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The Effect of Flurprimidol on Bud Flush, Shoot Growth, and on Endogenous Gibberellins and Abscisic Acid of Douglas-Fir Seedlings

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Abstract. For 4-month-old Douglas-fir [*Pseudotsuga menziesii* (Mirb.) Franco] seedlings under 17-h photoperiods in controlled environment conditions, flurprimidol (α-(1-methlyethyl-α-[4-(trifluoromethoxy)phenyl]-5-pyrimidine-methanol) decreased incidence of second flushing from 81 to 54% and significantly reduced height growth in the second year following low-temperature treatment, in comparison to untreated controls. For seedlings under 15-h photoperiods, flurprimidol had only limited early effects, but after a period of bud dormancy, shoot growth was reduced to one-third that of the untreated controls. Under both photoperiods flurprimidol significantly depressed abscisic acid levels and gibberellin-like bioactivity. These results suggest that growth reduction in this conifer by flurprimidol may well involve inhibition of gibberellin biosynthesis.

Late-summer bud flush is an unwanted occurrence in the production of Douglas-fir [*Pseudotsuga menziesii* (Mirb.) Franco] seedlings. In nurseries, bud set is often induced by mild water stress (Duryea 1984). However, maintaining bud set and limiting height growth can be difficult. If applied too late in the growing season, then fertilization or even normal irrigation can stimulate second flushing (Lavender 1984). Although undercutting or top pruning has been used to combat second flushing (Duryea 1984), an even wider array of techniques for stopping late flushing and encouraging development of the overwintering bud is needed. One possibility is the use of plant growth retardants.

Several chemical growth retardants are known to inhibit GA biosynthesis (Graebe 1987). Furthermore, published reports have shown that some chemical growth retardants can be active in very young conifers (Cheung 1975, Dunberg and Eliasson 1972, Pharis et al. 1967, Rietveld 1988, Weston et al. 1980, Wheeler 1987). However, these reports have largely concentrated on early "free" shoot growth of Pineaceae seedlings or the indeterminate growth of Cupressaceae and Taxodiaceae seedlings rather than on but set, second bud flush, or growth following dormancy. Hare (1984), however, has reported that flurprimidol could inhibit second flushing of loblolly (*Pinus taeda* L.) and slash (*P. elliotti* Engelm.) pines in a seed orchard. The objective of the present study was to determine whether flurprimidol can also inhibit second flushing of Douglas-fir seedlings, and also whether it impairs later growth. In addition, the hypothesis that this compound affects growth by altering levels of endogenous plant hormones was assessed. In particular, GA-like activity and ABA were evaluated.

Materials and Methods

Seedling Treatments

Douglas-fir seeds from a southern Oregon source were soaked overnight in mid-December and then stratified at 4°C for 6 weeks. The seeds were sown onto 2:1 peat/vermiculite in plastic containers (60 ml capacity). Germination and early growth of the seedlings occurred in a greenhouse under fluorescent lamps to provide a 16-h photoperiod. Fertilization, which began 2 weeks after emergence, was by nutrient solution made from a commercial mix (N:P:K 20:20:20) and applied at a concentration of 0.5 g L^{-1}.

In early June, 4 months after germination, 200 seedlings were selected for uniformity and randomly assigned to either a 15- or 17-h photoperiod under mixed fluorescent and incandescent

Abbreviations: GA, gibberellin; ABA abscisic acid; FPP, farnesyl pyrophosate; HPLC, high performance liquid chromatography; GC-EC, gas chromatography-electron capture; GC-MS, gas chromatography-mass spectrometry.

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lamps. Photosynthetically active radiation (PAR) was at least 120 $\mu E\ m^{-2}\ s^{-1}$. Because the critical photoperiod for bud flush and continued seedling shoot growth of Oregon-latitude Douglas-fir is 14 h (Downs 1962, Lavender 1981), these photoperiods were chosen to be either weakly or strongly promotive of growth. Photoperiods were applied in separate growth rooms, and temperature was set for a 22°/18°C day/night cycle.

After initiation of photoperiod treatments, 50 seedlings from each photoperiod were randomly selected for treatment with flurprimidol (EL-500, Lilly Research Laboratories, Indianapolis, IN, USA). Flurprimidol was prepared by dissolving 0.5 g of the technical material in 95% ethanol and then carefully diluting with distilled water to 0.5 L to avoid precipitation. Next, 5-ml aliquots containing 5 mg of flurprimidol in 1% ethanol were then applied via the root system. Two applications, 3 days apart, were made, giving 10 mg per seedling. As a control, a 1% solution of ethanol was applied in the same manner.

Seedling height, basal diameter, and bud flush were measured periodically throughout the summer. In late August, two foliage samples from each treatment combination were harvested for analysis of ABA and GA-like substances. Each sample contained the combined foliage from 12 seedlings. Foliage fresh weight was recorded, and the tissue then was frozen in a −60°C freezer.

On October 15, the photoperiod was changed to 8 h for both treatment sets. In early November, the temperature was lowered to 4°C for 12 weeks. Following chilling, the seedlings were subjected to a 16-h photoperiod at 22°/18°C (day/night). The seedlings promptly flushed, and after 2 months the new growth was harvested. The length of the new shoot was recorded, the number of needles tallied, and the dry weight (dw) measured. Stem unit count was determined by assuming each needle represented a stem unit. *Stem unit*, as defined by Doak (1935), is an internode plus its appendage. Average length and dw of a stem unit were computed by dividing the length and dw of the new shoot by the stem unit count.

Analysis for ABA and GA-Like Substances

Analytic procedures were adapted from Reeve and Crozier (1978) and Jones et al. (1980). All solvents were HPLC grade or distilled prior to use. The foliage samples were ground under liquid N_2 with a mortar and pestle, then placed in 100 ml of absolute methanol and left standing overnight at −20°C. Next, 40 mg of diethyldithiocarbamic acid (Sigma) were added as an antioxidant.

The methanolic extract was filtered, and the foliage residue was ground again and filtered twice with 100 ml of absolute methanol. Then 30 ml of a 0.5-M phosphate buffer at pH 9.2 were added to this methanolic extract, and the solution was reduced to the aqueous phase with a rotary evaporator held at 33°C. The pH of the aqueous extract was near 8.0. The sample was then centrifuged at 2000 rpm to precipitate the chlorophyll and hydrophobic materials, and the pellet was washed twice with a 0.5-M phosphate buffer at pH 8.0.

The combined supernatants were partitioned three times against equal volumes of n-hexane at near pH 8. The hexane was discarded. The aqueous phase was loaded onto a 3 × 13 cm column containing 30 ml polyvinylpolypyrrolidone (PVPP, Polyclar) and eluted under pressure from compressed air.The PVPP was washed with 50 ml of a 0.5-M phosphate buffer at pH 8.0, and the solutions were combined. The extract was next adjusted to pH 3.0 with HCl and loaded in stages onto a 1.5 × 10 cm column containing 5 ml of charcoal/celite (1:2). The charcoal column was washed with 25 ml of 20% aqueous acetone (discarded), and the plant hormones were eluted with 100 ml of 80% acetone. The acetone was removed in vacuo, and the acidic aqueous residue partitioned five times against ethyl acetate. The acidic ethyl acetate fractions were combined and frozen at −20°C to remove ice. We added 1 g Na_2SO_4 for final drying, and the sample was again filtered. The ethyl acetate was removed in vacuo, and the dry residue was stored at −60°C until HPLC.

A reverse-phase octadecyl-silica (C18) column (Beckman, 250 × 4.6 mm inside diameter, i.d.) was operated at 1 ml min^{-1} with a linear gradient of 10–100% methanol in 1% acetic acid over 23 min. The samples were injected in 50 µl of absolute methanol, and 1-ml fractions were collected from 0 to 30 min. Each fraction was dried and stored at −60°C until analysis.

Preliminary testing revealed that ABA eluted at R_t 18 or 19 min. These sample fractions were methylated with ethereal-diazomethane and analyzed by GC-EC (Varian 3700). The chromatograph was equipped with a ^{63}Ni electron-capture detector and a glass column (180 × 0.2 cm i.d.) packed with 2% OV-101. Temperatures were 210°C for the injector, 145°C for the column, and 260°C for the detector. The flow rate of the carrier gas, N_2, was 35 ml min^{-1}. The sample was diluted with 50 µl of methanol, and triplicate analyses of 8 µl were made. Quantification of putative ABA was by peak height interpolation from a curve prepared by using a methylated authentic ABA standard (Sigma). No internal ABA standard was used.

Analysis of GA-like substances was made by the dwarf rice (*Oryza sativa* L. cv Tan-ginbozu) microdrop bioassay (Murakami 1968) with the following modifications: Fractions 5 through 30 (except 18 and 19) were diluted with 20 µl of 50% ethanol, and 1-µl aliquots were applied to 2-day-old rice seedlings. Then, 8–10 rice seedlings were assayed for each fraction. After 3 days at 31°C, near 100% humidity, and 230 $\mu E\ m^{-2}\ s^{-1}$ PAR, the total elongation (length to tip of leaf) was measured and the amount of GA-like substances was interpolated from a standard curve prepared by using GA_3 (Sigma).

The presence of ABA in HPLC fractions R_t 18 or 19 min was confirmed by GC-MS. The GC used a 30 cm × 0.32 mm fused silica capillary column coated with DB-5 (J & W Scientific) and a 50°–255°C temperature gradient. Injections were splitless. The MS (Finnegan, model 4023) was operated at 70 eV. Recovery of ABA was approximated by adding a known quantity of ABA to a separate Douglas-fir foliage extract with predetermined ABA content and analyzing for total ABA. Gibberellin recovery was estimated by subjecting a solution of known GA_3 content to the extraction procedure and bioassaying the recovered materials. Recovery approximations were made in triplicate and were reasonably consistent for both plant hormones. Therefore, recovery was assumed to be similar among samples for both plant hormones.

Retention times for ABA, GA_3, GA_4, GA_9, and GA_{20} on the HPLC were determined by analysis of authentic standards. Authentic standards and confirmation by GC-MS were provided by Dr. Richard Pharis (University of Calgary, Alberta, Canada).

Statistical Analysis of Shoot Growth and Hormones

Differences for photoperiod and flurprimidol treatments were subjected to an analysis of variance (ANOVA) (SAS Institute Inc. 1985). The experiment was considered a completely randomized design. For the plant hormones, the standard deviation was proportional to the mean. Therefore, $\log_{10}$ transformations

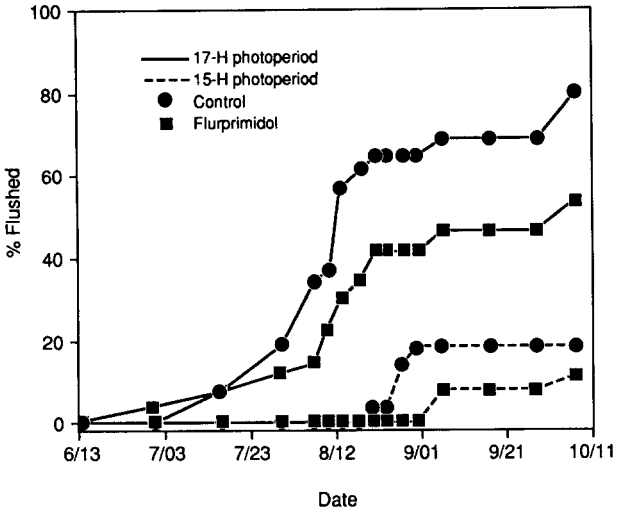

Fig. 1. The progression of second flushing for treated and control Douglas-fir seedlings under 15- or 17-h photoperiods.

were performed prior to ANOVA (Zar 1974). The association between bud flush and flurprimidol treatment was assessed by Chi-square 2 × 2 contingency tables (Zar 1974), with separate analyses for each photoperiod.

Results

Predormancy Growth

Transfer to the growth rooms from the greenhouse, where initial growth was accomplished, was sufficient to induce initial bud set for both photoperiods. As expected, many seedlings flushed subsequently, particularly under the longer photoperiod. However, this second flushing was not uniform and continued over the summer (Fig. 1). Prior to transfer to the 4°C, 8-h photoperiod, 81% of the untreated controls under the 17-h photoperiod had flushed a second time, but only 19% of those under the 15-h photoperiod had done so.

Although flurprimidol reduced the final percentage of flushing compared to the untreated control under both photoperiods, it did not eliminate second flushing. Over 50% of the flurprimidol-treated seedlings flushed under the 17-h photoperiod (Fig. 1). Chi-square analysis showed that the diminished flushing was significantly associated with flurprimidol treatment under the 17-h photoperiod (prob $\leq$ 0.04), but not under the 15-h photoperiod (prob $\leq$ 0.44).

Height growth closely reflected bud flush, with flurprimidol treatment having a greater effect under the 17- than the 15-h photoperiod (Table 1), thus showing a significant flurprimidol × photoperiod interaction. Diameter growth was significantly reduced by flurprimidol in both photoperiods with no significant interaction.

Postdormancy Growth

Flurprimidol had a large and significant effect on shoot growth following chilling, some 8 months after its application. For seedlings grown under 15-h photoperiods (these seedlings had a more normally timed bud set), the earlier flurprimidol treatment appreciably depressed post-chilling growth (Table 1), length of new shoots being reduced to one-third the length of the untreated control. Seedlings previously held under 17-h photoperiods had poor postdormancy growth and did not show large flurprimidol effects. Consequently, there was a significant flurprimidol × photoperiod interaction for new shoot length, dw, and stem unit count.

The average stem unit length and dw, and the weight-to-length ratio, did not show statistically significant treatment interactions ($p > 0.05$); however, they did show significant flurprimidol effects (Table 1). Furthermore, although most components of growth were reduced, the weight-to-length ratio was increased. Thus, flurprimidol appears to have caused a reallocation of growth from elongation toward another component, for example, foliage or stem diameter.

Analysis for Plant Hormones

The GA bioassay of tissue extracts from untreated control seedlings showed that most GA-like activity was at R_t 24 min where GA_9 elutes (Fig. 2). However, R_t 8 and 22 min were also significantly above the bioassay control. Tissue extracts from flurprimidol-treated samples were compared with the untreated controls for two indicators of GA-like activity. R_t 24 min alone, and total activity for all fractions (Table 1). For both indicators, there was an apparent depression of GA-like activity in the flurprimidol-treated seedlings, and this was statistically significant for the R_t 24 min fraction. Length of photoperiod had no apparent effect on GA-like activity. Recovery, approximated as noted earlier, was 66% for GA-like substances.

Abscisic acid was found by GC-MS in either R_t 18 or 19 min, so both fractions were analyzed by GC-EC and summed for quantification of the putative ABA. As with GA-like activity, levels of putative ABA showed a significant depression after flurprimidol treatment, although the decline in ABA was not as great as for GA-like bioassay (Table 1).

Table 1. Mean responses of Douglas-fir seedlings to combined photoperiod and growth-retardant treatments. Photoperiods were 17 and 15 h, and the growth-retardant treatments were control and flurprimidol.[a]

Seedling response	Control (17 h)	Flurprimidol (17 h)	Control (15 h)	Flurprimidol (15 h)
Predormancy growth				
Second bud flush (%)	81	54	19	12
Height growth (cm)	3.00[a]	2.57[a]	2.26[b,c]	2.07[c]
Diameter growth (mm)	1.44[a]	1.31[b]	1.42[a]	1.31[b]
Postdormancy growth				
Stem unit count	23.6[c]	25.7[c]	49.3[a]	39.4[b]
Height growth (cm)	1.27[b]	0.72[c]	3.10[a]	1.01[b,c]
Dry weight (mg)	26.5[b,c]	20.4[c]	86.6[a]	39.3[b]
Average stem unit length (mm)	0.57[a]	0.30[b]	0.65[a]	0.26[b]
Average stem unit dry weight (mg)	1.13[b]	0.80[c]	1.65[a]	0.98[b,c]
Weight-to-length ratio (mg mm^{-1})	2.05[b]	2.56[b]	2.87[b]	4.08[a]
Analysis for growth regulators				
GA-like substances, R_t (24 min) (pg g^{-1})	388[a]	23[b]	597[a]	17[b]
GA-like substances, total activity (expressed as pg GA_3 g^{-1})	502[a]	204[a]	903[a]	189[a]
ABA (ng g^{-1} fresh weight)	24.8[a]	11.9[b]	48.1[a]	13.3[b]

[a] Mean values in the same row followed by different letters are significantly different at the 5% level.

The photoperiod × flurprimidol interaction was not significant for ABA.

Confirmation that putative ABA was present at R_t 18 or 19 min was provided by GC-MS (Table 2). Major ions and intensities agreed closely with published spectra of methyl-ABA (Dorffling and Tietz 1983, Saunders 1978). Recovery of ABA, approximated as noted above, was 88%.

Discussion

Douglas-fir has previously been found responsive to the growth retardant paclobutrazol (Wheeler 1987) and possibly to AMO-1618 (Pharis et al. 1967). The present study has shown that shoot growth in Douglas-fir is also responsive to retardation by flurprimidol. Hare (1984) found that flurprimidol was highly effective in reducing shoot elongation of loblolly and slash pine propagules in a seed orchard. Moreover, of several compounds tested, flurprimidol was the most effective nonphytotoxic retardant. However, in Hare's study the dosage of flurprimidol was considerably above that of the other retardants.

Although flurprimidol did reduce second flushing in Douglas-fir, the effect on growth prior to dormancy was not striking, merely reflecting the effect on second flushing. In contrast, shoot growth after chilling was clearly inhibited by flurprimidol, particularly for seedlings grown under 15-h photoperiods. Under the 17-h photoperiod treatment, flurprimidol's effectiveness was less, although still significant. However, for seedlings not given

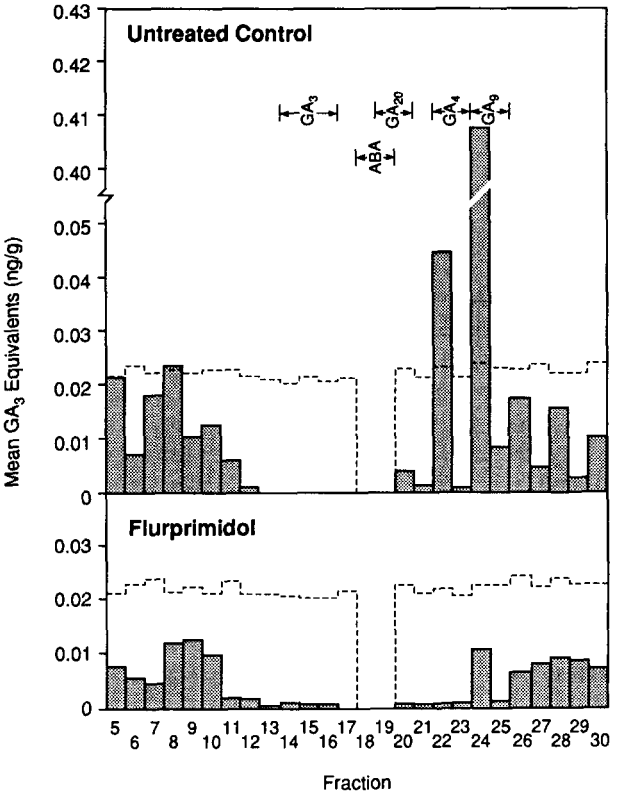

Fig. 2. Levels of gibberellin-like substances in HPLC fractions from foliage extracts from Douglas-fir seedlings. Seedlings were either treated or untreated with flurprimidol, and each treatment contained seedlings grown under either 15- or 17-h photoperiods. The *dotted line* is the 0.05% least significant difference (LSD) above the bioassay control. Retention times of authentic GA_3, GA_4, GA_9, GA_{20} and ABA are shown above GA-like activity.

Table 2. Data for confirmation of abscisic acid by GC-MS.

Source	Characteristic ions (mass-to-charge ratio, m/z)	Relative intensity (%)
Authentic standard	190	100.0
(R_t 12.59 min)	162	28.1
	134	28.8
	125	32.5
Putative	190	100.0
(R_t 13.00 min)	162	29.3
	134	27.4
	125	30.4

flurprimidol, photoperiod treatment during dormancy development also affected several postchilling growth components, with 17-h photoperiods depressing postchilling growth relative to the 15-h photoperiod. For seedlings given 17-h photoperiods, flurprimidol added only moderately to postchilling growth reduction.

In the production of seedlings for reforestation, spring growth must be maximized, and persistent growth-retardant effects are unwanted. Sterrett and Tworkoski (1987) reported long-term flurprimidol effects that may be attributed to persistence in the soil for up to 1–1.5 years. However, it should not yet be concluded that flurprimidol is inappropriate for conifer seedling production. The effects of low dosages of flurprimidol applied with other dormancy-inducing treatments remain to be examined.

In Douglas-fir, shoots are largely preformed in the overwintering bud, with subsequent growth the result of both cell division and cell elongation (Owens et al. 1985). In as much as GAs can affect elongation by stimulating both cell division and cell elongation in other plants (summarized in Jones 1973) and endogenous GA activity has been positively correlated with shoot elongation in conifers (Ross et al. 1983), the results of the present study suggest that flurprimidol may inhibit GA biosynthesis. This inference is not inconsistent with the results of other studies in which growth inhibitors were used (Graebe 1987). However, flurprimidol may have also reduced stem elongation by means other than inhibition of GA biosynthesis.

Working with Arizona cypress (*Cupressus arizonica* Greene), Kuo and Pharis (1975) first reported reduced levels of endogenous GA-like substances in conifers following treatment with a growth retardant. The results of the present study extend this relationship to flurprimidol and Douglas-fir. Moreover, these results support the hypothesis that bud flush for Douglas-fir is regulated by GA activity (see also Lavender et al. 1973). However, putative ABA levels were also reduced by flurprimidol. A similar pattern was reported by Norman et al. (1983) for growth retardants applied to the fungus *Cercospora rosicola* Passerini. Gibberellins and ABA have opposing effects in germinating cereals (Jacobsen and Chandler 1987), and GA_3 can counteract the inhibitory effect of ABA on release from bud dormancy in *Betula pubescens* (Wareing and Phillips 1983). Gibberellins and ABA are also antagonistic in the dwarf rice assay. However, a regulatory role for ABA in normal shoot elongation of higher plants has little supportive evidence (Powell 1982). Hence, reduced GA levels are probably more important for retardation of growth.

Two major zones of GA-like activity were found in chromatograms of extracts from seedlings not treated with flurprimidol (Fig. 2). Authentic GA_4 and GA_9 had retention times that closely correspond to these zones of GA-like activity. Doumas et al. (1992) reported most GA-like bioassay activity corresponded to retention times for GA_4 and GA_9, although in total six native GAs were found in Douglas-fir: GA_1, GA_3, GA_4, GA_7, GA_9, and GA_9-glucosyl ester.

It has been accepted that most growth retardants act, in part, by restricting GA biosynthesis (Graebe 1987). However, the inhibition of ABA biosynthesis indicates that flurprimidol has broader effects. Norman et al. (1986) showed that ancymidol inhibits terpenoid production prior to the biosynthesis of FPP in *Cercospora*. Since FPP is a precursor to both sesquiterpenes and diterpenes, this inhibition would account for the depression of ABA, GAs, and sterols if flurprimidol also had its major effect at this step. Coolbaugh and Hamilton (1976) reported a similar phenomenon in immature endosperm of wild cucumber [*Marah macrocarpus* (Greene) Greene], where ancymidol reduced the incorporation of mevalonic acid into ent-kaurene, the precursor to gibberellins. However, ancymidol can also inhibit several oxidation steps in the metabolism of ent-kaurene to GA_{12}, and this inhibition is much stronger (Coolbaugh et al. 1978).

The somewhat greater lowering of levels of GA-like substances relative to the modest diminishment of putative ABA in the present study suggests that the mechanism of action of flurprimidol in Douglas-fir may be similar to that of ancymidol in cucumber endosperm. Consequently, inhibition of GA biosynthesis by flurprimidol may be the primary means of retarding growth.

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times by GC-MS. His thoughtful and thorough review of the manuscript was particularly helpful. They also thank Lilly Research Laboratories for supplying samples of flurprimidol. Mention of commercial products does not constitute endorsement by Oregon State University to the exclusion of other products that may be suitable. This is Paper 2535 of the Forest Research Laboratory, Oregon State University.

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