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GROWTH SUBSTANCES ISOLATED FROM WOODY CUTTINGS OF *QUERCUS ROBUR* L. AND *JUGLANS REGIA* L.*

M. D. V. GESTO,† A. VÁZQUEZ,† J. MÉNDEZ,† E. VIEITEZ† and E. SEOANE‡

†Plant Physiology Department, CSIC; ‡Organic Chemistry Laboratory (Institute "Alonso Barba"), Apartado 122, Santiago de Compostela, Spain

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Abstract—A study of growth substances present in cuttings of *Quercus robur* L. and *Juglans regia* L. has been made by extraction with methanol, paper and thin-layer chromatography, u.v. and fluorescence spectroscopy and *Avena* coleoptile straight growth test. From *Q. robur*, scopoletin, vanillic, syringic, gentisic, salicylic and an unidentified acid (hydroxyaliphatic) were isolated and their growth-promoting properties studied. From *J. regia*, vanillic, syringic and three other not yet identified acids (one like *p*-coumaric, one similar to salicylic, and the other an inhibiting hydroxyaliphatic acid) were isolated and their growth properties studied. Moreover, hydroxyaromatic acids were obtained by alkaline hydrolysis of both plant extracts. Most of them probably come from the cleavage of glycosides.

INTRODUCTION

WE ARE trying to isolate and identify growth stimulators and inhibitors from plants, the woody cuttings of which are easy- and difficult-to-root when used for propagation. Our results on *Ribes rubrum*,[1] *Salix atrocinerea*,[2] *Platanus orientalis*[3] and *Castanea sativa*[4] have already been published. This paper is concerned with two other difficult-to-root plants *Quercus robur* and *Juglans regia*. No reports were found about growth substances in cuttings of these species.

RESULTS OF THE UNHYDROLYSED EXTRACT

Biohistogram and Compounds of the Acidic Fraction of Quercus robur

Biohistogram of this fraction in IAW showed a zone of strong growth inhibition (R_f 0·35–0·75) (Fig. 1) and one of growth stimulation in the eluate from zone R_f 0·10–0·35. Vanillic and syringic acids and scopoletin were isolated and identified in the latter zone. Indol-3-acetic-acid (IAA) was looked for, using u.v. and fluorescence spectroscopy and by chemical tests in every eluate with some growth stimulation, but with negative results. From the growth inhibition zone we have isolated gentisic, salicylic and another not yet identified acid, which is undetectable by indole and phenolic reagents. This substance is strongly growth inhibiting, has no u.v. absorption and (tentatively) appeared to be identical to the hydroxy-aliphatic acid reported as an inhibitor in *Castanea sativa*.[4]

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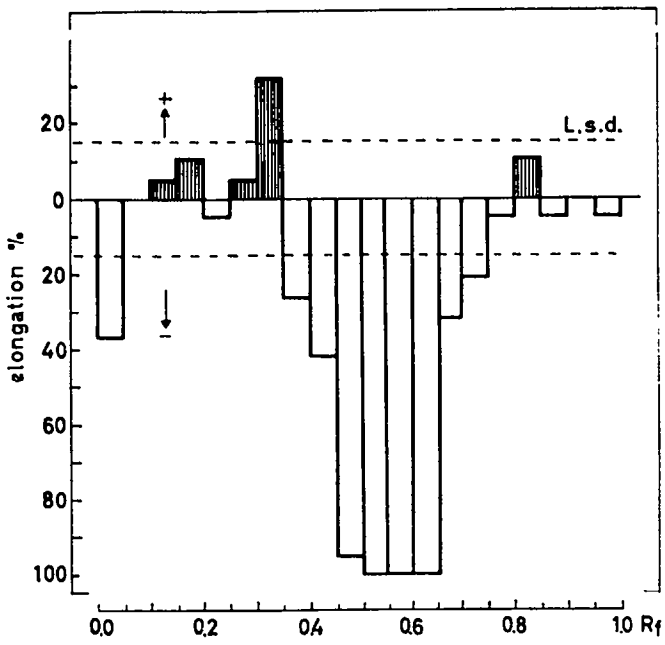

FIG. 1. BIOHISTOGRAM (*Avena* COLEOPTILE SECTION TEST) OF THE ACIDIC FRACTION OF UNHYDROLYSED EXTRACTS OF *Q. robur*. L.s.d. LEAST SIGNIFICANT DIFFERENCE.

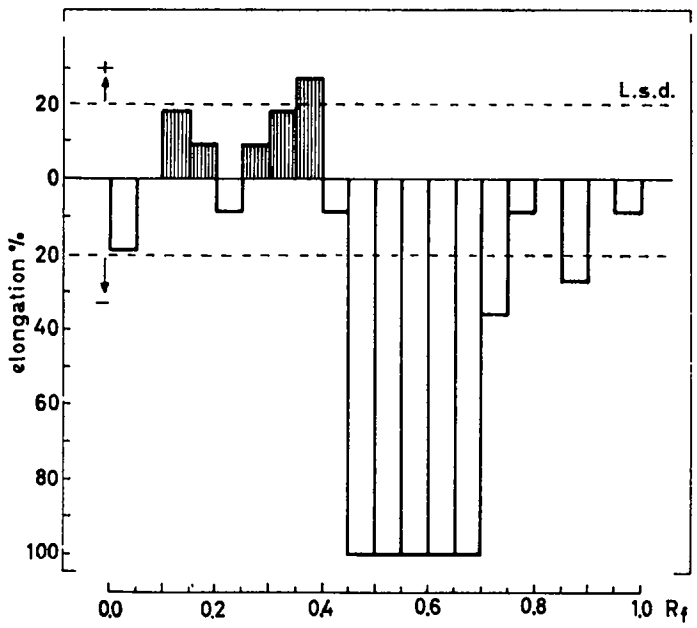

FIG. 2. BIOHISTOGRAM (*Avena* COLEOPTILE SECTION TEST) OF THE ACIDIC FRACTION OF UNHYDROLYSED EXTRACTS OF *J. regia*.

Biohistogram and Compounds of Acidic Fraction of Juglans regia

The biohistogram in IAW exhibited zones of weak growth stimulation (R_f 0·10–0·40) and of strong growth inhibition (R_f 0·45–0·75) (Fig. 2). From the stimulation zone, vanillic and syringic acids were isolated but IAA and *p*-hydroxybenzoic acid (PHB), if present, were undetectable. Moreover, another fluorescent phenolic acid, similar to *p*-coumaric in its R_f values and u.v. spectra, was isolated. From the inhibition zone, a substance like salicylic acid was obtained together with another inhibiting acid, which is undetectable by indole and phenolic reagents. The latter is probably the same hydroxyaliphatic acid, which was found in *Q. robur* and *C. sativa*. From *J. regia* it was isolated and purified by paper chromatography in three successive systems: IAW (R_f 0·45–0·75); butanol saturated with 1% ammonia (R_f 0·45–0·60) and 2% acetic acid (R_f 0·65–0·80).

Identification of Compounds

Vanillic acid was present in large, and syringic acid in small, concentration in *Q. robur*, but they were present in approximately the same concentration (8 mg/kg plant for each) in *J. regia*. The spots overlapped and were indistinguishable with the DQC spray. However, both acids can be recognized by their distinctive colour with DPNA, as a detecting reagent (dark-blue for syringic acid and red–violet for vanillic acid). They have been identified as reported,[4] and can be separated by paper chromatography in two additional systems: butanol saturated with 1% ammonia (syringic acid, R_f 0·07; vanillic acid, R_f 0·10) and 2% acetic acid (syringic acid, R_f 0·40; vanillic acid, R_f 0·43).

Gentisic acid was isolated by rechromatography of the zone at R_f 0·45–0·53 (about 700 μg/kg plant) and identified by its u.v. spectrum with λ_{max} 328 nm in neutral methanol, shifting to 326–328 nm by addition of alkali (gentisic acid λ_{max} 328 and 326 nm respectively), by its fluorescence spectra which gives with λ_{max} of excitation 334 nm a fluorescence peak at 455 nm either in neutral or in alkaline methanol (authentic sample had λ_{max} excit. 335 nm with fluorescence peak 453 nm), by its R_f values in four systems (Table 1) and that of its methyl ester. It was detected either by fluorescence, or by DQC (blue–violet or pink–brown).

Salicylic acid appeared in the same zone as the other acid with growth-inhibiting properties. They were separated chromatographically in a second solvent, and the salicylic acid was identified as previously[4] and by its R_f values in three systems (Table 1). Scopoletin was isolated by rechromatography of the zone at R_f 0·30–0·40 and purified by paper chromatography with 2% acetic acid (R_f 0·05–0·30). It is highly fluorescent under u.v. and becomes pink–blue with DQC; its u.v. spectrum in neutral methanol, like authentic scopoletin, has peaks at 295 and 340 nm, shifting to 395 nm by addition of alkali; its fluorescence spectrum with excitation maximum at 355 nm showed a peak at 435 nm in neutral methanol shifting with alkali to 395 and 475 nm respectively; its methyl ester, like an authentic sample, had R_f 0·71 in IAW; it is partially reduced by $LiAlH_4$ to an alcohol of R_f 0·74. All the derivatives give fluorescent spots under u.v. and violet with DQC.

The substance similar to salicylic acid from *J. regia* had identical u.v. spectrum to salicylic acid in neutral and alkaline methanol, the same R_f values in two systems; but differed in paper chromatography with 2% acetic acid: R_f 0·68 (salicylic acid, 0·58); and in TLC with butanol saturated with water: R_f 0·43 (salicylic acid, 0·50).

Biohistogram and Compounds of Neutral Fraction of Quercus robur

A strong growth-promoting activity was shown in the R_f 0·40–0·80 zone in IAW (Fig. 3), but no compounds were detected with either the indole or phenolic reagents. The substance

TABLE 1. R_f VALUES OF AUTHENTIC SAMPLES AND *Q. robur* AND *J. regia* ELUATES IN SEVERAL SOLVENTS

	Paper chromatography*				TLC	
	IAW	2% acetic acid	BA	BAW	BW	Plant
Eluate	- -	0·40	0·07	- - -	- -	
Syringic acid	- -	0·40	0·07	- - -	- - -	
Eluate	0·14	0·43	0·10	-	—	
Vanillic acid	0·14	0·43	0·10	- - -	—	*Q. robur* and *J. regia*
Eluate	0·21	0·46	—	—	0·48	
PHB	0·21	0·46	—	- -	0·48	
Eluate	0·34	0·56	0·17	- -	—	
MHB	0·34	0·56	0·17	-	—	
Eluate	0·49	0·51	0·22	-	0·44	
Gentisic acid	0·49	0·51	0·22	- - -	0·44	
Eluate	0·60	0·58	0·40	0·92	- - -	
Salicylic acid	0·60	0·58	0·40	0·92	—	*Q. robur*
Eluate	—	0·40	—	0·80	0·0–0·30	
Protocatechuic acid	—	0·42	—	0·83	0·0–0·32	
Eluate	0·34	0·25	0·45	—	0·72	
Scopoletin	0·34	0·25	0·45	—	0·72	
Eluate	0·23	0·30	0·20	—	0·46	
PCA	0·23	0·30	0·19	—	0·46	
Eluate	0·18	0·24	0·15	—	0·32	*J. regia*
Ferulic acid	0·18	0·25	0·15	—	0·32	

* IAW: Isopropanol/ammonia/water (10:1:1); BA: butanol saturated with 1% ammonia; BAW: butanol/acetic acid/water (4:1:5); BW: butanol saturated with water.

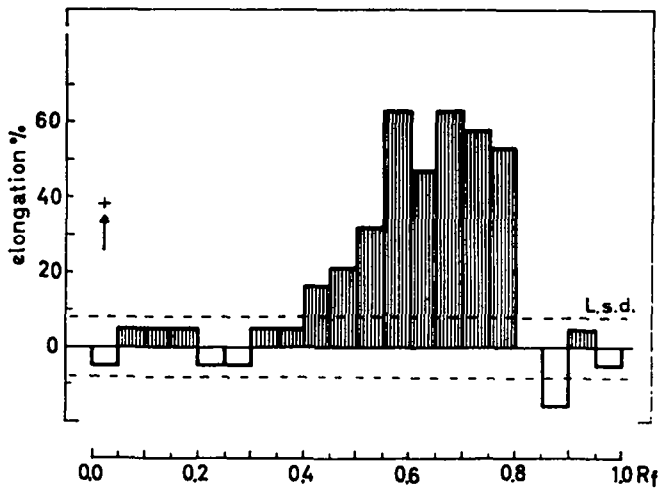

FIG. 3. BIOHISTOGRAM (*Avena* COLEOPTILE SECTION TEST) OF THE NEUTRAL FRACTION OF UNHYDROLYSED EXTRACTS OF *Q. robur*.

responsible for this growth activity is resistant to alkaline hydrolysis but labile when refluxed with acid (6 N HCl). Although this might suggest it was an indole nitrile, the products obtained by $LiAlH_4$ reduction were indetectable by indole reagents.

RESULTS OF HYDROLYSATE FRACTIONS

Acidic Fraction of the Quercus robur *and* Juglans regia *Hydrolysate*

According to the growth-stimulating and inhibiting zones of acidic biohistogram in IAW, bands of the chromatogram were cut and their eluates rechromatographed for identification. Protocatechuic, syringic, vanillic, PHB and *m*-hydroxybenzoic (MHB) acids were isolated and identified in *Q. robur*. In the same way syringic, vanillic, ferulic, PHB and *p*-coumaric acids were isolated from the different zones of chromatograms of *J. rejia*, and another substance similar to MHB (F_{36}) was also isolated.

Identification of Compounds. PHB has been identified in *Q. robur* and *J. regia* by comparing R_f values with those of an authentic sample in four solvent systems (Table 1), using DQC (blue–green) or DPNA (pink) for detection.

p-Coumaric acid was identified by its R_f values in four systems (Table 1), detecting the spots by their u.v. fluorescence, blue–violet colour with DQC, and blue–gray with DPNA; by its u.v. spectrum: λ_{max} 291–300 nm, shifting to 333–336 nm with alkali; by the R_f value (0·80) of its reaction product with diazomethane. All the values were identical with those of authentic samples.

m-Hydroxybenzoic acid (MHB) has been identified as previously reported[4] and by R_f values (Table 1); a second acid (F_{36}) with little higher R_f (0·36) to MHB in IAW was detected with DQC (blue). All the constants for F_{36} were similar but different from MHB.

Protocatechuic acid was identified by comparison with authentic sample in three systems (Table 1). It produces brown–gray spots with DQC and orange with diazotizated sulfanilic acid. Ferulic acid was identified as described elsewhere[4] and by its R_f values (Table 1).

GROWTH ACTIVITY OF THE ISOLATED COMPOUNDS

Growth stimulation and inhibition of the isolated substances were studied. A good agreement between pure specimens and chromatogram eluates of the plant extracts was obtained and the concentration limits, between which either growth-stimulation or inhibition occurs, have been well established for the authentic samples. Biological activity of mixtures of IAA and pure specimens were studied in the same way.

Vanillic, p-*hydroxybenzoic, salicylic and* m-*hydroxybenzoic acids.* These acids have shown the same growth stimulation and inhibition as their pure specimens, as reported previously.[1,4]

p-*Coumaric acid. p*-Coumaric acid was completely inactive at concentrations lower than 60 μg/ml, from which a weak growth inhibition starts and it increases progressively, reaching 45% at 150 μg/ml. Inhibition was accompanied by some cell plasmolysis; with the higher concentrations all cells were plasmolysed and dead.

Mixtures of IAA and p-*coumaric acid. p*-Coumaric acid was tested at various concentrations in the presence of 1, 10, 20 μg/ml of IAA. IAA growth stimulation did not change by addition of *p*-coumaric acid in concentrations lower than 50 μg/ml; stimulation was progressively reduced with 50–100 μg/ml, and inhibition was given in all cases with amounts of *p*-coumaric acid higher that 100 μg/ml.

Syringic acid. Concentrations lower that 10 μg/ml showed no activity, but a weak growth

stimulation started at this point, and increased with concentration to reach a maximum at 80–100 μg/ml (L.s.d.: 9% at level 5%). A slight toxicity was noted at 125 μg/ml, which increased with further increases in concentration.

Mixtures of IAA and syringic acid. In the presence of 0·025 μg/ml of IAA, a synergistic action was observed with syringic acid between 10 μg/ml and 100 μg/ml. With 0·1 μg/ml IAA the highest synergistic action was observed with 50 μg/ml of syringic acid. No positive synergistic action was detected with 1 μg/ml IAA at any concentration of the syringic acid (Fig. 4).

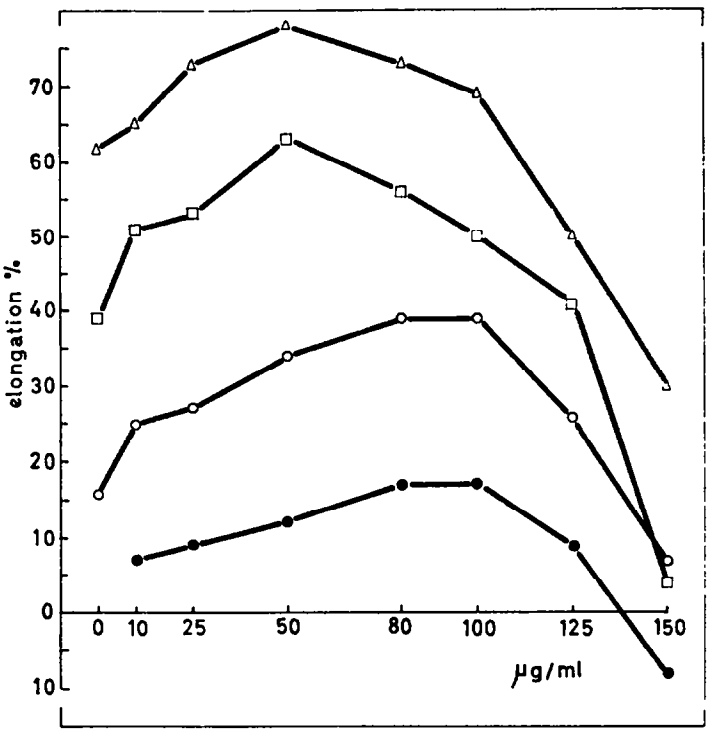

FIG. 4. BIOLOGICAL ACTIVITY (*Avena* COLEOPTILE SECTION TEST) OF SYRINGIC ACID (10–150 μg/ml) WITH AND WITHOUT IAA.

●——● No IAA. ○——○ 0·025 μg/ml IAA. L.s.d. 11% at level 5%. □——□ 0·1 μg/ml IAA. L.s.d. 12% at level 5%. △——△ 1 μg/ml IAA. L.s.d. 23% at level 5%.

Gentisic acid. Gentisic acid began to show a weak growth stimulation at 10 μg/ml, which increased with the concentration to reach a maximum at 80–100 μg/ml and then decreased. Gentisic acid became toxic and a growth inhibitor with concentrations higher than 150 μg/ml.

Mixtures of IAA and gentisic acid. In the presence of 0·025 and 0·1 μg/ml of IAA the elongation of the coleoptiles obtained with mixtures of gentisic acid was the sum of those obtained with the components; therefore there was no synergistic activity. With 1 and 5 μg/ml IAA, however, elongation was lower than the summation of those corresponding to the components, especially with concentrations of gentisic acid higher than 80 μg/ml. Accordingly, there was a negative synergistic action.

DISCUSSION

Both plants studied belong to the group of species which are difficult-to-root by cuttings, and the biohistograms with one zone of weak growth stimulation and another of a strong growth inhibition were very similar (Figs. 1 and 2). As has been shown with pure specimens, the growth-promoting activity of vanillic, syringic and PHB acids in concentrations of 10–100 μg/ml could explain the weak growth activity zone, where no IAA was found. In the same way the strong inhibition properties of salicylic and gentisic acids in concentrations higher than 150 μg/ml could account for the character of the inhibiting zone. But the action of the other inhibiting compound which is not detectable by indole and phenolic reagents, and is probably a hydroxyaliphatic acid, has to be added.

Although the biohistograms of the acidic fraction of the plants already studied, *Ribes rubrum*, *Salix atrocinerea*, *Platanus orientalis* and *Castanea sativa*, show similar inhibition zones, they are quantitatively different, since the inhibition is very prominent in *C. sativa*, *Quercus robur* and *Juglans regia*, and rather weak in the three easy-to-root plants. Consequently it is not unreasonable to suspect that inhibitors account for the lack of rooting ability by the cuttings of the other three species.

As far as activity in the neutral fraction is concerned, because of the similarity in biohistograms of easy- and difficult-to-root plants, and because of the unsuccessful attempts in characterizing the nature of the substances responsible for activity, no proper conclusions can be made.

EXPERIMENTAL

Extraction, separation and chromatographic analysis. 3 kg of fresh woody cuttings of *Quercus robur* and 2·25 kg of those of *Juglans regia*, previously frozen at −45°, were sliced and extracted with methanol at 0–2° for 24 hr. Extraction, concentration, fractionation and chromatographic analysis were carried out as previously reported.[1]

Acidic, phenolic and neutral substances were separated from their solution in ether by successive extraction with aqueous solutions of Na_2CO_3 and NaOH. Alkaline substances contained in the early methanolic aqueous extracts, acidified with HCl, were separated by ether extraction, after basification with an excess of NaOH.

Hydrolysis of the aqueous residue. The aqueous residue, after removing ether soluble compounds, promoted the growth of *Avena* coleoptiles and therefore was hydrolysed with 0·5 N Ba $(OH)_2$ and the hydrolysate was fractionated as above.[1]

Bioassay. The straight growth of *Avena* coleoptile sections was used either with chromatogram pieces or with the substances eluted from a chromatogram zone. Percentages of elongation in the biohistograms were calculated according to:

$$I\% = \frac{dL_T - dL_C}{dL_C} \cdot 100$$

Where dL_T and dL_C are the absolute elongations of the treated and control coleoptile sections, respectively.[5] This formula is different from others used before.[1-3]

Paper chromatography. Unless otherwise specified isopropanol/ammonia/water (10:1:1) (IAW) on Whatman paper No. 1 was throughout used as descending solvent. The most commonly used reagents were a 0·1% ethanolic 2,6-dichloroquinonechlorimide solution (DQC) with aqueous saturated borax solution overspray,[6] and diazotized *p*-nitroaniline (DPNA)[7] for phenols, and Ehrlich for indole compounds.[6]

Fluorescence spectra. A Zeiss spectrophotofluorometer ZFM 4C with two monochromators and a Xenon arc-lamp was used to run the activation and fluorescence spectra. Standard compounds were used in the concentrations of 5 μg/ml or 1 μg/ml.

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