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Effects of Prohexadione on Cambial and Longitudinal Growth and the Levels of Endogenous Gibberellins A_1, A_3, A_4, and A_9 and Indole-3-acetic Acid in *Pinus sylvestris* Shoots

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Abstract. Prohexadione, a gibberellin (GA) biosynthesis inhibitor, was applied in ethanol around the circumference at the midpoint of the previous year terminal shoot of dormant *Pinus sylvestris* seedlings. After cultivating the seedlings under environmental conditions favorable for growth for 10 weeks, longitudinal and cambial growth were measured, and the endogenous levels of GA_1, GA_3, GA_4, GA_9, and indole-3-acetic acid (IAA) were determined by combined gas chromatography-mass spectrometry, using deuterated GAs and [$^{13}C_6$]IAA as internal standards. Prohexadione application inhibited elongation and xylem and phloem production in the current year terminal shoot and xylem production in the previous year terminal shoots. Concomitantly, in both ages of shoots the cambial region contents of GA_1, GA_3, and GA_4 were decreased, whereas the level of GA_9 was increased. However, the IAA content was not altered in the terminal bud on the current year terminal shoot or in the cambial region of the current year or previous year terminal shoots. The results provide additional evidence that: (1) GAs are involved in the regulation of cambial growth, as well as longitudinal growth, in *Pinus sylvestris* shoots; (2) they act directly, rather than indirectly, by altering the IAA level; and (3) the $GA_9 \rightarrow GA_4 \rightarrow GA_1$ pathway is a major route of GA biosynthesis in conifer species.

It is well established that gibberellins (GAs) play a major role in the control of shoot elongation in non-woody angiospermous species (e.g., Kobayashi et al. 1994, Zeevaart et al. 1993), and accumulating evidence indicates that they also are important for the regulation of cambial growth, as well as longitudinal growth, in shoots of conifers and woody angiosperms (Junttila 1991, Little and Pharis 1995). In the case of conifers, it has been demonstrated that: (1) GA_1, GA_3, GA_4, GA_7, GA_8, GA_9, GA_{12}, GA_{15}, GA_{20}, GA_{29}, GA_{34}, GA_{51}, and GA_9-glucosyl ester occur naturally in shoots, or more specifically in the stem, cambial region, or needles, which suggests the existence of the early nonhydroxylation pathway of GA biosynthesis (Doumas et al. 1992, Lorenzi et al. 1976, 1977, Moritz 1992, 1995, Moritz et al. 1989b, Odén et al. 1987, Pharis et al. 1992, Wang et al. 1995a); (2) the application of GA_1, GA_3, GA_4, or $GA_{4/7}$ stimulates shoot elongation and secondary xylem and phloem production, provided indole-3-acetic acid (IAA) is also available (Junttila 1991, Little and Pharis 1995, Moritz 1995, Pharis et al. 1991, Wang et al. 1995b); and (3) the endogenous levels of individual GAs vary with genotype, time of year, rate of shoot elongation, photoperiod, environmental stress, and type of bud being formed (Lorenzi et al. 1975, Moritz 1995, Moritz et al. 1990 a, 1990b, Odén et al. 1994, Pharis et al. 1992). However, it is not known for conifer shoots which GAs are active per se in the regulation of cambial or longitudinal growth or where they are synthesized, transported, and catabolized. Furthermore, it is unclear whether GAs act directly or indirectly by affecting the levels of other phytohormones involved in the control of shoot growth, particularly IAA (Wang et al. 1992, 1995b), whose presence is known to be essential (Little and Pharis 1995).

The application of prohexadione, an acylcyclohexanedione type of plant growth retardant, has

Abbreviations: GA, gibberellin; IAA, indole-3-acetic acid; HPLC, high performance liquid chromatography; GC, gas chromatography; SIM, selected ion monitoring; MS, mass spectrometry.

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been shown to inhibit shoot elongation in many species of woody and nonwoody angiosperms (Junttila 1993b, Junttila et al. 1991, Nakayama et al. 1991, Ross et al. 1993, Zeevaart et al. 1993). It acts by inhibiting late steps in GA biosynthesis, notably 3β-hydroxylation and thus the conversion of GA_{20} to GA_1, which appears to be the primary GA active per se for shoot elongation in angiosperms (Kobayashi et al. 1994, Nakayama et al. 1990, 1992, Zeevaart et al. 1993). To a lesser extent, however, prohexadione also inhibits 2β-hydroxylation, a step known to inactivate GAs (Griggs et al. 1991, Nakayama et al. 1990, 1992). In addition, acylcyclohexanedione treatment has been observed to increase markedly the levels of abscisic acid and cytokinins, but it has little, if any, effect on the IAA content (Adams et al. 1992, Grossman et al. 1994). There are no reports concerning the influence of acylcyclohexanedione application on cambial growth and endogenous phytohormone levels in the shoots of any woody species or on shoot elongation in conifers.

In the work reported here, we used prohexadione to extend our investigation of the roles of GAs in the regulation of cambial and longitudinal growth in shoots of the conifer *Pinus sylvestris* (Wang et al. 1992, 1995a, 1995b). The effects of prohexadione on xylem and phloem production, shoot elongation, IAA content, and the levels of GA_1, GA_3, and GA_4, which may be active per se in conifer shoot growth, and of GA_9, which is a major GA precursor in conifers (Moritz 1995, Moritz and Odén 1990, Moritz et al. 1989a, Odén et al. 1995, Wang et al. 1995a), are presented.

Materials and Methods

Plant Material and Application of Prohexadione

The *P. sylvestris* (L.) seedlings were 3 years old and dormant at the start of the experiment and had been cultivated in 2-liter pots at the nursery of the Forest Research Institute, Sävar, Sweden. In late winter, the seedlings were transferred from the nursery to an unlighted greenhouse having a minimum temperature of 5°C and allowed to thaw. Three groups of 10 seedlings were selected so that the average length of the previous year (1-year-old) terminal shoot on the main stem was the same for each group. The seedlings in one group were treated with about 100 μL/seedling of 0, 0.1, or 1 mg of prohexadione/liter of ethanol. The prohexadione was prepared by dissolving prohexadione calcium (BX-112; calcium 3,5-dioxo-4-propionylcyclohexanecarboxylate, Kumiai Chemical Industry Co., Ltd., Tokyo, Japan) in 0.1 M hydrochloric acid, extracting into ethyl acetate, and evaporating to dryness. The prohexadione solutions were applied with a small paintbrush to a 2-cm band around the circumference of the previous year terminal at its midpoint, after removing the needles and periderm with a scalpel. After covering the application site with aluminum foil, the seedlings were cultured in a controlled environment chamber having a photoperiod of 18 h, a photon flux density of about 300 μmol m^{-2} s^{-1} from Osram HQI-TS 400 W/DH metal halogen lamps, a day/night temperature of 23/13°C, and a relative humidity of about 75%. The seedlings were watered daily and fertilized weekly with 0.5% Superbra S (Hydro Supra AB, Landskona, Sweden), which contained 6.5% nitrogen, 1.0% phosphorus, and 4.7% potassium. At weekly intervals, the length of the current year terminal shoot on the main stem was measured, and the prohexadione solutions were reapplied. After 10 weeks of culture, the seedlings were harvested to measure cambial growth and the endogenous contents of IAA and GAs.

Measurement of Cambial Growth

Transverse handcut sections were obtained at the midpoint and 3 cm from the apical and basal ends of the current year and previous year terminal shoots. They were stained in a saturated aqueous solution of phloroglucinol in 20% hydrochloric acid and mounted in glycerol. The radial widths of xylem and phloem were measured at eight equidistant positions around the circumference of each section. In the previous year terminal shoot, the middle measurement position was located at the center of the prohexadione application point, the xylem measurement started from the last formed latewood tracheid in the previous year annual ring, and the phloem measurement included the width of phloem produced in the current and previous years.

Measurement of IAA and GAs

IAA and GAs were measured in the cambial region of the current year and previous year terminal shoots, and IAA was also quantified in the terminal bud developing at the apex of the current year terminal shoot. For current year terminal shoots, the cambial region sample was obtained from the stem segment located between the apical and basal positions of cambial growth measurement, minus the transverse section used to measure cambial growth at the middle position. For previous year terminal shoots, cambial region samples were collected at two locations: (1) between 1 cm below the prohexadione application site and the basal position of cambial growth measurement; and (2) between 1 cm above the prohexadione site and the apical position of cambial growth measurement. IAA was measured separately in the two previous year cambial region samples, whereas the GAs were quantified after pooling them. For both current year and previous year terminal shoots, the cambial region was collected by peeling the bark, scraping the exposed surface on the xylem side with a scalpel, stripping the surface tissues on the bark side using fine nosed forceps, and combining the scrapings and strippings for each segment. Thus, the cambial region sample contained differentiating xylem, the cambium, current year phloem and, in segments from the previous year terminal shoot, previous year phloem. All terminal bud and cambial region samples were frozen in liquid nitrogen and stored at −80°C until used.

IAA was measured as described by Sundberg (1990). In brief, each sample was ground in liquid nitrogen, extracted for 2 h at 4°C in 0.05 M sodium phosphate buffer, pH 7.0, containing 0.02% diethyldithiocarbamic acid (Sigma) as antioxidant and 50 ng of [$^{13}C_6$]IAA (Cambridge Isotopes Laboratories) as internal standard. The extract was purified by neutral and acidic diethyl ether partitioning, and the acidic ether portion was methylated and subjected to reversed phase high performance liquid chromatography (HPLC). The HPLC mobile phase consisted of 50% meth-

anol in 1% acetic acid and was delivered at a flow rate of 1 ml min^{-1} by Waters model 501 pumps and model 680 gradient controller (Waters Associates, Milford, MA, USA). The sample was introduced by a Waters 712 WISP onto a 10 cm × 8 mm (inner diameter) 4-mm Nova-Pak C_{18} cartridge fitted in an RCM 8 × 10 module. The IAA-methyl ester fraction was collected, silylated, and measured by gas chromatography-selected ion monitoring-mass spectrometry (GC-SIM-MS). Chromatography was done on a 25 × 0.25 mm (inner diameter) SE-30 column with a film thickness of 0.25 μm (Quadrex Company, New Haven, CT, USA) using He as a carrier gas at a flow rate of 1 ml min^{-1}. The GC-MS was performed with a Hewlett-Packard 5890 GC linked by a direct inlet to a Hewlett-Packard 5770 mass selective detector and 9133 data system (Hewlett-Packard, Palo Alto, CA, USA). The ions 202, 208, 261, and 267 were detected using a dwell time of 100 ms, and the endogenous content of IAA was calculated by the isotope dilution equation as modified by Cohen et al. (1986).

To measure GAs, samples were extracted in 80% aqueous MeOH containing 0.02% (w/v) diethyldithiocarbamic acid as antioxidant and 10 ng of 17,17-$[^2H_2]GA_9$, 100 pg of 17,17-$[^2H_2]GA_4$, 100 pg of 71,17-$[^2H_2]GA_3$, and 100 pg of 17,17-$[^2H_2]GA_1$ as internal standards. After extraction for 4 h at 4°C in darkness, the MeOH was filtered off, and the residue was washed with fresh MeOH and reextracted under the same conditions. Both filtrates were combined, and MeOH was evaporated under reduced pressure at 35°C. The aqueous residue was adjusted to about 5 ml with 0.5 M sodium phosphate buffer, pH 8.0, and applied to a polyvinylpolypyrrolidone column (20 × 10 cm, inner diameter). The column was eluted with 0.1 M sodium phosphate buffer, pH 8.0, and the 0–75-ml fraction was collected, acidified to pH 2.7 with 6 M HCl, and extracted five times with 75 ml of ethyl acetate. The combined ethyl acetate phase was decreased to a small volume under reduced pressure at 35°C. The samples were then applied to a QAE-Sephadex A-25 column (Pharmacia, Uppsala, Sweden) that had been preequilibrated with 50 ml of water, pH 8.0. The free GAs were eluted with 50 ml of 0.2 M formic acid, which was run through a 100-mg Bond Elute C_{18} column (Sorbent AB, Vastra Frolunda, Sweden) that had been washed with MeOH and 0.2 M formic acid. The column was eluted with 5 ml of MeOH to collect the free GAs. The MeOH was evaporated to dryness in a SpeedVac concentrator (Savants Instruments, Farmingdale, NY, USA), and the samples were subjected to HPLC. The LC consisted of two M 501 pumps (Waters Associates) connected to the column through a Waters U6K injector with a 250-μl loop. The pumps were controlled by a Waters M 680 gradient controller. The samples were purified by a reverse phase column packed with 5 mm of Nucleosil C_{18} (200 × 4.6 mm, inner diameter). The mobile phase consisted of a linear gradient from water and acetic acid (99:1%, v/v) to MeOH and acetic acid (99:1%, v/v). The samples were run at a flow rate of 1 ml min^{-1}, and 60 1-ml fractions were collected. Fractions known to contain the GAs of interest were methylated with ethereal diazomethane and, after evaporation under a stream of N_2, trimethylsilylated in 20 μl of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide and 10 μl of pyridine at 70°C for 30 min. The derivatization mixture was then reduced to dryness and dissolved in *n*-heptane. Samples were injected in the splitless mode into a Hewlett-Packard 5890 GC fitted with a fused silica glass capillary column (25 m × 0.25 mm, inner diameter) with a chemically bonded 0.25-μm SE-30 stationary phase (Quadrex). The injector temperature was 270°C. The column temperature was held at 60°C for 2 min, then increased by 20°C min^{-1} to 200°C, and by 4°C min^{-1} to 260°C. The column effluent was introduced into the ion source of a JMS-SX102 mass spectrometer (JEOL, Tokyo, Japan). The interface and the ion source temperatures were 260°C. Ions were generated with 70 eV at an ionization current of 300 μA. Quantification was made by selected reaction monitoring (Moritz and Olsen 1995). The acceleration voltage was 10 kV, and the precursor ions were selected by magnetic switching. The daughter ions formed in the first field-free region were detected by switching the magnetic field and the electrostatic field simultaneously. The dwell time was 100 ms, and the reactions *m/z* 418 to *m/z* 284 and *m/z* 420 to *m/z* 286 for GA_4, *m/z* 298 to *m/z* 270 and *m/z* 300 to *m/z* 272 for GA_9, *m/z* 506 to *m/z* 448 and *m/z* 508 to *m/z* 450 for GA_1, *m/z* 504 to *m/z* 446 and *m/z* 506 to *m/z* 448 for GA_3 were recorded. Helium was used as the collision gas. All data were processed by a JEOL MS-MP7010D data system.

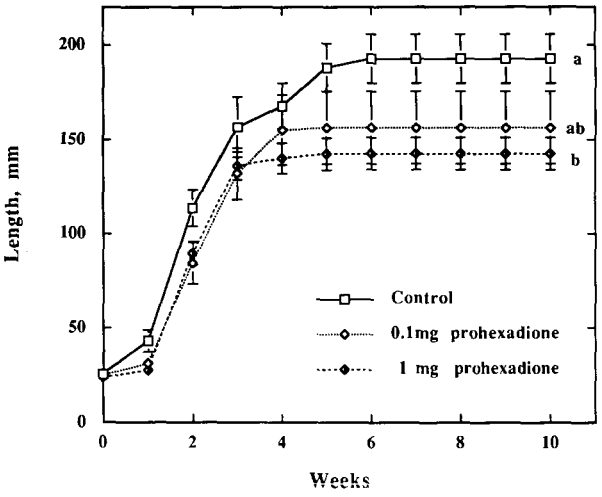

Fig. 1. Progress of longitudinal growth in the current year terminal of 3-year-old seedlings treated laterally at the middle of the previous year terminal shoot with 0, 0.1, or 1 mg of prohexadione/liter of ethanol for 10 weeks. Mean ± S.E., $n = 10$.

Statistics

The significance of the difference between means was determined using a *t* test or analysis of variance and Duncan's multiple range test, as appropriate. In the figures, within each measured variable and measurement position, means accompanied by the same letter are not significantly different ($p \leq 0.05$).

Results

Prohexadione application inhibited both longitudinal and cambial growth, the 1 mg/L concentration having the greatest effect. The reduction in shoot extension was evident early in the elongation period (Fig. 1). Xylem production was inhibited along the entire length of the current year and previous year terminal shoots, but most noticeably at and below the application point, whereas phloem production was decreased only at the middle and base of the current year terminal shoot (Fig. 2). Treatment with 1 mg of prohexadione/liter of ethanol, relative to the control, inhibited the elongation of the current year

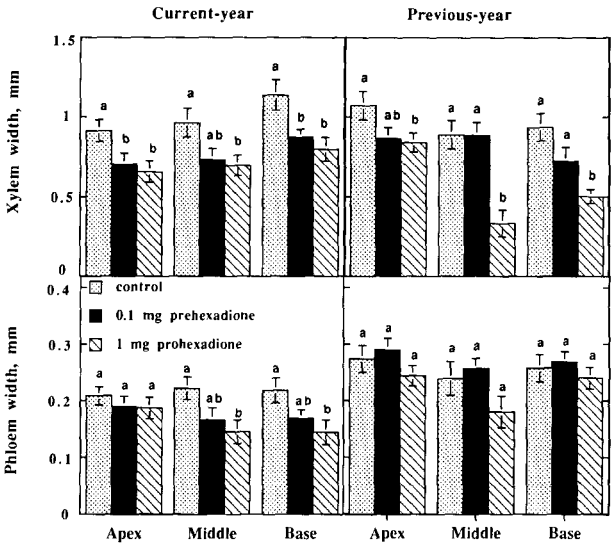

Fig. 2. Radial width of xylem and phloem in the current year and previous year terminal shoots of 3-year-old seedlings treated laterally at the middle of the previous year terminal shoot with 0, 0.1, or 1 mg of prohexadione/liter of ethanol for 10 weeks. Mean ± S.E., n = 10.

terminal shoot and its distal terminal bud by 27 and 32%, respectively, and the production of xylem and phloem by 28–62% and 7–35%, respectively.

The 1 mg/L concentration of prohexadione did not affect the level of IAA either in the terminal bud forming on the current year terminal or in the cambial region of the current year or previous year terminal shoots (Fig. 3). In contrast, it increased the level of GA_9 and decreased that of GA_1, GA_3, and GA_4, the extent of change being 61–86% in the current year terminal and 20–50% in the previous year terminal shoot (Fig. 4).

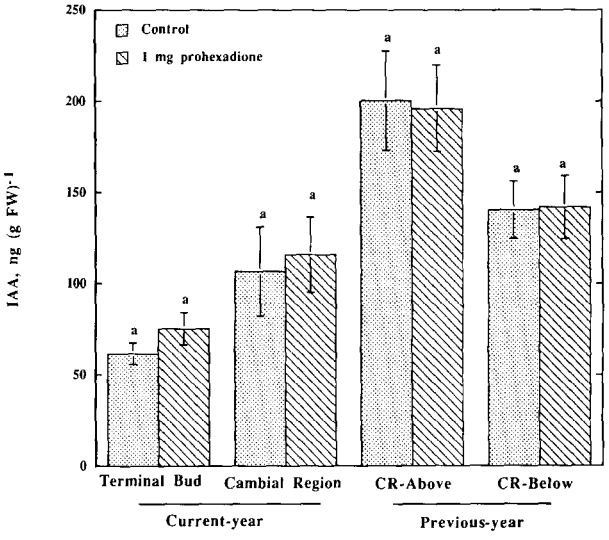

Fig. 3. IAA levels in the terminal bud and cambial region of the current year terminal shoot, and in the cambial region (*CR*) of the previous-year terminal shoot above and below the prohexadione application point, in 3-year-old seedlings treated laterally at the middle of the previous year terminal shoot with 0 or 1 mg of prohexadione/liter of ethanol for 10 weeks.

Discussion

The lateral application of prohexadione in ethanol to the previous year terminal shoot of *P. sylvestris* seedlings inhibited xylem production in that shoot both above and below the application point, as well as longitudinal growth and xylem and phloem production distally in the current-year shoot (Figs. 1 and 2). Thus the prohexadione was taken up, presumably into the vascular system, and distributed acropetally and basipetally. Prohexadione also inhibited xylem and phloem production above and below the application site when applied laterally to previous year *P. sylvestris* terminals that had been debudded and treated apically with 1 mg of IAA/g of lanolin (Q. Wang, C.H.A. Little and P.C. Odén, unpublished results). Considered together, these results indicate that applied prohexadione is able to inhibit longitudinal and cambial growth concomitantly but also can inhibit cambial growth independently, hence the prohexadione-induced decrease in cambial growth is not mediated by that in shoot elongation. This is the first report that cambial growth in a woody species, as well as shoot elongation in a conifer, are inhibited by an acylcyclohexanedione-type growth retardant. These compounds have also been observed to reduce shoot extension in a wide variety of woody and nonwoody angiospermous species (Junttila 1993b, Junttila et al. 1991, Nakayama et al. 1991, Ross et al. 1993, Zeevaart et al. 1993), whereas growth retardants known to interfere with early steps in GA biosynthesis, such as AMO-1618, ancymidol, chlormequat chloride, flurprimidol, and paclobutrazol, have

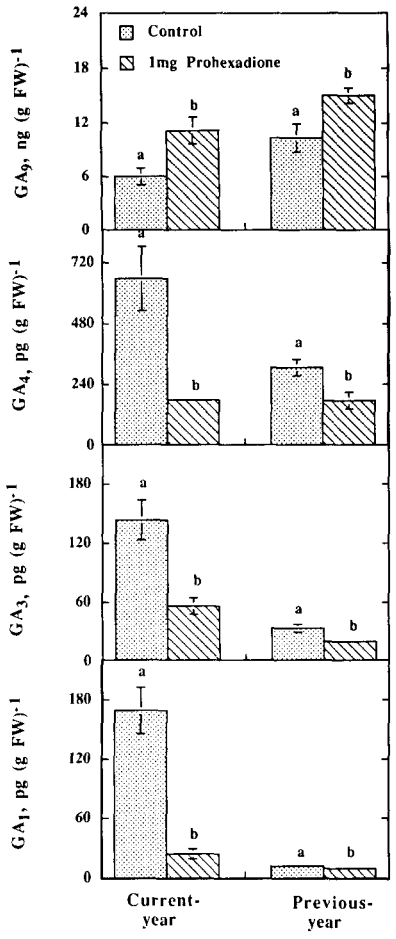

Fig. 4. Levels of GA_1, GA_3, GA_4, and GA_9 in the cambial region of the current year and previous year terminal shoots of 3-year-old seedlings treated laterally at the middle of the previous year terminal shoot with 0 or 1 mg of prohexadione/liter of ethanol for 10 weeks. Mean ± S.E., $n = 5$.

been found to inhibit longitudinal growth in several conifers (Graham et al. 1994, Hare 1984, Weston et al. 1980).

Prohexadione treatment decreased the levels of GA_1 and GA_3 to a similar extent, on a percentage basis (Fig. 4), which is consistent with the hypothesis that they are active per se in the control of shoot growth in *P. sylvestris*. The GA_4 content was similarly decreased, but as deuterated GA_4 is readily converted to deuterated GA_1 in this species (Wang et al. 1995a), as well as other woody species (Junttila 1993a, Moritz et al. 1989a, Odén et al. 1995), it probably is not active per se. The percentage reduction in GA_1 and GA_3 in the current year terminal shoot was significantly greater than that in either longitudinal or cambial growth (Figs. 1 and 2), which may reflect the importance of other factors besides GAs for shoot growth in *P. sylvestris*. IAA would be one such factor, as it is known to be required for both cambial and longitudinal growth (Little and Pharis 1995), and, indeed, prohexadione did not decrease its content (Fig. 3). Similarly, shoot elongation was inhibited, and the GA content was markedly reduced, whereas the shoot IAA level was not changed or was inconsistently affected when cimectacarb was sprayed on *Hordeum vulgare* seedlings (Adams et al. 1992), and prohexadione calcium was applied hydroponically to seedlings of *Triticum aestivum* and *Brassica napus* (Grossman et al. 1994). Furthermore, we have observed that applying $GA_{4/7}$ laterally to debudded, previous year *P. sylvestris* terminal shoots treated apically with exogenous IAA promoted cambial growth without elevating the cambial region IAA content (Wang et al. 1995b). Accordingly, we conclude that GAs stimulate cambial and longitudinal growth in *P. sylvestris* shoots directly rather than indirectly by altering the IAA level.

Metabolic studies with deuterated GA_4 and GA_9 have revealed that $GA_9 \rightarrow GA_4 \rightarrow GA_1$ is a major pathway of GA biosynthesis in the shoots of conifers (Moritz and Odén 1990, Moritz et al. 1989a, Odén et al. 1995, Wang et al. 1995a). This is confirmed by the finding that prohexadione treatment increased the GA_9 level and decreased the contents of GA_4 and GA_1 (Fig. 4), which is attributable to inhibition of the 3β-hydroxylation of GA_9 to GA_4. However, $[^2H]GA_9$ is also metabolized to $[^2H]GA_{20}$ in *P. sylvestris* shoots (Wang et al. 1995a); thus prohexadione treatment would inhibit the 3β-hydroxylation of GA_{20} to GA_1 if this step occurs in conifers, as it does in angiosperms (Fujioka et al. 1990, Kobayashi et al. 1994, Zeevaart et al. 1993). When $[^2H]GA_{20}$ was fed to *P. sylvestris* shoots, however, only $[^2H]GA_{29}$ was formed (Wang et al. 1995a); therefore, whether conifer shoots can convert GA_{20} to GA_1 remains speculative.

The decrease in the cambial region level of GA_3 (Fig. 4) conceivably can be attributed to prohexadione inhibiting three different biosynthetic steps: (1) the conversion of GA_9 to GA_4, if GA_3 is formed from GA_4 via GA_7 or GA_1; (2) the conversion of 2,3-dehydro-GA_9 to GA_7, if GA_3 if formed from GA_9 via GA_7; and (3) the conversion of GA_5 to GA_3, if the $GA_{20} \rightarrow GA_5 \rightarrow GA_3$ pathway found in angiospermous species (Fujioka et al. 1990, Smith et al. 1991) also exists in conifers. However, the formation of $[^2H]GA_3$ from $[^2H]GA_1$, $[^2H]GA_4$, $[^2H]GA_9$ or $[^2H]GA_{20}$ has not been observed, nor has endogenous GA_5 been identified, in the shoots of conifers; thus the biogenesis of GA_3 in these species awaits clarification.

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