

Gibberellin-like Substances From Vegetative Tissue of a Conifer, Arizona Cypress¹

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Abstract. Vegetative shoots of Arizona cypress (*Cupressus arizonica* Greene) were found to contain an estimated 40 to 70 μg of gibberellin-like activity per kg. Based on elution patterns of silicic acid and celite partition columns, mobilities on thin layer chromatograms and specificity of the cucumber, d-3 dwarf maize, dwarf pea, and barley half seed bioassays it was possible to determine that the tissue contained at least 5 acidic, ethyl acetate-soluble gibberellin-like substances. The major one would appear to be GA_3 . In addition, GA_9 , GA_4 , and/or GA_7 -like compounds, and 2 unidentified gibberellin-like substances are present.

Gibberellin A_3 (GA_3) was originally thought to have little effect as a growth regulator on conifers (25). However, a number of Japanese workers have reported that GA_3 induces flowering in the Cupressaceae and Taxodiaceae (3,8,9). Exogenous application of gibberellins A_1 , A_3 , A_4 , A_7 , A_9 , and A_{13} will induce precocious flowering in Arizona cypress (*Cupressus arizonica* Greene) (17,18,19). Furthermore, AMO-1618, an inhibitor of gibberellin biosynthesis, effectively retards growth of Arizona cypress. This retardation can be overcome by simultaneous GA_3 treatment (21). In addition, GA_3 has been found to control apical dominance in both Arizona cypress and redwood (*Sequoia sempervirens*) (20,24).

Since it appears that gibberellins are implicated in a number of the growth and differentiation processes of Arizona cypress it was of interest to determine whether or not endogenous gibberellins occur in this conifer. This paper reports on the extraction, purification, separation, and detection by bioassay of at least 5 acidic gibberellin-like substances from non-flowering leafy shoots of Arizona cypress.

Methods

Growth of Material. Shoot tips of Arizona cypress (*Cupressus arizonica* Greene) 5 to 10 cm long were harvested periodically from 2 year old plants grown in a cooled greenhouse under the following photoperiod and temperatures: at least 18 hr of

natural and supplemental incandescent light, (supplemental light 0400 to 0900 hr, and 1600 to 2200 hr) and at approximately 22° from 0500 to 1900 hr and 18° from 1900 to 0500 hr. The harvested material was frozen in liquid N_2 , stored at -90° until lyophilization, and kept at -20° until extraction.

Extraction, Purification, and Partition Column Chromatography. One kg of lyophilized tissue, consisting of approximately 80 % leaf and 20 % recently formed stem tissue, was macerated for 3 min in a Waring Blendor with 75 % cold methanol (1 liter per 100 g of tissue). The extract was filtered through cheesecloth and centrifuged for 10 min at 22,000g. The pellets were washed with methanol and discarded. Methanol was removed from the supernatant *in vacuo*; an aqueous green syrup remained. Phosphate buffer (0.5 M pH 8) was added to the syrup (1 liter per kg tissue) and the pH adjusted to 8.0 with KOH. The solution was then filtered through Selas silver filters until the now yellow-brown solution was clear. The aqueous extract was washed by partitioning against a total of 1.5 liters of ethyl acetate at each of the following pH's: 8, 7, 6, and 5. The acid fraction was then extracted by adjusting the aqueous extract to pH 2.5 with HCl and partitioning against ethyl acetate (5 times; 3:2 v/v ethyl acetate: aqueous phase). The acidic ethyl acetate-soluble material was then dried with anhydrous Na_2SO_4 and taken to dryness *in vacuo*. This yielded 1.05 g of an oily residue. The residue was placed on top of a 450×40 mm silicic acid partition column (23) filled with 250 g of acid washed Silica AR CC-4, 100 to 200 mesh (Mallinckrodt), and the column eluted stepwise; first with increasing concentrations of ethyl acetate in hexane (Fractions 1-26), then with 1 % followed by 5 % methanol in ethyl acetate (Fractions 27 and 28) and finally with methanol alone (Fraction

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29). Five hundred ml fractions were collected, taken to dryness *in vacuo* and tested for biological activity (see below).

A further 2 kg batch of lyophilized material was extracted as above, the aqueous phase was then washed with ethyl acetate at pH 8 seven times (2.5:1 v/v ethyl acetate: aqueous phase) prior to the separation of the ethyl acetate-soluble acid fraction at pH 2.5. The 8.5 g of acidic components were then further purified by a 20-tube countercurrent extraction (carried out with a Pope 200 ml manual countercurrent extractor). The lower phase was 1.5 M pH 5.5 phosphate buffer saturated with ethyl acetate and the upper phase was ethyl acetate saturated with buffer. Based on the barley half seed α -amylase test (see below) the gibberellin-like material was concentrated in tubes 11 to 16. The peak of activity was in tube No. 14 which contained 156 mg of dry weight and bioassay activity of 28 to 60 μ g of GA_3 equivalents. A 1/20 aliquot was dissolved in phosphate buffer saturated ethyl acetate and pipetted onto the top of a 200 $\times$ 19 mm phosphate buffered celite column (10). The column was filled with 20 g Celite 435 and developed with 500 ml of ethyl acetate and ten 50 ml fractions were collected (Fractions 1-10). The column was then washed with 200 ml of acetone (Fraction 11). All fractions were taken to dryness *in vacuo* prior to being tested for biological activity (see below).

Thin Layer Chromatography. Certain of the fractions from the partition columns that were found to contain gibberellin-like activity were rechromatographed on thin layers of either Kieselguhr G with benzene/acetic acid/water, 8:3:5 v/v (13) or Silica gel G with benzene/n-butanol/acetic acid, 75:20:5 v/v (5). The thin layers were eluted with methanol and/or ethyl acetate which was evaporated to dryness *in vacuo* and redissolved in the appropriate amount of solvent for bioassay. For seedling assays this was a 0.05 % aqueous "Tween 20" solution.

Bioassays. The following assays were used: Progress No. 9 dwarf pea stem elongation (11); *Zea mays* d-3 dwarf mutant leaf sheath elongation (22); hypocotyl elongation of *Cucurbita sativa* var. Straight Eight (1) and the barley half seed reducing sugar (16) and α -amylase tests (6).

Results

Both dwarf corn and dwarf pea assays of eluates from the silicic acid partition column revealed a major peak of gibberellin-like activity (approximately 20 μ g GA_3 equivalents) in fractions 24 and 25 (fig 1). Standards GA_1 and GA_3 are eluted in this zone. In addition the dwarf corn assay detected minor activity in fractions 5 to 10 (GA_4 and GA_7 appear in this region) and 22. The barley half seed reducing sugar and α -amylase tests (fig 2) corroborated the results of the pea and corn assays. Additional biological activity was detected in fraction numbers 2 and 3 (sugar release), 13 (α -amylase

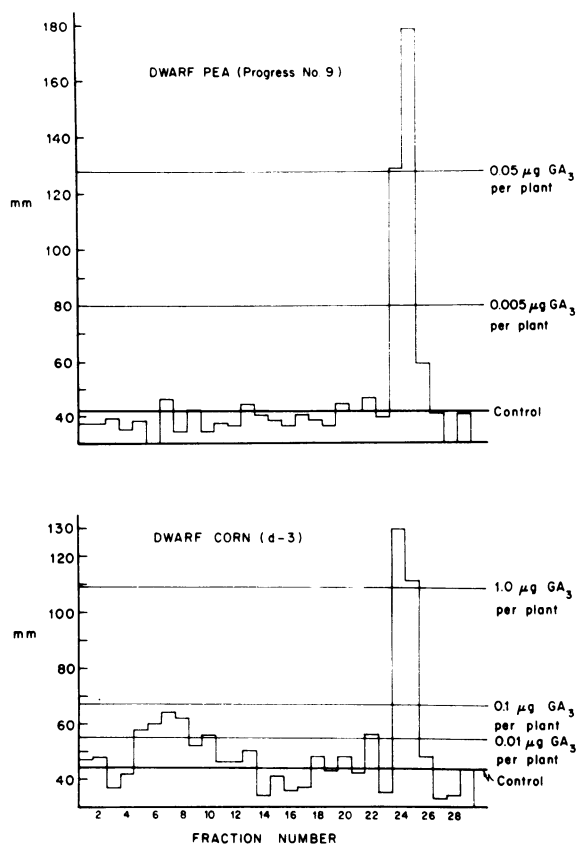


FIG. 1. Progress No. 9 dwarf pea and d-3 maize mutant bioassays of a silicic acid partition chromatogram of the acid fraction extracted from 1 kg of Arizona cypress foliage. Four seedlings per treatment, 1/10 of extract per maize seedling and 1/160 per pea seedlings.

release), 20 (sugar release) and 26 to 29 (reducing sugar and α -amylase release). The cucumber hypocotyl assay showed significant stimulation in fractions 2 and 3, 13, and 14, and 21 (fig 2). Since GA_3 is very active in the cucumber test (1) and is eluted from a silicic acid column with 2 % ethyl acetate in hexane it is possible that the activity in fractions 2 and 3 could be due to the presence of a GA_3 -like compound.

The biological activity in the fraction 13 and 14 region detected by the cucumber hypocotyl and barley half seed α -amylase test could not be correlated with any of the characterized gibberellins available to us. Fractions 27 to 29 which were somewhat toxic to pea and corn seedlings, showed major gibberellin-like activity in the barley half seed reducing sugar and α -amylase assays when tested at high dilutions. It should be noted that the barley half seed sugar assay is sensitive to solvent residues and other organic compounds (2). The α -amylase measurements can therefore be considered to be the most accurate for a survey application such as this.

Since fraction numbers 24 and 25 contained the largest amount of gibberellin-like material, further

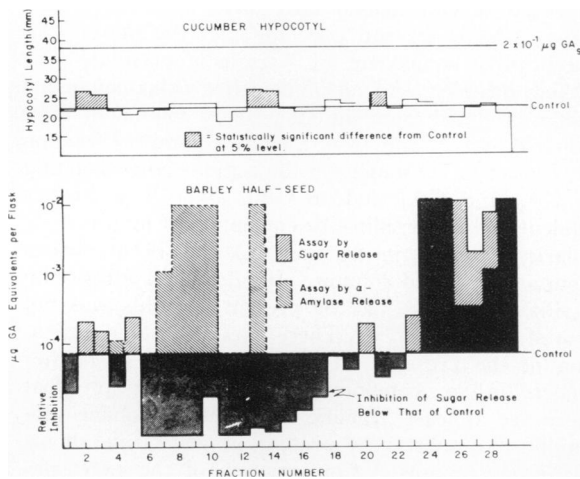


FIG. 2. Cucurbit hypocotyl and barley half seed reducing sugar and α -amylase test of a silicic acid partition chromatogram of the acid fraction extracted from 1 kg of Arizona cypress foliage. Twelve cucumber seedlings per treatment, 1/72 of extract per seedling. Barley half seed assays were carried out in duplicate. Reducing sugar estimations — 1/5 of extract per flask except fractions 24 and 25 where 1/50 was used. 1/30 of extract per flask for α -amylase tests, except fractions 24 and 25 where quantities are extrapolated from thin layer chromatography data of figure 3.

purification and characterization was attempted with these fractions. Thin layer chromatography on Kieselguhr G with a solvent of benzene/acetic acid/water (8:5:3 v/v) separates GA_1 from GA_3 (13). Figure 3 shows the gibberellin-like activity of the combined fraction numbers 24 and 25 after chroma-

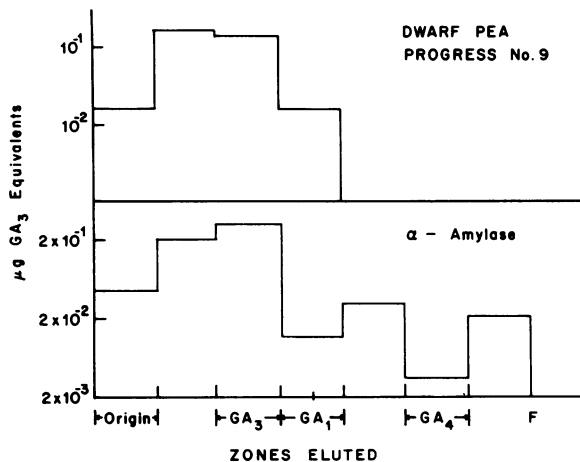


FIG. 3. Progress No. 9 dwarf pea and barley half seed α -amylase bioassays of 1/6 of fractions 24 and 25 from a silicic acid partition column of the acid fraction extracted from 1 kg of Arizona cypress foliage, following chromatography on a thin layer of Kieselguhr G with benzene/acetic acid/water (8:3:5 v/v). Three pea seedlings per treatment, 1/72 of extract per seedling. Barley half seed α -amylase assay carried out in duplicate, 1/16 of extract per flask.

tography in the above solvent system. The chromatogram was developed 3 times before separation of GA_1 and GA_3 (which were co-chromatographed on the sides of the plate) was achieved and streaking became minimal. Both the dwarf pea and barley half seed α -amylase assays showed high activity at the $GA_3 R_F$ and activity of a lower order at the GA_1 position. The barley assay also picked up a peak of activity that moved more slowly than GA_4 but more rapidly than GA_1 , and another peak that was less polar than GA_4 . The presence of GA_3 in the extract was also indicated by a characteristic yellow-green fluorescence, at the appropriate R_F after spraying the Kieselguhr layer with 50 % aqueous sulphuric acid (13). Upon heating the fluorescent spot turned pink, indicating that the fluorescence was either quenched by the presence of impurities or that the GA_3 may have been degraded during chromatography. Since a total of 13.2 μg of GA_3 equivalents were applied to the chromatogram and only 0.82 μg could be recovered, the latter possibility may be indicated.

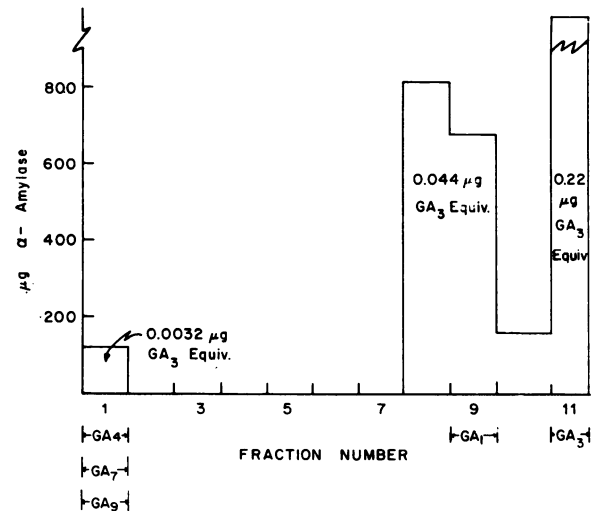


FIG. 4. Barley half seed α -amylase bioassay on a celite partition chromatogram of a portion of the acid fraction, containing 1.4 μg of GA_3 equivalents extracted from 2 kg of Arizona cypress foliage. Fractions 1 to 10 tested singly using 9/10 of extract, fraction 11 tested in triplicate at a 35-fold dilution.

Further evidence for the presence of GA_3 in Arizona cypress foliage was obtained from the extract of the 2 kg batch of lyophilized tissue that was purified by countercurrent fractionation. A portion of this extract containing approximately 1.4 μg of GA_3 equivalents, was chromatographed on a phosphate buffered celite column. Eluates from the column were tested for gibberellin-like activity with the barley half seed α -amylase assay (fig 4). Three areas of activity were observed. Fraction 11 contained 0.22 μg of GA_3 equivalents, although this is probably a low estimate as large quantities of inhibitors were present in this fraction. Samples of GA_3 are eluted from control columns in fraction 11.

Fraction 1 (GA_1 , GA_7 , and GA_9 zone) contained 0.0032 μg of GA_3 equivalents, and a third peak of activity equivalent to 0.044 μg GA_3 was detected in fractions 8 and 9. GA_1 is usually eluted from a celite column in fraction 9.

A 1/60 aliquot of fraction 11 was rechromatographed on a thin layer of silica gel G with benzene/n-butanol/acetic acid (75:20:5 v/v). Barley half seed α -amylase tests on eluates from the developed chromatogram indicated 1 minor and 2 major peaks of activity (fig 5). The peak at R_F 0.6 to 0.7 co-chromatographed with GA_3 . It was not possible to correlate the activity at R_F 's 0.1 to 0.2 and 0.3 to 0.4 with any of gibberellins A_1 to A_9 or A_{13} . However, it is probable that the very polar gibberellin-like compound at R_F 0.1 to 0.2 is equivalent to the activity eluted from the silicic acid column in fractions 28 and 29 (fig 2).

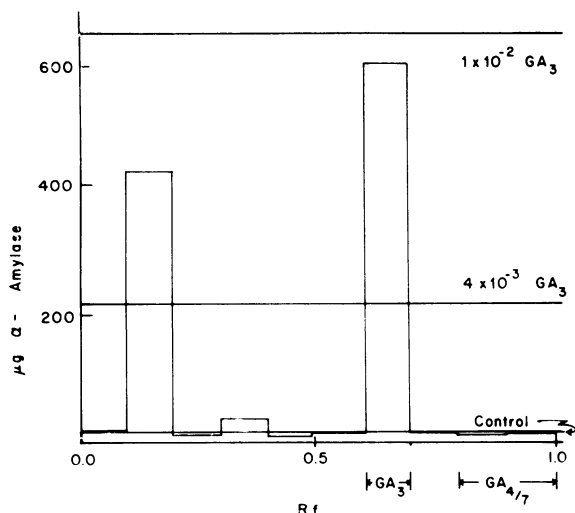


FIG. 5. Barley half seed α -amylase bioassay of 1/60 of fraction 11 from a celite partition chromatogram of a portion of the acid fraction extracted from 2 kg of Arizona cypress foliage, following chromatography on a thin layer of silica gel G with benzene/n-butanol/acetic acid (75:20:5 v/v). Fractions diluted 22-fold and tested in duplicate.

Conclusions

The occurrence of gibberellin-like substances in Arizona cypress has been demonstrated. Gibberellin-like substances in conifers have been reported previously in berries of *Juniperus* (7), new shoots of *Larix* (4, 15) and developing embryos (12), pollen (14), and vegetative shoots (15) of *Pinus*.

Since chemical characterization was not attempted, correlation of the gibberellin-like substances with the known gibberellins is based on a combination of elution patterns from partition columns, mobilities on thin layer chromatograms and bioassay specificity. It is therefore quite possible that the gibberellin-like substances may not be identical to the characterized

gibberellins with which they have been correlated. On this basis we can conclude that the major gibberellin-like component of Arizona cypress is GA_3 . Others include: A) an unidentified polar compound that was eluted from the silicic acid column in fractions 28 and 29 and from a celite column in fraction 11, B) active substances in the regions corresponding to GA_9 and GA_4 and/or GA_7 , and C) a further unidentified gibberellin-like compound of intermediate polarity detected in fractions 13 and 14 of eluates from a silicic acid column. In addition a gibberellin A_1 -like compound may be present, but this was not proved unequivocally. There was also some indication of the presence of trace quantities of further gibberellin-like compounds. Thus it appears that there are at least 5 acidic ethyl acetate-soluble gibberellin-like substances present in vegetative shoots of Arizona cypress. Confirmation of the exact nature of these compounds must await chemical characterization.

Substances interfering with the gibberellin bioassays were abundant in eluates from the silicic acid partition column. Purification by countercurrent distribution followed by chromatography on a buffered celite partition column and thin layers of silica gel yielded a cleaner preparation.

Based on bioassay data the total amount of gibberellin-like material is estimated to be approximately 40 to 70 μg of GA_3 equivalents per kilogram of lyophilized tissue, which is in the same range of concentration as many herbaceous plants.

Acknowledgments

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