Flower stimulation in young miniaturized seed orchards of Douglas-fir (*Pseudotsuga menziesii*)[1]

Marilyn L. Cherry, Thimmappa S. Anekonda, Michael J. Albrecht, and Glenn T. Howe

Abstract: We studied flower stimulation in two young miniaturized seed orchards of Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco var. *menziesii*) in Oregon. In experiment 1, female and male flowering were substantially enhanced when the trees were treated 2–4 years after grafting with stem girdling plus stem-injected gibberellin $A_{4/7}$ (GA 0.25× rate = ProCone™ at 0.084 µL·mm^{-2} scion cross-sectional area). Comparable results were obtained the following year when the same trees were retreated with 1× GA. In experiment 2, female and male flowering were significantly enhanced when 3-year-old trees were treated with girdling plus either 1× GA, 1.5× GA, or 2× GA. Some treatments had higher mortality and less height growth than the control in the year of cone development. We recommend using a combination of girdling and 1× GA biennially once trees are large enough to produce large per-hectare seed yields and withstand the stress of flower stimulation. At the study orchards, this seems to be about 5 years postgrafting, just before the sixth growing season. Yields were estimated to be 272 963 seeds·ha^{-1} at age 4 years, or 143 095 seeds·ha^{-1} annually with stimulation occurring every 2 years. Yields should increase as orchards age, with full stocking, and with higher planting densities.

Résumé : Nous avons étudié la stimulation de la floraison dans deux jeunes vergers à graines miniatures de douglas vert (*Pseudotsuga menziesii* (Mirb.) Franco var. *menziesii*) en Oregon. Dans l'expérience 1, la production de fleurs femelles et mâles a été substantiellement améliorée lorsque les arbres furent traités en annelant la tige et en y injectant de la gibbérelline $A_{4/7}$ (taux de GA de 0,25× = ProCone™ à 0,084 µL·mm^{-2} de surface transversale du greffon) deux à quatre ans après avoir été greffés. Des résultats comparables ont été obtenus l'année suivante lorsque les mêmes arbres furent traités à nouveau avec de la GA 1×. Dans l'expérience 2, la production de fleurs femelles et mâles a été améliorée de façon significative lorsque des arbres de trois ans furent annelés puis traités soit avec de la GA 1×, 1,5× ou 2×. Avec certains traitements, il y a eu plus de mortalité et la croissance en hauteur a été réduite comparativement au traitement témoin l'année du développement des cônes. Nous recommandons d'utiliser l'annélation combinée à l'injection de GA 1× sur une base bisannuelle, une fois que les arbres sont assez gros pour produire un rendement élevé de graines à l'hectare et supporter le stress d'une stimulation de la floraison. Dans les vergers à graines utilisés pour cette étude, il semble que cela se produit environ cinq ans après le greffage, juste avant la sixième saison de croissance. Les rendements ont été estimés à 272 963 graines·ha^{-1} à l'âge de quatre ans, ou de 143 095 graines·ha^{-1} annuellement avec une stimulation de la floraison à tous les deux ans. Les rendements devraient augmenter à mesure que les vergers à graines vieillissent, avec une densité relative adéquate et des densités de plantation plus élevées.

[Traduit par la Rédaction]

Introduction

Genetically improved seedlings of coastal Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco var. *menziesii*) are typically grown from seed produced in conventional, wind-pollinated orchards consisting of large, widely spaced grafted trees that are intensively managed for seed production (Howe et al. 2006). In older orchards, flower stimula-

tion can be used to increase seed production and reduce seed production costs (Ross and Bower 1989; Philippe et al. 2004). Flower stimulation typically refers to the process of enhancing the production of male and female strobili (hereafter referred to as flowers) on trees that are already competent to flower. Even when flower-stimulating treatments are used in conventional orchards, commercial levels of seed production are often not obtained until the grafts are at least

M.L. Cherry,[2] T.S. Anekonda,[3] and G.T. Howe. Pacific Northwest Tree Improvement Research Cooperative, Oregon State University, Department of Forest Science, Corvallis, OR 97331-5752, USA.
M.J. Albrecht. Roseburg Forest Products, 34937 Tennessee Road, Lebanon, OR 97355, USA.

[1]Paper No. 3616 of the Forest Research Laboratory, Oregon State University, Corvallis, Ore., USA.
[2]Corresponding author (e-mail: marilyn.cherry@oregonstate.edu).
[3]Present address: Neurological Sciences Institute, Oregon Health Sciences University, 505 NW 185th Avenue, Beaverton, OR 97006, USA.

7–10 years old, and many first-generation orchards have taken 10–15 years to produce useful amounts of seed (Cress and Daniels 1990).

Financial returns from tree improvement could be increased by reducing the lag between orchard establishment and seed production. One way to do this is to greatly increase planting density and begin flower stimulating treatments soon after grafting, thereby obtaining large per-hectare seed yields from young grafts that each produce a moderate number of seeds. This is one potential advantage of miniaturized seed orchards (MSOs), which are orchards planted at close spacings, and then maintained at a height of approximately 2–4 m (Sweet 1995). The goal of most MSOs is to facilitate controlled pollination, thereby allowing breeders to increase genetic gains by producing elite full-sib families and eliminating pollen contamination. Other advantages may include earlier seed crops and reduced management costs. Because the crowns are closer to the ground, it should be more efficient and less costly to collect seed, manage pests, protect against frosts, and delay flowering using overhead irrigation.

Although Douglas-fir MSOs are now being established (Howe et al. 2006), their potential advantages are unproven, and methods of MSO establishment and maintenance have not been optimized. For these reasons, the Pacific Northwest Tree Improvement Research Cooperative undertook a long-term study of Douglas-fir MSOs (Howe et al. 2001). A key goal of this research is to develop flower-stimulating treatments for Douglas-fir MSOs that can be used to (i) shorten the time between grafting and seed production and (ii) reduce pollen contamination early in the life of the orchard by increasing pollen production.

In Douglas-fir and other conifers, flowering can be increased by using stem injections of gibberellic acid (i.e., $GA_{4/7}$, a mixture of GA_4 and GA_7), wounding the trees with stem girdles or root pruning, and fertilizing with calcium nitrate (Ebell 1972; Ross et al. 1985; Wheeler et al. 1985; Woods 1989). These treatments are applied in the spring, and mature seeds are available in the fall of the next year. Therefore, flower-stimulating treatments are usually applied every other year. Douglas-fir responds to exogenous $GA_{4/7}$ by flowering at a younger age, producing a greater number of reproductive buds, and increasing shoot elongation (Pharis et al. 1980; Ross 1983; Pharis et al. 1987). GA is typically most effective when it is combined with girdling, root pruning, or fertilization (Ross et al. 1980, 1985; Pharis and Ross 1986; Pharis et al. 1987). These combined treatments appear to have an additive or synergistic effect, particularly in clones and families that tend to flower poorly (Ross et al. 1985; Webber et al. 1985; Pharis and Ross 1986).

Although flower-stimulating treatments increase seed production, they can also have adverse effects. GA applications may cause damage, and stress-inducing treatments such as girdling can retard shoot elongation, reduce seed yields, and predispose the trees to insect attack (Ross et al. 1980; Pharis et al. 1987; Woods 1989). When GA and girdling are used together, GA may enhance shoot elongation and partially counteract the reduction in elongation caused by girdling (Webber et al. 1985).

Although flower-stimulating treatments have been tested on trees as young as 4 years old from grafting (Ross et al. 1980), most treatments have been optimized for older trees (Pharis et al. 1987), and techniques for stimulating flowering on very young (e.g., 2-year-old) grafts have not been reported. Furthermore, because very young grafts may be particularly susceptible to the adverse effects of these treatments, flower-stimulating treatments used in young MSOs should be carefully evaluated. Therefore, our objectives were to (i) determine whether flower stimulation is effective on Douglas-fir grafts as early as 2 years from grafting; (ii) develop flower-stimulating treatments for very young grafts by evaluating girdling and GA treatments alone and in combination; (iii) examine whether early flower stimulation adversely affects tree health, seed yield, or seed quality; and (iv) obtain preliminary seed yield projections for young Douglas-fir MSOs with and without flower stimulation.

Materials and methods

Seed orchards

We studied two clonal seed orchards at the Roseburg Forest Products Regeneration Center in the Willamette Valley of western Oregon. Both orchards were planted on a Newberg fine sandy loam, were not irrigated, and were routinely mowed and treated with herbicides to control competing vegetation. The Vaughn orchard was grafted in the spring of 1999 using scions collected from an existing seed orchard that had been established 15 years earlier. The Pacific Northwest Christmas Tree Association (PNWCTA) orchard was grafted in the spring of 1997 using scions collected from wild trees and an existing seed orchard that had been established 8–10 years earlier. All ortets were sexually mature and competent to flower when the scions were collected. Tree spacing is 2.44 m × 3.96 m (8 ft. × 13 ft.) in both orchards. All existing seed cones were removed from the study trees before the treatments were applied in the springs of 2001 and 2002.

Experiment 1: effects of GA and stem girdling, alone and in combination

Experiment 1 was designed to test the effects of GA stem injections and stem girdling, alone and in combination. In experiment 1, a randomized complete block design with subsampling was used to test flower-stimulating treatments in each orchard. Within each orchard (Vaughn and PNWCTA), flower-stimulating treatments were replicated across nine clones, using four ramets per clone. For each clone, the ramets studied were randomly chosen from all available ramets, excluding unhealthy trees. The flower-stimulating treatments consisted of stem injections of $GA_{4/7}$ (GA), girdling (G), a combination of girdling and $GA_{4/7}$ (G + GA), and an untreated control (C). One of the nine clones in the PNWCTA orchard was excluded from all statistical analyses (except for survival) because of poor health and high mortality. The poor performance of this clone seemed to be unrelated to the treatments, because it was first observed before the treatments were applied.

The first treatments were applied in the spring of 2001, 2 years after the Vaughn orchard was grafted and 4 years after the PNWCTA orchard was grafted. The trees were girdled on 18 April 2001 using a small hacksaw to make two

double-overlapping, half-circumferential cuts near the base of the tree, about 5 cm below the graft union. The distance between the girdles was about 1.5 times the stem diameter. The incision extended through the vascular cambium just into the outermost xylem.

GA was injected into the base of the scion just above the graft union on 16 May 2001, near the time of vegetative bud burst. We measured the scion basal area immediately above the graft union, drilled a single 6 mm hole about 2 cm into each stem at a slight angle, and injected ProCone™ (4% $GA_{4/7}$; Valent BioSciences, Libertyville, Illinois) at a $0.25\times$ rate of $0.084 \mu L \cdot mm^{-2}$ scion cross-sectional area. Drill holes were left untreated. A Jencons Sealpette micropipette (5–50 μL; Jencons Scientific Inc., Bridgeville, Pennsylvannia) was used for the stem injections in the younger Vaughn orchard, whereas a larger-volume Eppendorf Repeater Plus pipette was used in the PNWCTA orchard (Eppendorf, Westbury, New York). In the spring of 2002, all treatments were repeated on the same trees, except that the GA rates were quadrupled ($1\times = 0.336 \mu L \cdot mm^{-2}$ scion cross-sectional area). These trees were girdled on 11 April 2002, and GA was injected between 7 May and 13 May 2002 near the time of vegetative bud burst.

Tree heights were measured at the end of the 2000 growing season (before the first treatments were applied) and every year thereafter. These measurements were used to calculate relative growth rate (RGR), which is the ratio of annual stem growth to height at the beginning of the growing season. For each tree, we estimated the number of female (N_F) and male (N_M) flowers in the springs of 2002 and 2003 (i.e., 1 year after each treatment was applied) and in the spring of 2004 (i.e., 2 years after the last treatment). These data were then used to calculate the percentage of trees in each plot (treatment × clone × orchard combination) that had female (FEM%) or male (MALE%) flowers. For each tree, we also calculated the age at which we observed the first female flower (FEMAGE) or male flower (MALEAGE). Because we did not measure flowering in 2000, our estimates of FEMAGE and MALEAGE are based on the assumption that no 1-year-old grafts flowered. We counted the number of mature seed cones in the fall of 2003 and used N_F in the spring of 2003 to calculate the percentage of cones that aborted (ABORT%). Tree mortality (MORT%) was calculated based on tree survival after the 2005 growing season.

Experiment 2: effects of GA dosage in combination with girdling

Experiment 2 was designed to test the effects of GA dosage in combination with girdling. Experiment 2 had the same design as experiment 1, but we applied a different set of treatments in a single orchard (Vaughn) and year (2002). We tested the same nine clones that were tested in experiment 1, but we used a different set of randomly selected ramets. On 11 April 2002, we girdled all trees except for the controls and then injected GA on 13 May 2002, near the time of vegetative bud burst. In addition to the untreated controls, we tested three GA rates ($1\times = 0.336 \mu L \cdot mm^{-2}$, $1.5\times = 0.504 \mu L \cdot mm^{-2}$, and $2\times = 0.672 \mu L \cdot mm^{-2}$ scion cross-sectional area) in combination with girdling.

Tree heights were measured before the treatments were applied and every year thereafter, but RGR was not calculated in experiment 2, because some of these trees had been topped before this study was initiated. We counted N_F and N_M in spring 2003 and again in 2004 to examine treatment carryover effects. During the 2003 growing season, the trees in the control and $GA_{1\times}$ treatments were treated with a foliar spray of Asana® XL ($0.75 mL \cdot L^{-1}$ of water; DuPont, Wilmington, Delaware) to control cone and seed insects. We collected mature seed cones from these trees in fall 2003, extracted the seeds, and calculated the number of filled seeds and mean seed mass per cone.

Statistical analyses

Separate analyses were carried out for each experiment, year, and trait. Based on an evaluation of the residuals, N_F and N_M were log-transformed ($\ln(N + 0.5)$). Percentages (FEM%, MALE%, ABORT%, and MORT%) were arcsine square root transformed before analysis. No transformations were needed for RGR, FEMAGE, MALEAGE, number of cones, number of seeds per cone, and seed mass.

Analyses for experiment 1 were conducted using the following mixed effects model for each year and trait:

$$[1] \quad Y_{ijkl} = \mu + \text{orchard}_i + \text{treatment}_j + \text{orchard} \times \text{treatment}_{ij} + \text{clone(orchard)}_{ik} + \text{treatment} \times \text{clone(orchard)}_{ijk} + \varepsilon_{ijkl}$$

where orchard_i is the fixed effect of the ith orchard (Vaughn or PNWCTA), treatment_j is the fixed effect of the jth treatment, $\text{orchard} \times \text{treatment}_{ij}$ is the interaction of the ith orchard and jth treatment, $\text{clone(orchard)}_{ik}$ is the random effect of the kth clone in the ith orchard, $\text{treatment} \times \text{clone(orchard)}_{ijk}$ is the interaction between the jth treatment and the kth clone in the ith orchard, and ε_{ijkl} is the residual error. The mean square for treatment × clone(orchard) was used as the error term for testing all treatment differences.

The analogous model for experiment 2 in the Vaughn orchard was

$$[2] \quad Y_{jkl} = \mu + \text{treatment}_j + \text{clone}_k + \text{treatment} \times \text{clone}_{jk} + \varepsilon_{jkl}$$

The mean square for treatment × clone was used as the error term for experiment 2.

These analyses were conducted using the SAS PROC GLIMMIX procedure (version 9; SAS Institute Inc., Cary, North Carolina). Because the PNWCTA orchard was planted in clonal rows, the variation removed by the clone(orchard) term consisted of inherent variation among clones, environmental variation among seed orchard rows, and their interaction. In the Vaughn orchard, the clones were randomly located throughout the orchard. Therefore, the variation removed by the clone(orchard) term did not contain any systematic variation among orchard rows. Despite this small difference in orchard design, the two orchards were included in a single analysis to increase statistical precision and control type 2 error.

Except for MORT%, differences among treatment means were tested using a two-tailed Tukey–Kramer multiple comparison test. Analyses were conducted across orchards and

Table 1. Number of Douglas-fir flowers per tree 1 year after the trees in experiment 1 were given flower-stimulating treatments in spring 2001 and 2002.

| Orchard | Age from grafting (years) | | Female flowers/tree (N_F) | | | | Male flowers/tree (N_M) | | | |
	When last treated	When last measured	C	G	GA*	G + GA	C	G	GA	G + GA
Spring 2002, 1 year after the GA$_{0.25\times}$ treatment										
Vaughn	2	3	0.4a	2.0ab	2.4ab	7.2b	1.5a	13.6a	10.6a	17.6a
PNWCTA[†]	4	5	1.2a	1.0a	6.0ab	13.8b	37.6a	62.6ab	96.2bc	153.3c
Combined	2–4	3–5	0.8a	1.5a	4.2a	10.5b	19.5a	38.1a	53.4ab	85.5b
Spring 2003, 1 year after the GA$_{1\times}$ treatment										
Vaughn	3	4	9.5a	9.2a	17.5a	24.9a	139.4a	51.6a	215.2b	197.0ab
PNWCTA[†]	5	6	2.2a	8.6a	19.4a	61.5b	123.8a	446.1ab	1594.3b	3452.5c
Combined	3–5	4–6	5.9a	8.9a	18.4b	43.2b	131.6a	248.9a	904.8b	1824.7b

Note: Values are least-squares means of untransformed data. Statistical tests are based on log-transformed data (ln(N + 0.5)). For each row and flower sex, means followed by the same letter are not significantly different according to the Tukey–Kramer multiple comparison test (p = 0.05). C, control; G stem girdled; GA, stem injection with GA$_{0.25\times}$ or GA$_{1\times}$; G + GA = stem injection with GA$_{0.25\times}$ or GA$_{1\times}$ plus girdling.
*GA rate was 0.25× and 1× (where 1× = ProCone™ at 0.336 µL·mm^{-2} scion cross-sectional area) for the 2001 and 2002 treatments, respectively.
[†]PNWCTA, Pacific Northwest Christmas Tree Association.

Table 2. Percentage of Douglas-fir trees with flowers 1 year after the trees in experiment 1 were given flower stimulating treatments in spring 2001 and 2002.

| Orchard | Age from grafting (years) | | Percentage of trees with female flowers (FEM%) | | | | Percentage of trees with male flowers (MALE%) | | | |
	When last treated	When last measured	C	G	GA*	G + GA	C	G	GA	G + GA
Spring 2002, 1 year after the GA$_{0.25\times}$ treatment										
Vaughn	2	3	5.6a	20.4ab	24.1ab	40.7b	12.0a	24.1a	25.0a	35.2a
PNWCTA[†]	4	5	14.6a	14.6a	28.1ab	51.0b	61.5a	61.5a	87.5a	89.6a
Combined	2–4	3–5	10.1a	17.5a	26.1a	45.9b	36.7a	42.8ab	56.2ab	62.4b
Spring 2003, 1 year after the GA$_{1\times}$ treatment										
Vaughn	3	4	33.3a	41.7ab	85.2c	69.4bc	51.8a	60.2ab	90.7c	78.7bc
PNWCTA[†]	5	6	14.6a	21.9ab	46.9bc	79.2c	77.1a	87.5a	90.6a	100.0a
Combined	3–5	4–6	24.0a	31.8a	66.0b	74.3b	64.5a	73.8a	90.7b	89.3b

Note: Values are least-squares means of untransformed data. Statistical tests are based on arcsine square root transformed data. For each row and flower sex, means followed by the same letter are not significantly different according to the Tukey–Kramer multiple comparison test (p = 0.05). C, control; G, stem girdled; GA, stem injection with GA$_{0.25\times}$ or GA$_{1\times}$; G + GA, stem injection with GA$_{0.25\times}$ or GA$_{1\times}$ plus girdling.
*GA rate was 0.25× and 1× (where 1× is ProCone™ at 0.336 µL·mm^{-2} scion cross-sectional area) for the 2001 and 2002 treatments, respectively.
[†]PNWCTA, Pacific Northwest Christmas Tree Association.

within orchards (using the SLICE option of PROC GLIMMIX), and differences were declared significant at the 5% level of probability (p = 0.05). We used a one-tailed Dunnett's test to investigate whether MORT% was significantly less for the treated trees than for the controls. Differences in the cone and seed traits between the C and G + GA$_{1\times}$ treatments in experiment 2 were evaluated using a t test (p = 0.05).

Results

Flowering numbers and percentages

To evaluate the direct effects of the treatments, we measured flowering 1 year after the trees were treated. Compared with the other treatments in experiment 1, N_F and FEM% were significantly greater in the G + GA$_{0.25\times}$ treatment in 2002 and significantly greater in both the G + GA$_{1\times}$ and GA1X× treatments in 2003 (combined analyses in Tables 1 and 2). Similar treatment effects were observed for N_M and MALE%, except that there was a significant interac-

tion between the orchards for N_M in 2003 (p = 0.014). In this case, the GA$_{1\times}$ and G + GA$_{1\times}$ treatments were generally superior in both orchards, but the treatment differences were much larger in the older PNWCTA orchard (Table 1). Overall, the best flower enhancement treatment was G + GA, and the second most effective treatment was GA without girdling. In the combined analyses, N_F, FEM%, N_M, and MALE% were usually largest in the G + GA treatment and always greater in the GA treatment than in the control; these differences were often significant (Tables 1 and 2).

In experiment 1, N_M and MALE% were significantly greater in the older PNWCTA orchard (4- or 5-year-old grafts) than in the Vaughn orchard (2- or 3-year-old grafts) in both 2002 and 2003 (p < 0.021). Because these orchards are unreplicated (i.e., they occur on adjacent but separate sites), this difference could result from factors other than graft age. N_F and FEM% did not differ between the orchards in either year.

In experiment 2 (Vaughn orchard), N_F, FEM%, and N_M

Table 3. Number of Douglas-fir flowers per tree (N_F, females; N_M, males) and percentages of trees with flowers (FEM%, females; MALE%, males) in 2003, 1 year after the 3-year-old grafts in experiment 2 (Vaughn orchard) were given flower stimulating treatments in 2002.

| Trait | Treatment | | | |
	C	G + GA$_{1\times}$	G + GA$_{1.5\times}$	G + GA$_{2\times}$
N_M	3.5a	39.2b	23.6b	27.4b
FEM%	25.0a	63.0b	80.6b	70.4b
N_M	89.0a	593.5b	583.3b	446.7b
MALE%	61.1a	80.6ab	96.3b	100.0b

Note: Values are least-squares means of untransformed data. Statistical tests for N_F and N_M are based on log-transformed data ($\ln(N + 0.5)$), whereas tests for FEM% and MALE% are based on arcsine square root transformed data. For each row, means followed by the same letter are not significantly different according to the Tukey–Kramer multiple comparison test ($p = 0.05$). C, control; G + GA, stem girdling plus stem injection with GA$_{1\times}$, GA$_{1.5\times}$, or GA$_{2\times}$, where 1× is ProCone™ at 0.336 µL·mm^{-2} scion cross-sectional area.

Table 4. Carryover effects of flower-stimulating treatments in experiment 1: number of Douglas-fir flowers per tree in 2004, 2 years after the trees were given their last flower stimulating treatment in spring 2002.

| | Age from grafting (years) | | Female flowers/tree (N_F) | | | | Male flowers/tree (N_M) | | | |
Orchard	When last treated	When last measured	C	G	GA*	G + GA	C	G	GA	G + GA
Vaughn	3	5	12.7a	4.0a	1.5a	0.2a	503.5a	599.7a	39.2ab	14.1b
PNWCTA[†]	5	7	2.2a	2.0a	15.3a	1.7a	587.1a	390.9a	282.6a	131.1a
Combined	3–5	5–7	7.5a	3.0a	8.4a	0.9a	545.3a	495.3a	160.9ab	72.6b

Note: Values are least-squares means of untransformed data. Statistical tests are based on log-transformed data ($\ln(N + 0.5)$). For each row and flower sex, means followed by the same letter are not significantly different according to the Tukey–Kramer multiple comparison test ($p = 0.05$). C, control; G, stem girdled; GA, stem injection with GA$_{0.25\times}$ or GA$_{1\times}$; G + GA, stem injection with GA$_{0.25\times}$ or GA$_{1\times}$ plus girdling.
*GA rate was 0.25× and 1× (where 1× is ProCone™ at 0.336 µL·mm^{-2} scion cross-sectional area) for the 2001 and 2002 treatments, respectively.
[†]PNWCTA, Pacific Northwest Christmas Tree Association.

Table 5. Carryover effects of flower stimulating treatments in experiment 2 (Vaughn orchard): number of Douglas-fir flowers per tree (N_F, females; N_M, males) and percentages of trees with flowers (FEM%, females; MALE%, males) in 2004, 2 years after the 3-year-old grafts were given flower stimulating treatments in 2002.

| Trait | Treatment | | | |
	C	G + GA$_{1\times}$	G + GA$_{1.5\times}$	G + GA$_{2\times}$
N_F	8.7a	1.9a	1.6a	0.8a
FEM%	41.7a	6.5b	15.7ab	12.0ab
N_M	483.6a	14.0b	27.6b	17.7b
MALE%	83.3a	25.0b	33.3b	40.7b

Note: Values are least-squares means of untransformed data. Statistical tests for N_F and N_M are based on log-transformed data ($\ln(N + 0.5)$), whereas tests for FEM% and MALE% are based on arcsine square root transformed data. For each row, means followed by the same letter are not significantly different according to the Tukey–Kramer multiple comparison test ($p = 0.05$). C, control; G + GA, stem girdling plus stem injection with GA$_{1\times}$, GA$_{1.5\times}$, or GA$_{2\times}$, where 1× is ProCone™ at 0.336 µL·mm^{-2} scion cross-sectional area.

were always significantly greater in the G + GA treatments (1×, 1.5×, and 2×) than in the control (Table 3), and there were no significant differences among the G + GA treatments. Similar results were observed for MALE%, except that the G + GA$_{1\times}$ treatment was not significantly greater than the control.

To evaluate the indirect (carryover) effects of the treatments, we also measured flowering 2 years after the last treatments were applied. In experiment 1, there were no treatment differences for N_F (Table 4), FEM%, or MALE%

(data not shown). However, there was a significant reduction in N_M for the trees that had received the G + GA treatments in 2001 and 2002 (combined analysis, Table 4). The two treatments with the greatest N_M in 2003 (GA and G + GA; Table 1) had the lowest N_M in 2004 (Table 4). In experiment 2, we found no differences in N_F, but FEM% was significantly lower in the G + GA$_{1\times}$ treatment compared with the control (Table 5). In the same experiment, N_M and MALE% were significantly lower in the three G + GA treatments compared with the control. The three treatments with the

Fig. 1. Age of Douglas-fir grafts when they first flowered in the Vaughn orchard (FEMAGE and MALEAGE in experiment 1). For each flower sex, bars with the same letter are not significantly different according to the Tukey–Kramer multiple comparison test ($p = 0.05$). C, control; G, girdled; GA, $GA_{4/7}$ stem injection; G + GA, girdled plus $GA_{4/7}$ stem injection. GA rates were 0.25× in 2001, followed by 1× in 2002, where 1× is ProCone™ at 0.336 µL·mm^{-2} scion cross-sectional area.

Fig. 2. Cone abortion (ABORT%) in experiment 1 (Vaughn and PNWCTA orchards). Values are least-squares means of untransformed data. Bars with the same letter are not significantly different according to the Tukey–Kramer multiple comparison test ($p = 0.05$) based on an analysis of arcsine square root transformed percent data. C, control; G, girdled; GA, $GA_{4/7}$ stem injection; G + GA, girdled plus $GA_{4/7}$ stem injection. GA rates were 0.25× in 2001, followed by 1× in 2002, where 1× is ProCone™ at 0.336 µL·mm^{-2} scion cross-sectional area.
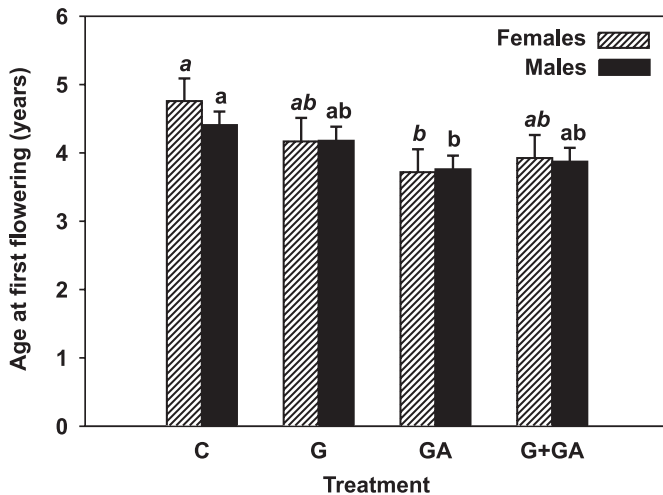
greatest N_M and MALE% in 2003 (the G + GA treatments; Table 3) had significantly lower N_M and MALE% in 2004 (Table 5).

Years to flowering

In experiment 1, we began measuring flowering when the grafts in the Vaughn orchard were 2 years old (the same year that the first treatments were applied). Therefore, we were able to obtain reasonable estimates of the age of first flowering for each treatment by assuming that no flowering occurred in the first year after grafting. Both FEMAGE and MALEAGE were significantly lower in the GA treatment compared with the control (Fig. 1).
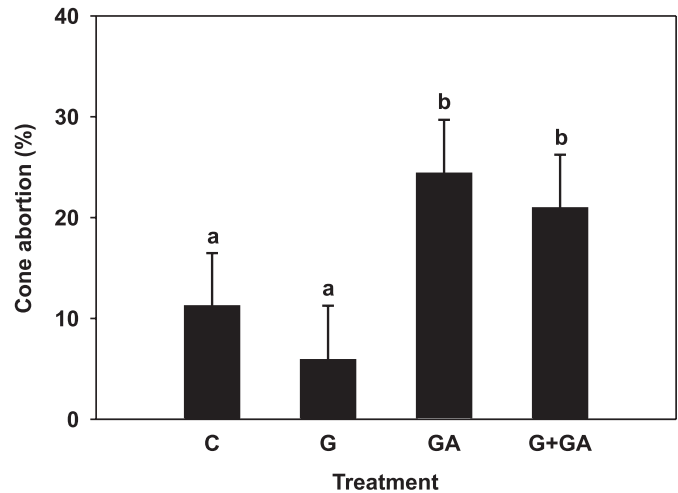
Seed production

ABORT% varied among treatments in experiment 1 but not in experiment 2. In experiment 1, ABORT% was significantly greater in the GA and G + GA treatments compared with the control and girdling treatments (Fig. 2).

In experiment 2, we compared measures of seed quantity and quality between the control and G + GA_{1x} treatment. Seed quality was not measured in the other treatments because we could not treat the whole orchard with insecticides to control seed and cone insects. The G + GA_{1x} treatment did not adversely affect the number of seeds per cone. The mean number of filled seeds per cone was 10.9 for the controls and 10.6 for the trees in the G + GA_{1x} treatment ($p = 0.930$; Table 6). Seeds from the G + GA_{1x} treatment, however, weighed slightly less (12.5 mg) than seeds from the controls (14.6 mg; $p = 0.049$). Estimated cone and seed yields are presented in Table 6.

Relative growth rate and tree mortality

We studied the potential adverse effects of the treatments by comparing RGR and MORT% among the treatments. In

experiment 1, the F test for RGR during the 2002 growing season was significant ($p = 0.029$), but none of the individual treatment comparisons were significant using the Tukey–Kramer multiple comparison test ($p > 0.070$; Fig. 3). In general, the GA and G + GA treatments tended to have higher RGRs, and there was no significant interaction between the orchards ($p = 0.567$; Fig. 3). These results indicate that the stimulation treatments did not adversely affect growth during the growing season in which the second treatments were applied. In contrast, RGR during the 2003 growing season was significantly lower in the GA and G + GA treatments compared with the G and control treatments ($p < 0.0001$; Fig. 3). This indicates that the stimulating treatments resulted in significantly less growth in the treatments that had the largest numbers of developing cones.

Mortality of the stimulated trees was always greater than the mortality of the controls. In experiment 1, however, these differences were only significant for the girdling (G) treatment compared with the control ($p = 0.021$; Fig. 4A), and in experiment 2, they were only significant for the G + $GA_{1.5x}$ treatment ($p = 0.005$; Fig. 4B).

Discussion

Flowering can be enhanced on trees as early as 2 years from grafting

Our results clearly demonstrate that female flowering can be enhanced on trees as early as 2 years from grafting using a combination of girdling and GA. Even at this young age, the G + $GA_{0.25x}$ treatment at the Vaughn orchard had 18 times as many female flowers and more than 7 times as many flowering trees as did the controls. Overall, the G + GA treatment increased N_F more than 13-fold in 2002 and almost sevenfold in 2003. The treatments also enhanced

Table 6. Preliminary estimates of cone and seed yields for a Douglas-fir miniaturized seed orchard (2.44 m × 3.96 m; 1035 trees·ha^{-1}).

Treatment	Age 6 survival (%)*	Seed† Mass (mg)	Seed† No. per cone	No. per flowering tree† Cones	No. per flowering tree† Seeds§	No. per live tree Cones	No. per live tree Seeds§	Seeds·ha^{-1} (age 4)‡ Actual survival	Seeds·ha^{-1} (age 4)‡ 100% survival	Seeds·ha^{-1} (ages 4 + 5)/2‡ Actual survival	Seeds·ha^{-1} (ages 4 + 5)/2‡ 100% survival
Control	97.2	14.6a	10.9a	13.4a	66.6a	3.4	16.6	16 739	17 221	29 174	30 014
G + GA$_{1\times}$	83.3	12.5b	10.6a	63.2b	497.5b	40.2	316.6	272 963	327 687	143 095	171 783

Note: Estimates are based on measurements made in experiment 2, assuming different rates of graft survival. All data are for 4-year-old grafts, except for survival (age 6 years) and the per-hectare seed yields averaged over 2 years (i.e., 4- and 5-year-old grafts that were stimulated at age 3 years). Control, untreated control; G + GA$_{1\times}$, stem girdling plus stem injection with 1× GA, where 1× is ProCone™ at 0.336 µL·mm^{-2} scion cross-sectional area.

*We used survival at age 6 years to project per-hectare seed yields, whereas the data used to estimate per-tree seed yields were measured at ages 4 years and 5 years.

†For each column, means followed by the same letter are not significantly different ($p < 0.05$).

‡Estimates of the number of seeds per hectare at age 4 years were calculated as follows: (number of seeds per live tree) × (1035 grafted trees·ha^{-1}) × (% survival at age 6 years). Seed yield estimates were made based on actual survival and 100% survival of grafted trees. Because we did not count seeds and cones 2 years after treatment (age 5 years), we estimated seed yields for age 5 years using flower counts. Therefore, estimates of the mean number of seeds per hectare at ages 4 and 5 years were calculated as follows: [(seeds·ha^{-1} (age 4 years)) + (seeds·ha^{-1} (age 4 years)) × (flowers per live tree (age 5 years)/flowers per live tree (age 4 years))]/2.

§The number of seeds per tree does not equal the product of seeds per cone and cones per tree because of a negative correlation (–0.32; $p = 0.086$) between these variables.

Fig. 3. Relative growth rate (RGR) in experiment 1 (Vaughn and PNWCTA orchards). Bars with the same letter are not significantly different according to the Tukey–Kramer multiple comparison test ($p = 0.05$). C, control; G, girdled; GA, GA$_{4/7}$ stem injection; G + GA, girdled plus GA$_{4/7}$ stem injection. GA rates were 0.25× in 2001, followed by 1× in 2002, where 1× = ProCone™ at 0.336 µL·mm^{-2} scion cross-sectional area. RGR was measured during the season of flower bud differentiation (2002) and cone development (2003).
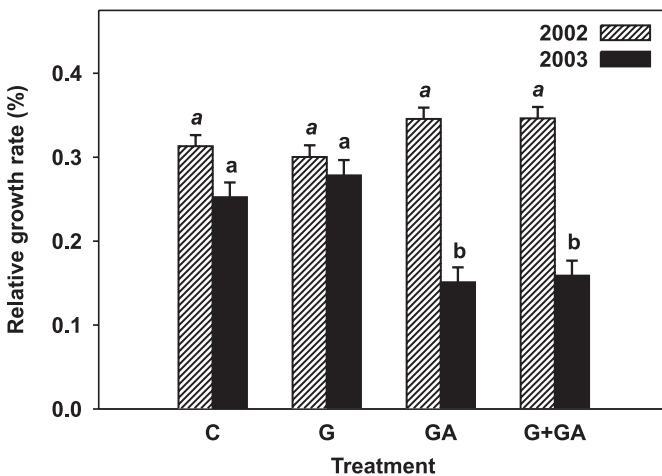
male flowering, and the response to flower stimulation seemed to increase with orchard age (Table 1). Overall, the G + GA treatment increased N_M over fourfold in 2002 and almost 14-fold in 2003. In experiment 2, N_F was about 7–11 times greater in the G + GA treatments, whereas N_M was approximately 5–7 times greater. These levels of flower stimulation are large enough to be important in practice. In fact, the G + GA$_{1\times}$ treatment has been used operationally in these orchards since age 4 years.

Flowering might have been even greater in the G + GA treatment if we had treated the 2-year-old grafts with a higher concentration of GA in 2001. For larger Douglas-fir trees (diameter at breast height (DBH) = 5–20 cm), the rec-

ommended rate of ProCone™ is 0.18–0.36 µL·mm^{-2} stem cross-sectional area (ProCone™ label; Valent BioSciences). In 2001, we used about one-quarter of this rate (0.25×; 0.084 µL·mm^{-2}). Because GA at the 0.25× rate did not cause any overt problems, we increased the rate of GA to 1× in 2002 and retreated the same trees. This 1× rate is within the recommended range cited above. Although it is uncommon to stimulate trees in successive years, we chose to treat these trees aggressively so that subtle adverse effects of the treatments could be detected.

Considering trees of all ages, the best treatments for enhancing female and male flowering were the G + GA treatments (Tables 1–3). In experiment 1, the second most effective treatment was GA without girdling. The GA treatment also reduced the age at which the grafts first produced female and male flowers: the mean age of female flowering was advanced by 1 year, and the mean age of male flowering was advanced by 0.65 years compared with the control. Overall, these results are consistent with those of other studies indicating that GA stem injections enhance flowering but are most effective when they are combined with girdling (Ross et al. 1980, 1985; Pharis and Ross 1986; Pharis et al. 1987). Although girdling is clearly effective on older trees (Woods 1989), our results were ambiguous. Flowering was almost always greater in the girdling treatment than in the control; however, none of these comparisons was statistically significant (Tables 1 and 2).

In experiment 2, we tested ProCone™ amounts ranging from 0.336 µL·mm^{-2} (1×) to 0.672 µL·mm^{-2} (2×) in combination with girdling. These treatments consistently enhanced flowering compared with the control, but the treatments did not differ among each other. Therefore, considering the potential adverse effects of GA, we recommend using the G + GA$_{1\times}$ treatment for enhancing flowering in young grafts.

Seed yield and quality

The GA and G + GA treatments increased the rate of cone abortion in experiment 1 (Fig. 2) but not in experiment 2 (data not shown). Furthermore, seed masses were slightly lower in the G + GA$_{1\times}$ treatment than in the untreated controls; however, this difference was small, and the number of

Fig. 4. (A) Tree mortality (MORT%) in experiment 1 (Vaughn and PNWCTA orchards). C, control; G, girdled; GA, $GA_{4/7}$ stem injection; G + GA, girdled plus $GA_{4/7}$ stem injection. GA rates were 0.25× in 2001, followed by 1× in 2002, where 1× is ProCone™ at 0.336 µL·mm^{-2} scion cross-sectional area. (B) Tree mortality (MORT%) in experiment 2 (Vaughn orchard). C, control; G + GA, stem girdling plus stem injection with $GA_{1\times}$, $GA_{1.5\times}$, or $GA_{2\times}$, where 0.25× is the same as in experiment 1. Values are least-squares means of untransformed data. Bars with the same letter as that of the control do not significantly differ from the control according to the one-tailed Dunnett's test (p = 0.05) based on an analysis of arcsine square root transformed data.
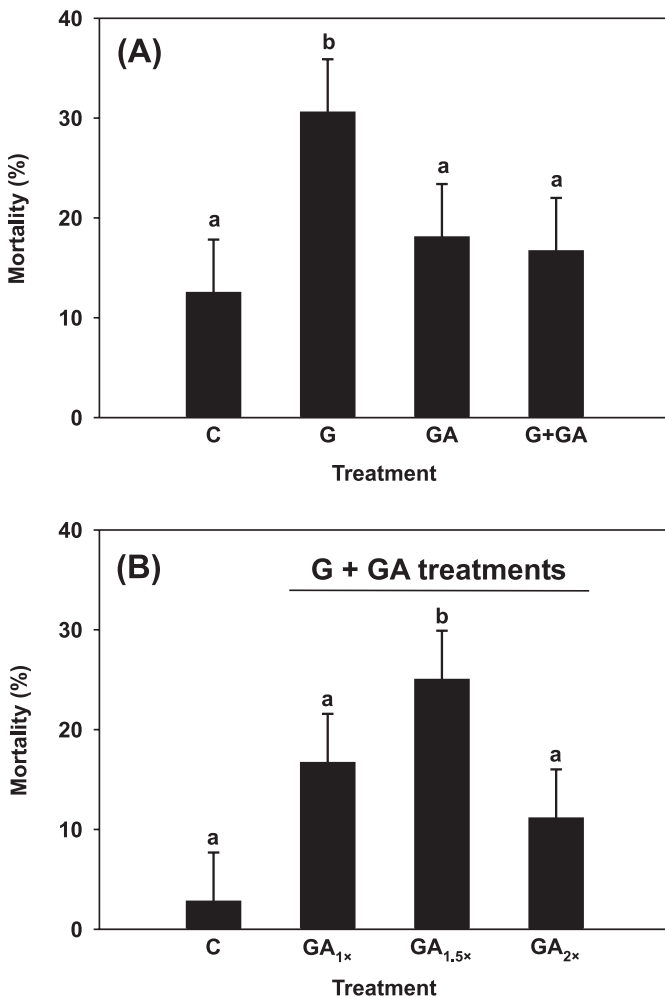
filled seeds per cone was not significantly different between the treated and untreated trees. Ross et al. (1980) also reported higher cone abortion rates for GA-treated trees and suggested this could be a direct response to GA or an indirect effect of intercone competition for nutrients. In previous studies, girdling decreased the number of filled seeds per cone (Ross et al. 1980) and resulted in a small decrease in seed mass (Woods 1989).

The flower-stimulating effects of the treatments far outweighed any increase in cone abortion. Assuming 1035 trees·ha^{-1} and a graft survival rate of 83.3% we estimated that 272 963 seeds·ha^{-1} could be harvested when the grafts are

4 years old (Table 6). This compares with 16 739 seeds·ha^{-1} without flower stimulation and a graft survival of 97.2%. Assuming that the trees are treated every second year, and that each seed weighs 12.5 mg, annual production would be 143 095 seeds·ha^{-1} (1.8 kg·ha^{-1}), which is about five times the production without flower stimulation (Table 6). If survival could be increased to 100% (e.g., by irrigating and replacing dead trees), annual production could be as high as 171 783 seeds·ha^{-1} or 2.1 kg·ha^{-1}. Furthermore, seed yields should increase when the trees become older and larger and when higher MSO planting densities are used. For example, we are now testing MSO planting densities of 417, 1250, and 3333 trees·ha^{-1}, which would lead to annual yields of 0.9, 2.6, and 6.9 kg·ha^{-1}, respectively, assuming the same number of seeds per tree and the same graft survival as we measured in experiment 2.

By stimulating male flowering, pollen production should increase and pollen contamination should decline. Pollen contamination, which is the pollination of seed orchard parents by nonorchard trees, is often 30%–40% in mature conventional Douglas-fir orchards (Slavov et al. 2005) and is expected to be even higher in very young orchards.

Carryover and adverse effects on tree health

Flower-stimulating treatments may have additional carryover effects 2 years after they are applied. In Douglas-fir, for example, girdling increased flowering 2 years poststimulation (Woods 1989). In our experiments, however, we observed a negative relationship. The treatments with the greatest male flowering 1 year after the last stimulation (i.e., the G + GA treatments of 2002) had the lowest male flowering the following year (Tables 4 and 5). The same trend was observed for female flowering, but these differences were rarely significant (Tables 4 and 5).

Direct damage from flower-stimulating treatments may become evident shortly after the treatments are applied or may only become apparent after repeated treatments. In experiment 1, we applied treatments in two consecutive years to ensure that subtle adverse effects would be detected. Treatments may also have indirect effects that adversely affect tree health and survival, such as those resulting from the heavy production of pollen and cones. Tree health and vigor were evaluated by measuring RGR and tree mortality, and cone and seed health were evaluated by measuring seed cone abortion, numbers of filled seed per cone, and seed mass.

We observed no immediate adverse effects of the treatments. In experiment 1, for example, we measured RGR during the 2002 growing season (immediately after the trees were treated for the second time) and found no differences among the treatments. During the following season, however, RGR was significantly lower in the GA and G + GA treatments, presumably because resources were allocated to seed and cone production at the expense of vegetative growth. Our results also suggest that flower stimulation will increase tree mortality. The higher mortality of the G + $GA_{1.5\times}$ treatment in experiment 2, in particular, leads us to caution against using dosages higher than 1×. The G + $GA_{1\times}$ treatment had a survival rate of 83.3% versus 87.5% for the control across both orchards in experiment 1 and 83.3% compared with 97.2%, respectively, for the control in experiment 2. It is unclear what caused the mortality. Because the

treatments with heavy cone production had much lower RGRs in 2003 and lower cone production in 2004, the stress to the tree associated with cone production may have contributed to this excess mortality. Stem girdling in two consecutive years could have also had negative consequences (Fig. 4A). Orchard survival might have been greater, and treatment differences lower, if the orchards were irrigated. If flower-stimulating treatments are used in young orchards, irrigation and fertilization are recommended to partially mitigate the physiological stresses caused by the treatments themselves or by the subsequent cone crops (Ross and Bower 1991).

Implications for seed orchard management

Although seed production can be enhanced on 2-year-old grafts, it is unlikely that flower stimulation will be desirable or cost effective until the trees are at least 4 or 5 years old. Because seed production is limited by crown size, it is desirable to maximize vegetative growth for the first few years. Unfortunately, flower stimulation had a negative effect on RGR during the year of cone production and will delay crown development. In contrast, it is desirable to inhibit vegetative growth in MSOs once sufficient crown size is achieved, which seems to be about 6 years old for these orchards but may occur earlier in MSOs with higher planting densities. Another reason to delay flower stimulation is the increased mortality of the treated trees. Based on experience with conventional orchards, the adverse effects of flower stimulation should become less pronounced as the trees become older and larger. Because the timing of financial returns is an important driver in financial analyses, there is an important tradeoff between speeding financial returns by practicing very early flower stimulation, versus delaying flower stimulation to minimize tree mortality and other adverse effects. Our data should be valuable for quantifying these tradeoffs. For the type of orchards we studied, it appears that flower stimulation should begin about 5 years after grafting, at the beginning of the sixth growing season.

Flowering varied substantially among the clones. Considering all stimulated trees in experiment 2, for example, clonal means ranged from 0.5 to 63.6 for N_F and from 8.3% to 81.2% for FEM%. In the same experiment, clonal means ranged from 47.9 to 914.1 for N_M and from 60.4% to 100% for MALE%. Other researchers have reported large clonal differences in flowering and have concluded that GA is most effective on clones that are predisposed to flower (Ross and Pharis 1976, 1986). These data suggest that early flower stimulation may be cost effective for some clones but not others.

Further research is needed to understand the interactions between crown management and flower stimulation in MSOs. In particular, it will be important to understand how the timing and severity of pruning affects flowering and seed production. In addition, flower stimulation in MSOs might be improved if foliar application of GA can be optimized for Douglas-fir rather than relying on stem injections. Furthermore, it might be possible to substitute root pruning for stem girdling, resulting in additional cost savings, but these treatments have not been optimized for MSOs. Despite these operational constraints, the G + GA$_{1×}$ treatment we describe is being used operationally in the Vaughn and PNWCTA orchards.

References

Cress, D.W., and Daniels, J.D. 1990. Forest seed orchards of western Oregon, western Washington and northern California. Daniels and Associates Consultants, Centralia, Wash.

Ebell, L.F. 1972. Cone-induction response of Douglas-fir to form of nitrogen fertilizer and time of treatment. Can. J. For. Res. **2**: 317–326.

Howe, G.T., Anekonda, T.S., and Slavov, G. 2001. Pacific Northwest Tree Improvement Research Cooperative annual report, 2000–2001 [online]. Oregon State University, Corvallis, Ore. Available from http://www.fsl.orst.edu/pnwtirc/reports/report_pdf/00-01.pdf [accessed 15 March 2006].

Howe, G.T., Jayawickrama, K.J., Cherry, M.L., Johnson, G.R., and Wheeler, N.C. 2006. Breeding Douglas-fir. *In* Plant breeding reviews. Vol. 27. *Edited by* J. Janick. J. Wiley & Sons, Hoboken, N.J. pp. 245–353.

Pharis, R.P., and Ross, S.D. 1986. Flowering of Pinaceae family conifers with gibberellin A$_{4/7}$ mixture: how to accomplish it, mechanisms and integration with early progeny testing. *In* Proceedings, Conifer Tree Seed in the Inland Mountain West Symposium, 5–6 August 1985, Missoula, Montana. *Edited by* R.C. Shearer. USDA Forest Service, Intermountain Research Station, Ogden, Utah.

Pharis, R.P., Ross, S.D., and McMullan, E. 1980. Promotion of flowering in the Pinaceae by gibberellins III. Seedlings of Douglas-fir. Physiol. Plant. **50**: 119–126.

Pharis, R.P., Webber, J.E., and Ross, S.D. 1987. The promotion of flowering in forest trees by gibberellin A$_{4/7}$ and cultural treatments: a review of possible mechanisms. For. Ecol. Manage. **19**: 65–84.

Philippe, G., Lee, S.J., Schute, G., and Heois, B. 2004. Flower stimulation is cost-effective in Douglas-fir seed orchards. Forestry, **77**: 279–286.

Ross, S.D. 1983. Enhancement of shoot elongation in Douglas-fir by gibberellin A$_{4/7}$ and its relation to the hormonal promotion of flowering. Can. J. For. Res. **13**: 986–994.

Ross, S.D., and Bower, R.C. 1989. Cost-effective promotion of flowering in a Douglas-fir seed orchard by girdling and pulsed stem injection of gibberellin A$_{4/7}$. Silvae Genet. **38**: 189–194.

Ross, S.D., and Bower, R.C. 1991. Promotion of seed production in Douglas-fir grafts by girdling + gibberellin A$_{4/7}$ stem injection and effect of retreatment. New For. **5**: 23–34.

Ross, S.D., and Pharis, R.P. 1976. Promotion of flowering in the Pinaceae by gibberellins I. Sexually mature, non-flowering grafts of Douglas-fir. Physiol. Plant. **36**: 182–186.

Ross, S.D., and Pharis, R.P. 1986. Status of flowering in conifers: a constraint to tree improvement? *In* Proceedings, Canadian Tree Improvement Association 20th Meeting, 19–22 August 1985, Quebec City, Que. *Edited by* F. Caron, A.G. Corriveau, and T.J.B. Boyle. Canadian Forest Service, Chalk River, Ont. pp. 11–28.

Ross, S.D., Pharis, R.P., and Heaman, J.C. 1980. Promotion of cone and seed production in grafted and seedling Douglas-fir seed orchards by application of gibberellin $A_{4/7}$ mixture. Can. J. For. Res. **10**: 464–469.

Ross, S.D., Webber, J.E., Pharis, R.P., and Owens, J.N. 1985. Interaction between gibberellin $A_{4/7}$ and root-pruning on the reproductive and vegetative process in Douglas-fir. I. Effects on flowering. Can. J. For. Res. **15**: 341–347.

Slavov, G.T., Howe, G.T., and Adams, W.T. 2005. Pollen contamination and mating patterns in a Douglas-fir seed orchard as measured by simple sequence repeat markers. Can. J. For. Res. **35**: 1592–1603.

Sweet, G.B. 1995. Seed orchards in development. Tree Physiol. **15**: 527–530.

Webber, J.E., Ross, S.D., Pharis, R.P., and Owens, J.N. 1985. Interaction between gibberellin $A_{4/7}$ and root-pruning on the reproductive and vegetative process in Douglas-fir. II. Effects on shoot elongation and its relationship to flowering. Can. J. For. Res. **15**: 348–353.

Wheeler, N.C., Masters, C.J., Cade, S.C., Ross, S.D., Keeley, J.W., and Hsin, L.Y. 1985. Girdling: an effective and practical treatment for enhancing seed yields in Douglas-fir seed orchards. Can. J. For. Res. **15**: 505–510.

Woods, J.H. 1989. Stem girdling to increase seed and pollen production of coast Douglas-fir. British Columbia Ministry of Forests, Victoria, B.C. Res. Note 103.