Ann. Sci. For. (1989) 46 suppl., 259s-263s
Forest Tree Physiology, E. Dreyer *et al.*, eds

Study of endogenous plant growth substances in Douglas fir II. Gibberellin analysis

P. Doumas[1], J. Bianco[2] and M. Bonnet-Masimbert[1]

[1] *Station d'Amélioration des Arbres Forestiers, INRA Ardon, 45160 Olivet, and*
[2] *Laboratoire de Physiologie Végétale, Université de Nice, 06000 Nice, France*

Introduction

Flowering in Pinaceae conifers can be brought about by the application of less polar gibberellins (GAs), especially GA4/7 applied singly or in combination with other plant growth regulators (such as naphthyl acetic acid) or culture treatments, such as high temperature, water stress, girdling or root-pruning (Pharis and Ross, 1986). GAs seem to be essential in the flowering induction strategy. It is therefore important to know the endogenous GAs of a species before trying to interpret any physiological role of endogenously or exogenously applied GAs.

The level of endogenous GAs in plant tissues is generally very low (1–10 ng/g fresh weight). Consequently, selective methods must be used to analyze GAs. One course of action is to use selective GA immunoassays to detect immunoreactive components in high performance liquid chromatography (HPLC) eluates. Weiler and his coworkers (Weiler and Wieczoreck, 1981; Aztorn and Weiler, 1983a, b) have shown that immunological analyses of GAs could be effective and promising.

We have developed a procedure, combining HPLC separation and enzyme-linked immunosorbent assay (ELISA), which can recognize a limited number of GAs. We have analyzed the effect of flower-inducing treatments on GA levels from juvenile trees. This paper reports preliminary results on the analysis of several GA-like substances in elongating shoots of Douglas fir (*Pseudotsuga menziesii* Mirb.) with or without a flower-inducing treatment, independent of any flowering response on such juvenile trees.

Materials and Methods

Plant material

Experiments were performed at INRA, Orléans, France, on 4 yr old cuttings from one clone. Plants were subjected at the time of bud burst to 1 of 3 treatments: 1) control; 2) spray of GA4/7 (200 mg/1) plus naphthyl acetic acid (10 mg/1) and Aromox–C (a cationic detergent, 0.002% active ingredient) as a surfactant; 3) stem girdling (2 half girdles, 2 cm apart, close to the branch base). Elongating shoots were collected at different dates during the floral initiation time, frozen in liquid nitrogen, lyophilized and ground.

Extraction and purification

Shoot samples were homogenized in 80% methanol with 40 mg/l butylated hydroxy-toluene (BHT) as anti-oxidant and extracted at 4°C for 36 h. After filtration on a 0.45 μm Millipore filter, the samples were loaded onto 2 Sep-Pak C18 cartridges (Waters) and eluted with 80% methanol (40 mg/l BHT). The eluates then were evaporated under vacuum at 30°C. The residues were taken up with 500 μl of methanol–TEA acetate (20 mM) (1/1), pH 3.35, and were injected onto the HPLC column.

High performance liquid chromatography

The extracts were purified and fractionated with a reverse phase system consisting of a System Gold Beckman connected to a C18 column (250 x 4.6 mm; Merck LiChrospher 100 RP–18, 5 μm) eluted with mixtures of methanol and 20 mM TEA acetate buffer, pH 3.35. The following solvent gradient was used: 8% methanol used as the equilibrating solvent; a linear gradient was initiated to 80% over 37 min and then increased to 100% over 10 min. Flow rate was 1 ml/min. Fractions were collected every minute for 60 min, methylated and the GA-like activity was tested by binding it to anti–GA3 antibodies.

ELISA

Polyclonal anti–GA3 antisera were prepared by immunizing rabbits with GA3–BSA conjugates in their anhydride form. Samples and standards were methylated with ethereal diazomethane before ELISA. Microtitration plates were coated with GA3–BSA and ELISA was performed as described elsewhere (Bianco *et al.*, in preparation). In order to increase the rapidity of the test, anti–GA3 antibodies were directly labeled with peroxidase enzyme using the sodium periodate method. Absence of addition of a second antibody, such as peroxidase-labeled sheep anti-rabbit antibody reduced the number of steps and improved the efficiency of the method.

Results

ELISA parameters

An example of a standard curve obtained is shown in Fig. 1. The detection limit is 40 fmol of GA3 methyl-ester and the working range of the assay is between about 50

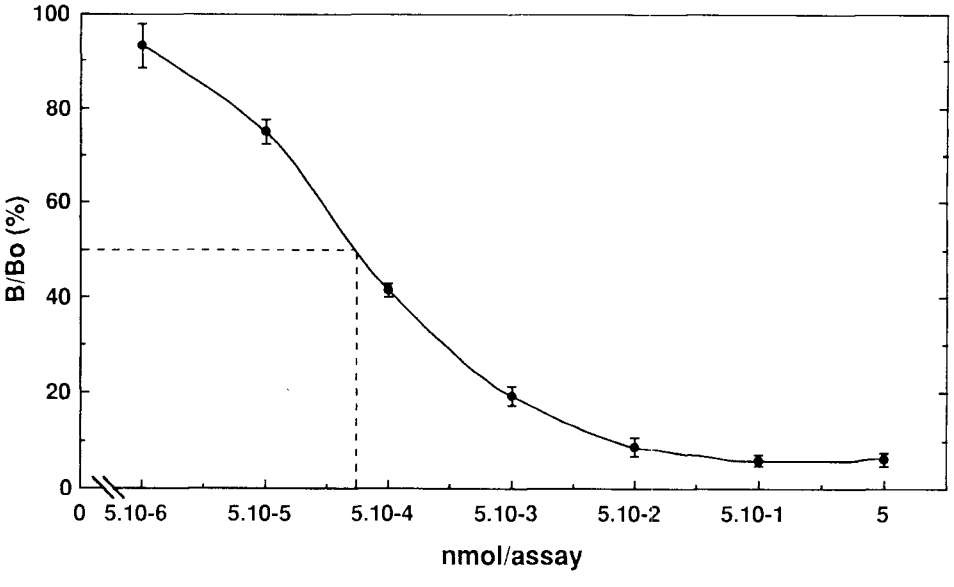

Fig. 1. Standard curve for ELISA with antiserum directed against GA3. *B* = binding of the tracer to the antibody in the presence of standards, and *Bo* = binding of the tracer in the absence of standards.

fmol and 50 pmol of GA3 methyl-ester per well. The anti–GA3 antibodies cross-react with GA1, GA5, GA7, GA8 and GA13.

Plant sample analyses

Elution of available authentic tritiated GA standards (GA3, GA4, GA5, GA8, GA9, GA20) from a reverse phase HPLC system is shown in Fig. 2. Under our conditions, we were able to separate several GAs in a timed program of 50 min. ELISA of individual fractions from plant extract HPLC eluates confirmed the presence of several peaks of cross-reactive material (Fig. 3). In the shoot sample from the control trees (Fig. 3A), 5 immunoreactive peaks appeared which have, respectively, a retention time of 8, 16, 21, 27 and 46 min. Only 3 of them co-eluted with GA standards: GA8 (8 min), GA3 (15–16 min) and GA5/20 (26–29 min). The profile of GA-like substances in the extract from GA4/7-sprayed plants (Fig. 3B) shows several immunoreactive peaks at 7, 16, 22, 28, 32, 37, 42 and 46 min. Some of them co-chromatographed with standards, *e.g.,* GA8, GA3, GA5/20, GA4 (39 min) and GA9 (41 min). In the shoot extract from stem-girdled trees (Fig. 3C), only 3 GA-like peaks were present at 15, 21 and 46 min, one of which co-migrated with the GA3 standard. Culture treatments induce a dramatic increase of GA levels.

Discussion and Conclusion

The results described above on the endogenous GAs of Douglas fir shoots provide a clear illustration of the utility of a combined HPLC–ELISA detection system for GAs. This method allows rapid, specific

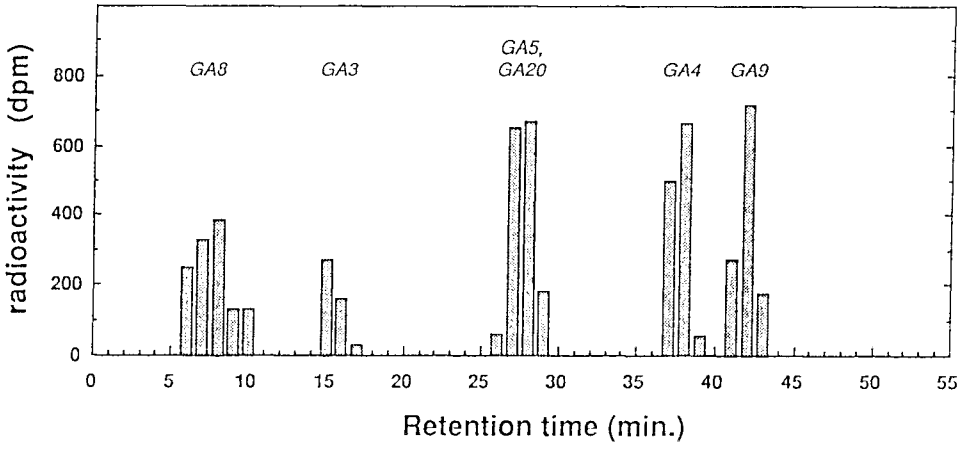

Fig. 2. Reverse phase HPLC of tritiated GAs.

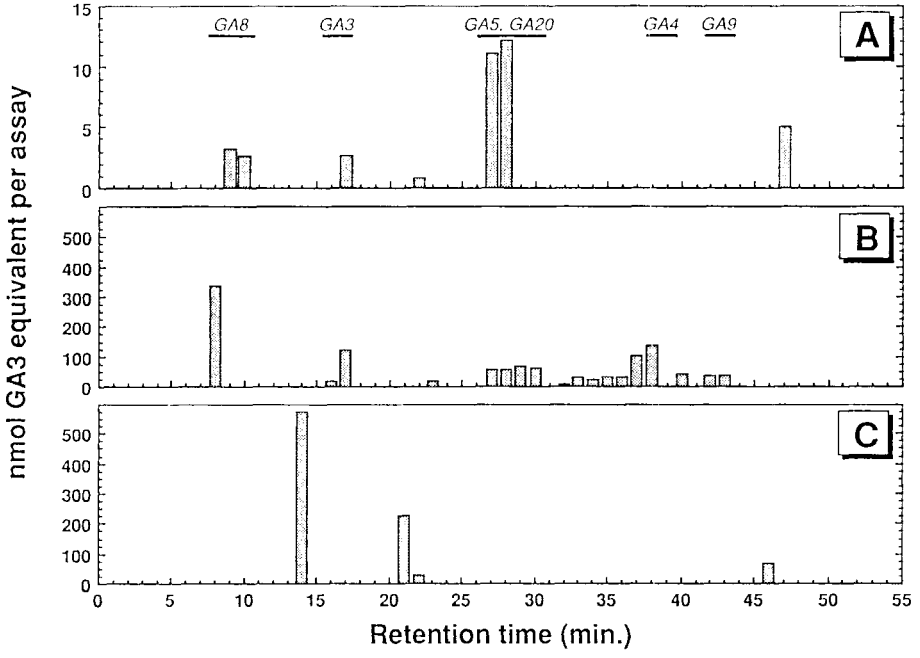

Fig. 3. Levels of GA-like compounds in purified extracts of *Pseudotsuga menziesii* shoots: (**A**: control; **B**: spray of GA4/7 and NAA; **C**: stem girdling). Extracts were chromatographed on reverse phase HPLC and activity was determined by ELISA with antiserum directed against GA3. (The scale for *Y*-axis is different in **A**, **B** and **C**.)

and sensitive detection, identification and quantification of some GAs. C18 purification and directly labeled antibodies decrease the number of steps required and improve the rapidity of the method.

These preliminary results suggest that untreated shoots contain at least 5 different GAs and that flower-induction treatments cause changes in GA patterns and tremendous increases of GA levels. The most interesting result was obtained for shoot samples from GA4/7-sprayed trees. This treatment induced an important modification of the original GA pattern observed. This result suggests that GA4/7 is directly metabolized in treated shoots and the quantity of more polar GAs is increased, as proposed by Pharis *et al.*, (1987). Thus, GA4/7 either may have a direct role in flowering or it may be an important precursor in the metabolism of other flower-inducing GAs.

This study represents only a preliminary assessment. Long-term analysis of GAs related to flowering and affected by culture treatments must continue.

References

Atzorn R. & Weiler E.W. (1983a) The immunoassay of gibberellins. I. Radioimmunoassay for the gibberellins A1, A3, A4, A7, A9 and A20. *Planta* 159, 1-6

Atzorn R. & Weiler E.W. (1983b) The immunoassay of gibberellins. II. Quantitation of GA3, GA4 and GA7 by ultrasensitive solid-phase enzyme immunoassays. *Planta* 159, 7-11

Pharis R.P. & Ross S.D. (1986) Hormonal promotion of flowering in Pinaceae family conifers. *In: Handbook on Flowering.* vol. 5. (Havely A., ed.), CRC Press, Boca Raton, FL, pp. 269-286

Pharis R.P., Webber J.E. & Ross S.D. (1987) The promotion of flowering in forest trees by gibberellin 4/7 and cultural treatments: a review of the possible mechanisms. *For. Ecol. Manage.* 19, 65-84

Weiler E.W. & Wieczorek U. (1981) Determination of fentomole quantities of gibberellic acid by radioimmunoassay. *Planta* 152, 159-167