ required for each tree was applied through stem injection with a micro-pipette, using a $GA_{4/7}$ solution with 30 mg $GA_{4/7}$ ml^{-1} dissolved in 95% ethanol. To apply the $GA_{4/7}$ solution, one or two holes, 2-3 cm in depth, were drilled into the stem at a slight angle at least 10 cm above the higher cut in girdled grafts, and at similar stem height in non-girdled grafts. Control grafts received an equivalent volume of 95% ethanol. Afterwards, holes were covered with bee wax to prevent pathogens and loss of $GA_{4/7}$ solution.

Traits measured and statistical analysis

Presence of male and female strobili was evaluated for each trial the spring after treatments were applied, including the following response variables: percentage of trees with (1) male and (2) female strobili; (3) percentage of branches with male strobili in the three lowest crown whorls; (4) average number of male strobili per branch (in the three lowest whorls); and (5) number of female strobili in the upper and lower half of the crown.

Separate analysis of variance (ANOVA) were carried out for each trial and variable, according to the experimental design and structure of treatments in each case, with the GLM procedure of SAS (SAS Institute, 1998). For FT_1, the data was first analyzed without including the control treatments with the following factorial model:

$$\begin{gathered} y_{ijkl} = \mu + CG_i + D_j + GA_k + Gi_l + CG_i{*}D_j + \\ + CG_i{*}GA_k + CG_i{*}Gi_l + D_j{*}GA_k + D_j{*}Gi_l + \\ + GA_k{*}Gi_l + CG_i{*}D_j{*}GA_k + CG_i{*}D_j{*}Gi_l + \\ + CG_i{*}GA_k{*}G_i + D_j{*}GA_k{*}Gi_l + \\ + CG_i{*}D_j{*}GA_k{*}Gi_l + e_{ijkl} \end{gathered}$$

where CG_i is the fixed effect of ith clone group, D_j is the fixed effect of jth application date, GA_k is the fixed effect of the kth dose of GA, Gi_l is the fixed effect of the lth girdling level, $CG_i{*}D_j$ to $CG_i{*}D_j{*}GA_k{*}Gi_l$ are the double, triple and quadruple interaction terms among the factors involved, and e_{ijkl} is the residual error. A second analysis of variance, including the control treatments, was done for data on the first application date, using a similar factorial model, but without the application date (D_j) effect.

An analogous approach was followed for FT_2. The data without the control treatment was analyzed with the model:

$$\begin{gathered} y_{ijl} = \mu + C_i + D_j + T_k + C_i{*}D_j + C_i{*}T_k + D_j{*}T_k + \\ + C_i{*}D_j{*}T_k + e_{ijkl} \end{gathered}$$

where C_i is the fixed effect of ith clone, D_j is the fixed effect of jth application date, T_k is the fixed effect of *k*th treatment, C_i*D_j, C_i*T_k, D_j*T_k and C_i*D_j*T_k are the double and triple interaction terms among the factors involved, and e_{ijkl} is the residual error. An additional analysis of variance was done for data on the first application date, considering the factors clone, treatment (including the control) and the interaction between them. Significant differences were declared with $p \leq 0.05$, and mean values were compared with the Tukey test. Response variables were transformed before the analysis of variance in order to meet the assumptions of normality and homogeneity of variances for ANOVA (Zar, 1996). The percentage of branches with male strobili was transformed with the arcsine square-root function; the average number of male strobili per branch and the number of female strobili per crown section and per tree were transformed with the square-root function $[(x+0.1)^{0.5}]$. Mean values presented in tables are in the original scale. To compare the percentage of trees with male or female strobili among clones, application dates and treatments, contingency tables and the chi-square (χ^2) test were used (Infante & Zárate, 2000).

Results

Percentage of trees with reproductive structures

Overall, 48% and 82% of grafts produced male and female strobili, respectively, in FT_1; the respective values in FT_2 were 79% and 66% (Table 1). No significant differences among girdling levels, $GA_{4/7}$ dose or application dates were detected with the chi-square test in both trials, but the percentage of trees with male and female strobili differed among clones (Table 1). All clones produced male and female strobili in the FT_2 with percentages varying from 67 to 100% for male and from 38 to 95% for female strobili (Table 1).

In FT_1, 92% of grafts receiving 1.27 g cm^{-2} $GA_{4/7}$ in September produced female strobili versus 75% in the control treatment; in FT_2 respective values when applied in July were 92 and 25% (Table 1). On the other hand, 96% of grafts produced female strobili in FT_1 when treatments were applied in September, and 71% when applied in October, whereas in FT_2, 96% of grafts produced female structures when treated in July, and 54% when treated in September (Table 1).

Production of male and female strobili

Significant differences ($p < 0.05$) among clones, but not among application dates, GA dose, girdling levels or their interactions, were found in the production of male and female strobili in FT_1 (Table 2); stem girdling and $GA_{4/7}$ application did not have a positive effect on differentiation of reproductive structures in this trial. In FT_2, however, a significant main effect ($p < 0.05$) of clones and application dates was found for all traits measured on male and female strobili (Table 2). Treatment effects and clone X treatment interaction were significant only for male strobili in this second trial. No other interaction effects were significant in the analysis across application dates. When treatments applied in early July were analyzed separately (to include the control), treatment effects were significant ($p < 0.05$) for both male and female strobili production, but clone differences were significant only for total and upper crown female strobili, and the interaction clone X treatment was not significant. Thus, except for the interaction effect detected in production of male strobili, the clones tested responded similarly to the treatments and application dates.

The timing of treatment application was critical for cone stimulation, as shown by the contrasting results in both trials. In $FT_{1,}$ when treatments were applied in late summer and early fall, none of the traits was affected. However, in FT_2, treatments applied in early summer (on July 3) increased substantially the production of both male and female strobili (5-fold increase in male strobili and 3-fold increase in female strobili per tree) as compared to the treatments applied in early September (Table 3).

In FT_2, combined application of $GA_{4/7}$ and partial stem girdling had a positive effect on induction of both male and female strobili, as compared to the application of $GA_{4/7}$ alone, but the effect was significant only for male ones. $GA_{4/7}$-treated grafts produced on average 71.9 male strobili per branch in 34.2 % of the branches in the lower whorls and 14.7 female strobili per tree, whereas grafts receiving the combined treatment produced 101.1 male strobili per branch in 48.7% of branches and 22.8 female strobili per tree (Table 3). This represents a 100 and 55% increase in the number of male and female strobili, respectively.

When applied in July, use of $GA_{4/7}$ and partial stem girdling increased production of male strobili by 84% and female strobili by 89 % with respect to the use of $GA_{4/7}$ alone. $GA_{4/7}$ applied in July also caused a 3-fold increase in the number of male strobili (as a result of almost doubling the percentage of branches with strobili and the number of strobili per branch), and a 13-fold increase in the number of female strobili per tree, as compared to the control (19.7 *vs*. 1.4, Table 3). Thus, the combined application of $GA_{4/7}$ and partial stem girdling in July caused a 5-fold increase in male strobili and over a 25-fold increase in female strobili per tree, compared to the control (Table 3). However, when $GA_{4/7}$ alone or

Table 1. Percentage of *P. patula* grafts (%) with male and female strobili in each trial (FT_1 and FT_2), by clone, $GA_{4/7}$ dose, application date, and girdling level

FT₁			FT₂		
Factor and level	**♂ strobili**	**♀ strobili**	**Factor and level**	**♂ strobili**	**♀ strobili**
"Low" production clones:			Clone:		
12	90	80	27	71	81
14	20	100	32	100	95
46	10	70	34	76	48
33	0	40	33	67	38
Group mean	30	73		—	—
"High" production clones:					
19	100	100		—	—
116	70	100		—	—
Group mean	85	100		—	—
Application date:			Application date:		
September	46	96	July	92	96
October	50	71	August	79	67
	—	—	September	71	54
$GA_{4/7}$ dose:			Treatment:		
1.27	50	79	$GA_{4/7}$	67	72
2.54	46	88	$GA_{4/7}$ + Gi	94	72
Stem girdling:					
Without	50	83		—	—
With	46	83		—	—
<u>Date 1 (September):</u>			<u>Date 1 (July):</u>		
$GA_{4/7}$ dose:			Treatment:		
0	50	75	Control	67	25
1.27	50	92	$GA_{4/7}$	83	92
2.54	42	100	$GA_{4/7}$ + Gi	100	100
Stem girdling:					
Without	50	94		—	—
With	44	83		—	—
Trial average:	**48**	**82**		**79**	**66**

$GA_{4/7}$ = gibberellic acid; Gi = stem girdling

combined with partial stem girdling were used in September, the production of male strobili was similar to the control, and the number of female strobili per tree was only 8.4 and 9.9, respectively (data not shown).

Production of male and female strobili also varied among clones in each trial. Overall, in FT_1 less male strobili per branch, but more female strobili per tree, were formed than in FT_2 (Table 4), primarily because of the different clones included in each trial and the variation in cone production in previous years. However, for clone 33 included in both trails, male and female strobili production increased in the second trial.

In FT_1, clones in the "high production" group produced on average over 10 times as much male and female strobili as those in the "low production" group (Table 4). Clone 19 was the most prolific, with over 110 male strobili per branch in 82.5 % of branches at each whorl and over 100 female strobili per tree (Table 4). In the "low production" group, clones 14 and 46 behaved mostly as females, with around 10 female strobili per tree, and clone 12 mostly as male. In FT_2, clones 27 and 32 were quite fertile with 100 or more male strobili per branch and almost 30 female cones, but clones 33 and 34 had much lower production of reproductive structures for both sexes (Table 4). The significant clone X treatment interaction detected for male strobili in FT_2 revealed a larger effect of the combined use of $GA_{4/7}$ and partial stem girdling, compared to the use of $GA_{4/7}$ alone, on the proportion of branches and number of male strobili per branch on clones 27 and 33 than on clones 32 and 34 (Figure 1).

Discussion

Injection of a commercial mixture of $GA_{4/7}$ combined with partial stem girdling were successful in stimulating male and female cone production in young *P.*

Table 2. Statistical significance values (p) obtained from analysis of variance for factors included in the flower induction trials of *P. patula*

Factor	df	♂ strobili: Branches with strobili (%)	♂ strobili: Strobili per branch (No.)	♀ strobili (No.): Upper crown	♀ strobili (No.): Lower crown	♀ strobili (No.): Total tree
FT_1 (not including controls)[¶]:						
Clon group (C)	1	0.004	0.041	<0.001	<0.001	<0.001
Date (D)	1	0.250	0.357	0.810	0.998	0.994
$GA_{4/7}$ (G)	1	0.366	0.385	0.057	0.067	0.062
Girdling (Gi)	1	0.997	0.836	0.707	0.587	0.858
C × D	1	0.957	0.827	0.111	0.422	0.250
C × G	1	0.400	0.602	0.069	0.069	0.069
C × Gi	1	0.685	0.949	0.757	0.358	0.599
D × G	1	0.621	0.637	0.468	0.721	0.568
D × Gi	1	0.300	0.267	0.365	0.808	0.903
G × Gi	1	0.921	0.520	0.255	0.624	0.474
FT_1 (September):						
Clon group (C)	1	0.001	0.018	<0.001	<0.001	<0.001
$GA_{4/7}$ (G)	2	0.252	0.768	0.122	0.254	0.182
Girdling (Gi)	1	0.698	0.975	0.950	0.819	0.844
C × G	2	0.277	0.773	0.057	0.062	0.068
C × Gi	1	0.911	0.742	0.723	0.339	0.681
G × Gi	2	0.570	0.268	0.320	0.750	0.652
C × G × Gi	2	0.122	0.058	0.180	0.626	0.516
FT_2 (not including controls)[¶]:						
Clone (C)	3	0.001	0.003	<0.001	<0.001	<0.001
Date (D)	2	0.002	<0.001	0.005	0.012	0.002
Treatment (T)	1	0.007	0.009	0.313	0.110	0.176
C × D	6	0.688	0.585	0.530	0.585	0.524
C × T	3	0.010	0.018	0.245	0.070	0.120
D × T	2	0.390	0.244	0.832	0.160	0.499
C × D × T	6	0.836	0.854	0.801	0.409	0.638
FT_2 (July):						
Clone (C)	3	0.084	0.089	0.013	0.080	0.025
Treatment (T)	2	0.005	0.012	0.001	0.003	0.001
C × T	6	0.379	0.595	0.267	0.178	0.217

[¶]Control treatments are not included in the analysis of variance; triple and quadruple interaction terms in FT_1 were not significant and are not included in the Table to keep it simple and save space. $GA_{4/7}$ = gibberellic acid.

patula clones. Even though similar enhancing effects of $GA_{4/7}$ applied alone or combined with other cultural treatments have been reported for several temperate, subtropical and tropical pine species (Greenwood, 1982; Sirikul & Luukkanen, 1987; Harrison & Slee, 1991; von Aderkas *et al.*, 2004; Kong & von Aderkas, 2004; Codesido & Merlo, 2007; Peng *et al.*, 2011) no previous information was found for *P. patula*, so it seems to be the first report of effective flowering induction with $GA_{4/7}$ and stem girdling for this species. The effects of treatments were primarily on the amount of strobili formed per tree. A trend to increase the proportion of trees with a positive response was also observed, particularly in FT_2, but the differences were not enough to be declared significant with the contingency chi-square test. The overall percentage of trees with female strobili was relatively stable in both trials; however, the percentage of trees with male strobili increased in the second trial. In addition, although all clones included in the FT_2 produced male and female strobili, the proportion of ramets forming cones varied widely among them, and the response in strobili induction in this study seems to be dependent on several factors.

The contrasting results obtained in FT_2 for treatments applied in July, compared to when they were applied in September, confirm that treatments in FT_1 were applied too late in the growing season. In that particular year most probably the buds were already differentiated by the time the physiological stimuli produced by injecting $GA_{4/7}$ or girdling the stem reached the tree crown. Previous studies of flowering induction in most conifers, particularly in *Pinus* species, have emphasized

Table 3. Effect of application date, $GA_{4/7}$ dose and partial stem girdling on the average number of male and female strobili formed in the two flower induction trials (FT_1 and FT_2) for *P. patula*

Factor and level	♂ strobili		♀ strobili (No.)		
	Branches with strobili (%)	Strobili per branch (No.)	Upper crown	Lower crown	Total tree
FT_1 (not including controls)¶:					
Application date:					
September	19.7 a†	23.6 a	11.1 a	22.8 a	33.9 a
October	35.3 a	46.8 a	10.0 a	21.5 a	31.5 a
$GA_{4/7}$ dose:					
1.27	30.2 a	45.0 a	14.0 a	28.0 a	42.0 a
2.54	24.7 a	25.4 a	7.2 a	16.2 a	23.4 a
Girdling:					
without	26.9 a	30.7 a	11.2 a	21.5 a	32.7 a
with	28.1 a	39.8 a	10.0 a	22.8 a	32.8 a
FT_1 (September):					
$GA_{4/7}$ dose:					
0	31.9 a	34.6 a	14.9 a	17.9 a	32.8 a
1.27	23.8 a	23.5 a	15.9 a	29.4 a	45.3 a
2.54	15.6 a	23.7 a	6.3 a	16.1 a	22.4 a
Girdling:					
without	25.8 a	28.9 a	12.3 a	22.6 a	34.9 a
with	21.8 a	25.6 a	12.5 a	19.7 a	32.2 a
FT_2 (not including controls)¶:					
Application date:					
July	53.9 a	114.8 a	16.6 a	11.9 a	28.5 a
August	45.1 a	98.1 a	10.5 ab	8.1 ab	18.6 ab
September	25.3 b	46.6 b	6.4 b	2.8 b	9.2 b
Treatment:					
$GA_{4/7}$	34.2 b	71.9 b	9.1 a	5.6 a	14.7 a
$GA_{4/7}$ + girdling	48.7 a	101.1 a	13.2 a	9.6 a	22.8 a
FT_2 (July):					
Treatment:					
control	27.1 b	48.9 b	1.0 b	0.4 b	1.4 b
$GA_{4/7}$	43.7 ab	101.8 ab	13.3 ab	6.4 ab	19.7 ab
$GA_{4/7}$ + girdling	64.1 a	127.8 a	19.9 a	17.3 a	37.2 a

¶Control treatments are not included in the analysis of variance;

†values in the same column and factor followed by the same letter are not significantly different (p = 0.05); $GA_{4/7}$ = gibberellic acid.

the importance of optimum application time of treatments to stimulate differentiation of reproductive structures (Greenwood, 1982; Wakushima *et al.*, 1997; Isaza, 2009). In those studies, the optimum period for GA application to promote flowering in *Pinus* species has ranged from March to September (Luukanen & Johanson, 1980; Greenwood, 1982; Harrison & Slee, 1991; Siregar & Sweet, 1996; Wakushima *et al.*, 1997; Isaza, 2008), depending upon the species and the particular environmental conditions where the studies were done.

We could not find any published information about the timing of differentiation for reproductive structures in *P. patula*, but the striking differences in the effectiveness of $GA_{4/7}$ and stem girdling found among both trials indicates that it must be occurring sometime in mid-summer, since treatments applied after August did not have a positive effect on sexual differentiation of primordia, and it commonly takes 1-3 weeks after injecting GA or doing the girdling in the stem to detect any changes in phytohormones or related metabolites in the tree crown (Kong *et al.*, 2008, 2012). In the region where the flowering induction was done, young trees of *Pinus patula* have a shoot growing period that extends from February up to December at least, with 4-5 successive and overlapping shoot elongation cycles (Gómez-Cárdenas *et al.*, 1998), so it is difficult to pinpoint the exact timing when the morphological changes associated with bud formation and differentiation are occurring in the terminal leader or first-order lateral branches. On the other hand, the optimum timing for $GA_{4/7}$ and stem girdling application might differ with any change in the

Table 4. Clone and trial differences in the number of male and female strobili formed in the two flower induction trials (FT_1 and FT_2) for *P. patula*

Clone, group and trial	♂ strobili		♀ strobili (No.)		
	Branches with strobili (%)	Strobili per branch (No.)	Upper crown	Lower crown	Total tree
FT_1:					
"Low" production clones:					
12	58.9	80.3	2.2	2.3	4.5
14	2.2	3.5	1.5	9.1	10.6
33	0.0	0.0	0.5	0.7	1.2
46	1.4	0.4	5.6	6.6	12.2
Group mean	15.6 b†	21.0 b	2.5 b	4.7 b	7.2 b
"High" production clones:					
19	82.5	112.5	31.5	70.6	102.1
116	25.2	13.9	27.4	38.5	65.9
Group mean	53.9 a	63.2 a	29.5 a	54.6 a	84.1 a
Trial average	**28.4**	**35.1**	**11.4**	**21.3**	**32.7**
FT_2:					
27	41.2 ab†	99.6 ab	17.8 a	8.9 ab	26.7 a
32	59.7 a	122.2 a	16.7 a	12.9 a	29.6 a
33	28.7 b	45.3 b	1.3 b	2.8 bc	4.1 b
34	27.9 b	57.4 b	3.1 b	1.6 c	4.7 b
Trial average	**39.4**	**81.1**	**9.7**	**6.6**	**16.3**

†Values in the same column and trial followed by the same letter are not significantly different (p = 0.05).

environmental growing conditions, size of trees, application method, or other factors influencing either the time of strobili differentiation or the distance from the application point in the stem to the crown, as has been shown in other *Pinaceae* species (Chalupka, 1980; Daoust *et al.*, 1995; Siregar & Sweet, 1996; Almqvist, 2003; Kong & von Aderkas, 2004; Isaza, 2008).

Compared across application dates, partial stem girdling was more effective on improving the effect of gibberellin in the production of male strobili than female ones, but when applied in July the combined treatment is effective for both sexes, or even more so for female strobili (compared to the control treatment). Thus, either the physiological stimuli produced by stem girdling and $GA_{4/7}$ injection differ (Pharis *et al.*, 1987; Ross, 1992; Dick, 1995; Kong *et al.*, 2012), or the differentiation of both type of reproductive structures does not occur at the same time in *P. patula*, as has been found in other conifers (Chalupka, 1980; Pharis *et al.*, 1987; Peng *et al.*, 2011; Kong *et al.*, 2012). Male strobili are mainly produced in the lower crown branches whereas female strobili are concentrated in the middle and upper crown branches (Chalupka, 1980), so differences among the effect of $GA_{4/7}$ alone or combined with partial stem girdling might also be related to the differing distance of the respective branches to the point on the stem where the treatment was applied. Though not statistically significant, data in Table 3 for FT_2 shows that the relative effect of the combined application of $GA_{4/7}$ and partial stem girdling on female strobili was higher in the lower section of the crown. An anatomical and phenological study aiming to determine the timing of differentiation for both male and female primordia in *P. patula* would allow to improve the effectiveness of the flowering induction treatments and even promote sex conversion of male to female primordia in the lower branches to have a more balanced sex ratio, as has been shown to occur in other pine species (Wakushima, 2004; Kong *et al.*, 2015).

The variation among clones in the amount of reproductive structures produced (either male or female strobili) in *P. patula* is common to most tree species studied so far (Longman, 1982; Harrison & Slee, 1991; Siregar & Sweet, 1996; Codesido & Merlo, 2007; Isaza, 2009; Peng *et al.*, 2011) and it is generally due to genetic variation among them in sexual precocity, fecundity or sex behavior (i.e., maleness or femaleness). Contrasting with studies in other pine species where the clonal response to the induction treatments varied between "good" and "poor" flower producer clones (Harrison & Slee, 1991; Siregar & Sweet, 1996; Codesido & Merlo, 2007; Isaza, 2008), in our study the effect of $GA_{4/7}$ and stem girdling on female strobili was similar for all the clones tested (as indicated by the lack of clone group (or clone) by treatment interaction in both trials), regardless of their precocity and reproductive output in previous years or during the year of measurement. We recognize that the inability to detect

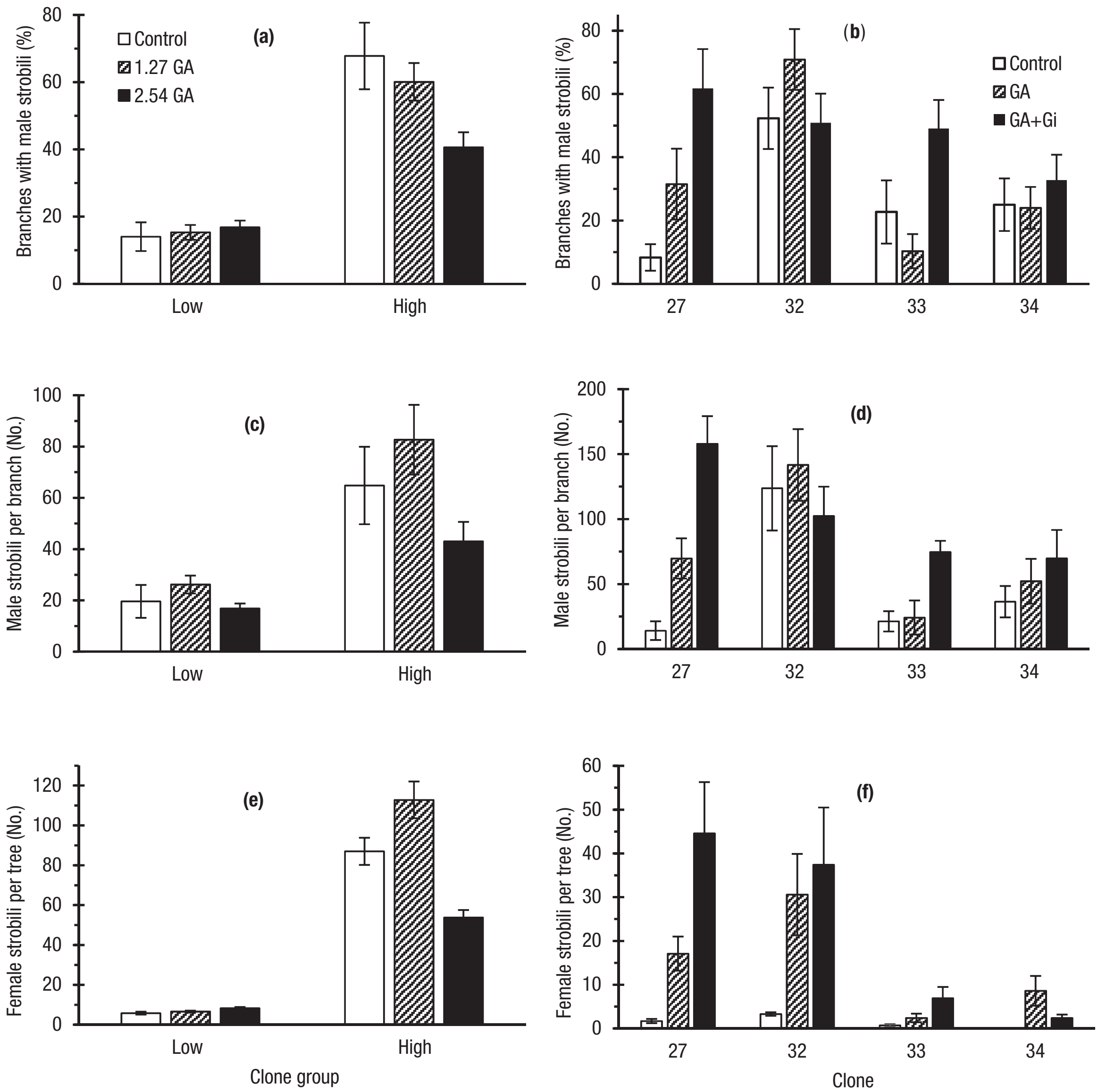

Figure 1. Effect of clone and treatment in the proportion of branches with male strobili (a, b), number of male strobili per branch (c, d), and number of female strobili per tree (e, f) in two separate flower induction trials for *P. patula*: FT_1 (a, c, e) and FT_2 (b, d, f). In both trials, the bars represent the mean value ± s. e. across application dates, except for the control, which was applied only in the first date. In FT_1, clones are grouped as "low" or "high" based on cone production in previous years, and treatments represent the $GA_{4/7}$ dose used in mg cm^{-2} cross-sectional area of stem. In FT_2, "GA" represents 1.27 mg cm^{-2} $GA_{4/7}$ and "Gi" is partial stem girdling (see text).

a significant clone by treatment interaction for female strobili, particularly in FT_2, might be partially due to the structure of treatments used, the limited number of replications available and the intrinsic variability in the response among ramets, since the relative change in production of female strobili due to the use of $GA_{4/7}$ combined with stem girdling, compared to the use of $GA_{4/7}$ alone, or to the control treatment included in July, varies among clones (Figure 1). Despite this, the significant clone X treatment interaction detected for male strobili and the variation among clones in relative amounts of female strobili produced in FT_2 due to the combined effect of $GA_{4/7}$ and stem girdling were not related to the average clonal fecundity in the measurement year.

The increase in the amount of reproductive structures observed in clone 33 (a "poor" producer clone) from FT_1 to FT_2 might be due to age or changes in environmental conditions from year to year but, most probably, it was due to the timing of induction treatments. In FT_2, clone 33 produced only 21.3 male strobili per branch and 0.7 female strobili per tree in the control treatment, but it produced 87.3 male per branch and 16.0 female strobili per tree when $GA_{4/7}$ and stem girdling were applied in July (data not included). Thus, these cultural treatments could be implemented as standard management practices in the seed orchard to enhance cone and seed production and to increase the genetic diversity in the seed crop by incorporating a larger number of clones, particularly those that would normally produce a low number of male or female strobili without the stimulus of the induction treatment.

Conclusions

Results from this study show that the combined use of $GA_{4/7}$ injected to the trunk and stem girdling effectively promote production of male and female strobili in young *P. patula* grafts, when they are applied on time to induce differentiation of the reproductive structures. Under the study conditions the most appropriate application date was in early July. Even though a broad clone variation in reproductive capacity was found in the study, the response to the induction treatments was not related to their fecundity in previous or during the evaluation years. Thus, the operational use of these cultural treatments would allow to accelerate and increase both seed production and genetic diversity in the seed crop of the seed orchard.

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