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Short Communication

Plant growth regulators G_1, G_2, G_3

Synthesis, extraction and determination of leaf content in *Eucalyptus grandis*

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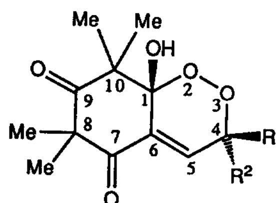
ABSTRACT

Endogenous concentrations of plant growth regulators G_1, G_2, G_3 from leaves of French-planted *Eucalyptus grandis* were determined. The leaves were extracted with methanol and the resulting extracts were purified by means of silica gel column chromatography and reversed-phase medium-pressure liquid chromatography. Improved synthesis, purification and physico-chemical characterization of the G factors, for use as external standards, were developed.

INTRODUCTION

Three closely related growth regulators designated as G substances occur in the leaves of *Eucalyptus grandis* and some other myrtaceous plants [1]. For some years there has been growing interest, especially in Australia [2,3], concerning these compounds. It has been suggested that in addition to auxin-like activity, the G factors affect stomatal conductance and photosynthesis [4], reduce water loss [5] and are probably involved in the frost resistance of *E. grandis* by controlling the active electron transport properties of membranes [6].

The G cyclic peroxides have been either isolated from *E. grandis* [2,7] or synthesized from precursor syncarpic acid [8] and their structures determined by X-ray analysis [9].



G	R_1	R_2
C_1	C_2H_5	CH_3
G_2	CH_3	C_2H_5
G_3	CH_3	CH_3

Me = CH_3

Endogenous concentrations of G factors have been determined for Australian *E. grandis*. The leaf content of G varies from 18.6 μg/g for juvenile trees to 7500 μg/g fresh weight for adult trees [6]. Although phytogeographical differences between the two hemispheres may affect the G content in *E. grandis*, to our knowledge no such data exist concerning the French-planted Eucalyptus. In connection with our current work on this topic [10,11], this paper reports on the research, extraction and isola-

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tion of the G substance from *E. grandis* and its research in *E. gunii* species.

EXPERIMENTAL

Plant material

Leaves from 4-month-old *E. grandis* seedlings were kindly provided by Afocel (Toulouse, France). For *E. gunii*, leaves were collected from two different clones of 4-month-old seedlings, one frost-resistant and the other frost-sensitive. All samples were grown in soil in a glasshouse.

Instrumentation

Medium-pressure liquid chromatography was performed on an Axxial apparatus (Axxial Modulo Prep, Martigues, France). Analytical chromatography was performed on a Waters Model 600 E apparatus equipped with a Rheodyne injector (20 μl) and a Waters Model 990 UV Specroflow detector. The detection wavelength was set at 240 nm. The column used was Nova-Pak silica (150 mm × 4.6 mm I.D.; 4-μm particle size) purchased from Waters. The eluent used was isooctane–dichloromethane (35:65) at a flow-rate of 1 ml/min.

NMR spectra were recorded in deuterochloroform on a Bruker AC 200 apparatus. IR and UV spectra were recorded on a Perkin-Elmer Model 883 and a Lambda 7 spectrophotometer, respectively. Melting points were measured on a Kofler bank.

Chemicals

The G_1, G_2, G_3 compounds used as external standards were chemically synthesized from syncarpic acid, which was obtained by a procedure developed in this laboratory [10]. Although the synthesis of G_1 and G_2 compounds has been described differently to that of G_3 [8], we obtained them under identical experimental conditions. Syncarpic acid (1 g, 5.5 mmol) in ethanol (40 ml) was added dropwise to a solution of the appropriate aldehyde in excess (110 mmol) [2-methylbutyraldehyde (9.4 g) for G_1 and G_2 and 2-methylpropionaldehyde (7.9 g) for G_3] in 10 ml of ethanol containing a catalytic amount of piperidine (0.2 ml). The mixture was allowed to stand for 15 min then solvent and excess of aldehyde were removed under reduced pressure.

G_3 was purified on a silica gel column (40 g, 70–200 μm Amicon, 500 mm × 20 mm I.D., eluent dichloromethane) to give 1.1. g (yield 75%) of isolated product, m.p. 170°C (lit. [8] m.p. 170–171°C); the purity was also checked by thin-layer chromatography (TLC) on a silica gel plate (R_F = 0.37, eluent dichloromethane).

A crude mixture of the two diastereoisomers G_1 and G_2 (75 mg) was purified by medium-pressure liquid chromatography on a silica gel column (20 g, 6–35 μm Amicon, 500 mm × 20 mm I.D.). The detection wavelength was set at 257 nm and the eluent used was dichloromethane–light petroleum (b.p. 45–60°C)–ethyl acetate (75:20:5) [TLC: R_F (G_1) = 0.35, R_F (G_2) = 0.29]. Intermediate fractions were repurified. The purity of the samples was greater than 97% from HPLC results. G_1 and G_2 were identified by their melting points: G_1, m.p. 100°C (lit. [8] m.p. 99–100°C); G_2, m.p. 127°C (lit. [8] m.p. 127–128°C). They were obtained in 46% (710 mg) and 31% (480 mg) total yield, respectively.

The IR spectra of G_1, G_2 and G_3 were recorded as 0.05 *M* solutions in chloroform [ν (cm^{-1}) $\pm$ 1]: G_1, 3578 (OH), 1724 (C_9=O), 1691 (C_7=O), 1636 (C=C); G_2, 3576 (OH), 1726 (C_9=O), 1691 (C_7=O), 1636 (C=C); G_3, 3570 (OH), 1725 (C_9=O), 1690 (C_7=O), 1638 (C=C).

The ^{1}H NMR spectra at 200 MHz and ^{13}C NMR spectra at 50.32 MHz were recorded in C^2HCl_3, with the following results [δ (ppm)]. G_1: ^{1}H NMR, 0.94 (t, J = 7.5 Hz, 3H), 1.03 (s, 3H), 1.30 (s, 3H), 1.34 (s, 3H), 1.35 (s, 3H), 1.44 (s, 3H), 1.71 (q, J = 7.5 Hz, 2H), 4.70 (s, 1H), 7.15 (s, 1H); ^{13}C NMR, 210.7 (C-9), 198.2 (C-7), 141.4 (C-5), 132.4 (C-6), 97.4 (C-1), 82.0 (C-4), 54.9 (C-8), 51.7 (C-10), 30.4 (CH_2), 26.6, 24.0, 21.6, 20.9, 15.2, 7.6 (6 × CH_3). G_2: ^{1}H NMR, 1.01 (dd, J = 7.5 Hz, 3H), 1.03 (s, 3H), 1.28 (s, 3H), 1.30 (s, 3H), 1.35 (s, 3H), 1.36 (s, 3H), 1.78 (m, J_{AB} = 13.5 Hz; J_{AX} = J_{BX} = 7.5 Hz, 2H), 3.70 (s, 1H), 7.20 (s, 1H); ^{13}C NMR, 210.7, 198.3, 143.0, 131.8, 97.3, 81.8, 55.0, 51.6, 29.3, 26.6, 24.1, 20.9, 20.1, 15.1, 7.7 (6 × CH_3). G_3: ^{1}H NMR, 1.00 (s, 3H), 1.08 (s, 3H), 1.25 (s, 6H), 1.28 (s, 3H), 1.43 (s, 3H), 4.09 (s, 1H), 7.09 (s, 1H); ^{13}C NMR, 210.8, 198.5, 143.2, 131.2, 97.3, 79.4, 54.9, 51.7, 26.6, 24.1, 23.9, 23.6, 20.9, 15.2 (6 × CH_3).

The UV spectra were recorded in ethanol G_{1-3} [λ_{max} (nm); ε (l mol^{-1} cm^{-1})]: G_1, 238, 6950; G_2, 239, 6950; G_3, 237, 6900.

Extraction and purification procedure

The extraction procedure was identical with that applied previously but the purification steps were significantly different [1,7]. Leaves of *E. grandis* (30 g) were pulverized with a pestle and mortar, then placed in methanol (750 ml) and stirred at room temperature for 15 h. The mixture was filtered through a Büchner funnel with a filter-paper and the filtrate was evaporated to dryness. The residue was then dissolved in water (100 ml) and the solution extracted with light petroleum (200 ml). The organic phase was dried over magnesium sulphate and evaporated to dryness. The residue (330 mg) was first separated by silica gel column chromatography [eluent cyclohexane (600 ml) then diethyl ether (200 ml)]. The G substances detected were collected along with other chlorophilic product and the solvent was evaporated (200 mg). Rapid filtration over reversed-phase silica [Amicon C_{18}, 90–130 μm, 20 g, eluent water–methanol (20:80), 150 ml] left 130 mg of product, which was purified by medium-pressure liquid chromatography (RSil C_{18}, 15–35 μm, 25 g, purchased from RSL Alltech, France) using water–methanol (25:75) as eluent (flow-rate 6 ml/min).

We thus obtained in two fractions (volume 25 ml each) the G substances along with a residual impurity that absorbs at the detection wavelength utilized for G_1–G_3 mixture analysis; it was eliminated by filtration of the mixture (50 mg) through a Sep-Pak silica cartridge [eluent ethyl acetate–dichloromethane–light petroleum (4:46:50), 4 ml]. The final purified extract contained principally G_1, G_2 and G_3 (Fig. 1) and was used for quantitative analysis.

The same purification procedure as described above was applied to two other samples of *E. grandis* leaves and was found to be reproducible.

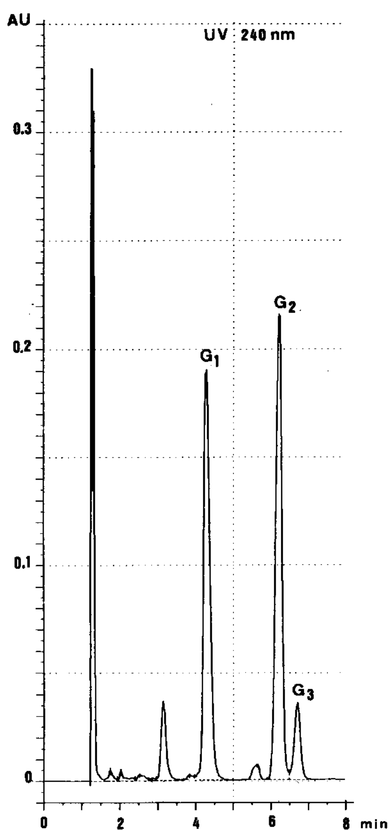

Fig. 1. HPLC of the final purified extract issued from leaves of *E. grandis*. Column, Nova-Pak (4 μm) (15 cm × 4.6 mm I.D.); detection, absorption at 240 nm; mobile phase, isooctane–dichloromethane (35:65); flow-rate, 1 ml/min; temperature, 20°C; injection, 20 μl of *ca.* 2.4 mg of purified extract in 10 ml of dichloromethane. Values on the ordinate are absorbance.

RESULTS AND DISCUSSION

The HPLC separation of an artificial mixture of G_1, G_2, G_3 was performed. Under the conditions described above, the capacity factors (k') obtained were $G_1 = 1.65$, $G_2 = 2.9$ and $G_3 = 3.2$.

Determination of each compound was possible using a G_1–G_3 mixture as external standards. Calibration was performed by plotting known concentrations of mixtures of G compounds *versus* peak area. The proportions of G were chosen in the range estimated from the purified extract ($[G_1]$, $[G_2]$ = $0.4 \cdot 10^{-4}$–$2.2 \cdot 10^{-4}$ *M*, $[G_3] = 0.7 \cdot 10^{-5}$ *M*). The results obtained for four concentration values included in these ranges showed very good linearity (correlation coefficient $r > 0.998$).

Quantitative analysis of a sample of the purified extract was then undertaken. For a 30-mg extract the following average values for three assays of G content were obtained: $G_1 = 7.7 \pm 0.15$, $G_2 = 8.6 \pm 0.12$ and $G_3 = 1.4 \pm 0.03$ mg. Thus the total leaf content of G substances in the *E. grandis* examined was calculated to be 600 μg/g fresh weight.

For a second sample of 60 mg of an extract, obtained before HPLC purfication using RSil, from 17 g of fresh leaves and checked by analytical chromatography, the $(G_1 + G_2)/G_3$ peak-area ratio

was found to be in the same range as that obtained with the preceding extract (9.1 *versus* 10.3), indicating a relative deviation of 12%.

Leaves of two *E. gunii* species were also treated following the same extraction and purification procedure, but we did not detect any G substances under the analytical conditions applied. Nevertheless, we observed major differences in the chromatograms of the two purified extracts of the *E. gunii* samples. Compounds derived from G substances may occur in these species through metabolic transformations so that it will be interesting to extract and identify the substances contained in the purified *E. gunni* extracts.

CONCLUSION

This improved synthesis, purification and characterization of G_1 and G_3 will enable us to examine their chemical behaviour. We have also developed a preparative method for the extraction of G compounds from the leaves of *E. grandis* and measured their content. This method is more convenient and consumes less solvent than previously reported methods.

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