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GROWTH SUBSTANCES ISOLATED FROM WOODY CUTTINGS OF *ALNUS GLUTINOSA* MEDIC. AND *FRAXINUS EXCELSIOR* L.

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Abstract—From the unhydrolysed extract of *Alnus glutinosa*, ferulic, *p*-coumaric, *p*-hydroxybenzoic and *trans*-cinnamic acids were isolated and identified by paper and thin-layer chromatography and by u.v. spectroscopy. From *Fraxinus excelsior*, vanillic, *p*-hydroxybenzoic, two unknown acids and scopoletin were detected in a similar way. Moreover, syringic, vanillic and *p*-hydroxybenzoic acids were characterized in the hydrolysate of both plants. Caffeic, isoferulic and the two unknown acids were present in the hydrolysate of *F. excelsior* and *p*-coumaric acid in that of *A. glutinosa*. Scopoletin and *p*-coumaric acid seem to be implicated in the rooting properties of woody plants.

INTRODUCTION

IN A systematic research for growth substances in easy- and difficult-to-root woody plants, we have already studied *Ribes rubrum*,[1,2] *Salix atrocinerea*,[3] *S. viminalis*,[4] *Ficus carica*,[4] *Platanus orientalis*,[5] *Castanea sativa*,[6] *Quercus robur*,[7] and *Juglans regia*.[7] The present paper is concerned with an easily rooting species (*Alnus glutinosa* Medic.) and a difficult-to-root one (*Fraxinus excelsior* L.). Among the naturally occurring inhibitors reviewed by Hemberg,[8] scopoletin (6-methoxy-7-hydroxycoumarin), a very common lactone in the plant kingdom, repressed root growth of *Avena* at a concentration as low as 30 μM. This effect was not observed in presence of IAA in the adventitious root formation test of mung bean cuttings,[9] but was found using the non-growing parts of *Avena* roots.[9] However, the compound had no effect in the *Avena* straight and curvature tests. Scopoletin, which was reported

[1] E. VIEITEZ, E. SEOANE, M. D. V. GESTO, M. C. MATO, A. VÁZQUEZ and A. CARNICER, *Physiol. Plantarum* **19**, 294 (1966).

[2] E. VIEITEZ, E. SEOANE, M. D. V. GESTO, M. C. MATO, A. VÁZQUEZ and A. CARNICER, *Anales Edafol. Agrobiol. Madrid* **25**, 69 (1966).

[3] E. VIEITEZ, E. SEOANE, M. D. V. GESTO, M. C. MATO, A. VÁZQUEZ, A. CARNICER and J. PEÑA, *Physiol. Plantarum* **20**, 232 (1967).

[4] A. VÁZQUEZ, J. MÉNDEZ, M. D. V. GESTO, E. SEOANE and E. VIEITEZ, *Phytochem.* **7**, 161 (1968).

[5] E. VIEITEZ, E. SEOANE, M. D. V. GESTO, A. VÁZQUEZ, M. C. MATO, J. MÉNDEZ and A. CARNICER, *Anales Edafol. Agrobiol. Madrid* **26**, 131 (1967).

[6] E. VIEITEZ, E. SEOANE, M. D. V. GESTO, A. VÁZQUEZ, J. MÉNDEZ, A. CARNICER and M. L. ARESES, *Phytochem.* **6**, 913 (1967).

[7] M. D. V. GESTO, A. VÁZQUEZ, J. MÉNDEZ, E. VIEITEZ and E. SEOANE, *Phytochem.* **6**, 1687 (1967).

[8] T. HEMBERG, *Handb. Pflanzenphysiol.* **14**, 1162 (1961).

[9] I. FERNQVIST, *Lantbrukshögsk. Ann.* **32**, 109 (1966).

in *Fraxinus* species,[10] showed an antagonism for auxins in the mesocotyl test[11] and analogous effect due to competitive inhibition was found for *trans*-cinnamic acid, a precursor of IAA,[8] in the pea section test. Caffeic acid, a promotor of root initiation,[9] has been reported in the hydrolysate of *F. excelsior* leaves.[12] No reports were found on growth substances in *A. glutinosa.*

RESULTS OF UNHYDROLYSED EXTRACTS

Biohistogram and Compounds of the Acidic Fraction of Alnus glutinosa

According to the bioassay (Fig. 1) or the chromogenic reactions with DPNA, three different zones on IAW chromatograms were eluted: R_f 0·10–0·20; R_f 0·20–0·30 (with erratic responses) and R_f 0·47–0·65 (strong inhibition). The eluates of the three zones were rechromatographed with 2 per cent acetic acid and several subzones were eluted according to the colour with DPNA. Ferulic acid and *p*-hydroxybenzoic acid (PHB) were identified from the first zone; *p*-coumaric acid and PHB were identified from the second zone. From the third zone, *trans*-cinnamic (3 mg/kg) acid was isolated.

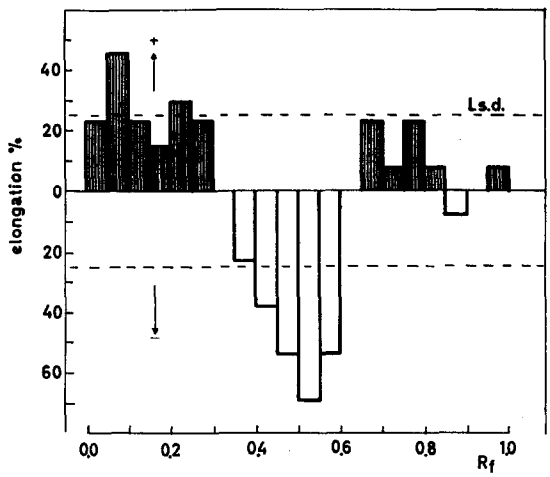

FIG. 1. BIOHISTOGRAM IN IAW (*Avena* COLEOPTILE SECTION TEST) OF THE ACIDIC FRACTION OF UNHYDROLYSED EXTRACT OF *A. glutinosa.* L.s.d. LEAST SIGNIFICANT DIFFERENCE, AT LEVEL 5 PER CENT.

Biohistogram and Compounds of the Acidic Fraction of Fraxinus excelsior

It has shown in IAW three different zones: R_f 0·00–0·35 of dubious activity; R_f 0·35–0·45 of growth stimulation and R_f 0·55–0·95 of strong growth inhibition (Fig. 2). The eluates of the first two zones were rechromatographed with 2 per cent acetic acid and subzones were made using u.v. fluorescence and a narrow strip sprayed with DQC. Almost pure scopoletin (about 110 μg/kg) was isolated together with vanillic (90 μg/kg) and PHB (60 μg/kg) acids. A further zone in 2 per cent acetic acid (R_f 0·35–0·43) gave a yellow fluorescence under u.v. light and two unknown compounds were isolated by further chromatography in butanol saturated with 1 per cent ammonia (unknown I, R_f 0·25–0·31; and unknown II, R_f 0·42–0·56). The eluate of the IAW zone, R_f 0·55–0·95, showed by rechromatography in butanol

[10] R. PARIS and A. STAMBOULI, *Ann. Pharm. Franc.* **18**, 873 (1960).
[11] J. P. NITSCH and C. NITSCH, *Bull. Soc. Botan. France* **108**, 349 (1961).
[12] E. C. BATE-SMITH, *J. Linn. Soc. Botany* **58**, 95 (1962).

saturated with 1 per cent ammonia a subzone R_f 0·55–0·95 which gave blue colour with DQC. By acidic hydrolysis of the eluate, an unidentified acid with bluish green colour with DQC at R_f 0·37 in IAW was obtained.

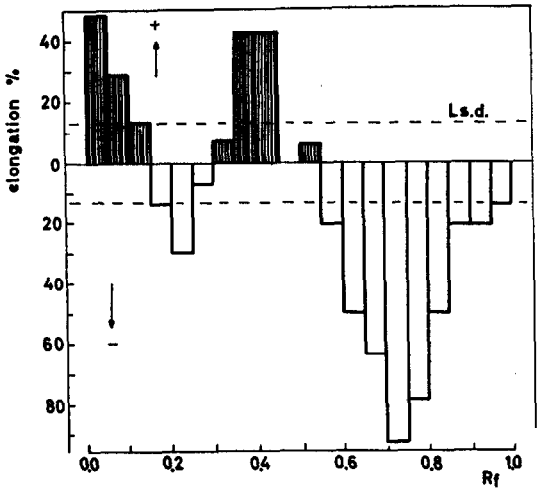

FIG. 2. BIOHISTOGRAM IN IAW (*Avena* COLEOPTILE SECTION TEST) OF THE ACIDIC FRACTION OF UNHYDROLYSED EXTRACT OF *F. excelsior*. L.s.d. LEAST SIGNIFICANT DIFFERENCE, AT LEVEL 5 PER CENT.

RESULTS OF HYDROLYSATE FRACTIONS

Biohistogram and Compounds of the Acidic Fraction of Alnus glutinosa

The zone R_f 0·37–1·00 in 2 per cent acetic acid was rechromatographed in IAW, and syringic, vanillic and PHB acids were characterized. The subzone in IAW, R_f 0·40–0·50, was rechromatographed in 2 per cent acetic acid and *p*-coumaric acid (2 mg/kg) was isolated and identified. From the band R_f 0·50–0·62, a fluorescent compound was isolated but not identified.

Biohistogram and Compounds of the Acidic Fraction of Fraxinus excelsior

This fraction gave inhibition along the entire length of the chromatogram in IAW. Caffeic acid (about 2·6 mg/kg) was isolated in an impure state, and purified by rechromatography with 2 per cent acetic acid. Syringic, vanillic, isoferulic (about 500 μg/kg) and PHB (400 μg/kg) acids were present at the zone R_f 0·10–0·25. They were separated by rechromatography with butanol saturated with 1 per cent ammonia. The two above-mentioned unknown acids were also found.

Identification of Compounds

Compounds were identified by their fluorescence under u.v. light, u.v. spectra (Table 1), chromatographic comparison with authentic samples in several systems (Table 2) and by the colour produced when sprayed with DSA and DQC.

The unknown I has a light blue fluorescence under u.v. light, changing to yellow on fuming with ammonia and gives gray-blue colour with DQC and light pink with DPNA or DSA. The u.v. spectra and R_f data are summarized in Tables 1 and 2. The unknown II is non-fluorescent; the u.v. spectra and R_f values are tabulated in Tables 1 and 2.

TABLE 1. ULTRAVIOLET SPECTRA IN METHANOL OF PURE SPECIMENS AND COMPOUNDS ISOLATED FROM *A. glutinosa* AND *F. excelsior*

Substance	λ_{max} nm	
	Neutral pH	Alkaline pH
Eluate	290; 315	300; 345
Caffeic acid	285; 320	303; 345
Eluate	273–274	268–269
trans-Cinnamic acid	271–272	265
Eluate	286–287	330–335
p-Coumaric acid	291	334–335
Eluate	295; 318–319	344–347
Ferulic acid	291; 314	306; 347–349
Eluate	285–290; 310–315	260; 290
Isoferulic acid	289; 315–320	259; 293
Eluate	225; 255; 295; 340	240; 395
Scopoletin	228; 255; 295; 345	240; 392–393
Unknown I	340	400
Unknown II	255; 305	270; 330

TABLE 2. R_f VALUES OF AUTHENTIC SAMPLES AND *A. glutinosa* AND *F. excelsior* ELUATES IN SEVERAL SOLVENTS

Substance	PC				TLC	Plant
	IAW	2% HAc	BA	EAW	BW	
Eluate	0·11	0·24	0·07		0·0–0·31	*F. excelsior*
Caffeic acid	0·11	0·24	0·07		0·0–0·33	
Eluate	0·62	0·59	0·42	0·73		*A. glutinosa*
trans-Cinnamic acid	0·56	0·58	0·43	0·76		
Eluate	0·27	0·30 0·49	0·18	0·56	0·52	*A. glutinosa*
p-Coumaric acid	0·27	0·30 0·52	0·19	0·58	0·52	
Eluate	0·17 0·24	0·21		0·53		*A. glutinosa*
Ferulic acid	0·17	0·25		0·53		
Eluate	0·23	0·46	0·12	0·60		*A. glutinosa*
PHB	0·24	0·46	0·12	0·61		*F. excelsior*
Eluate	0·29	0·25	0·12		0·32	*F. excelsior*
Isoferulic acid	0·29	0·24	0·11		0·31	
Eluate	0·34	0·25	0·45		0·72	*F. excelsior*
Scopoletin	0·34	0·25	0·45		0·72	
Eluate	0·15	0·38		0·57		*A. glutinosa*
Syringic acid	0·13	0·41		0·59		
Eluate	0·20	0·43		0·57		*F. excelsior*
Vanillic acid	0·17	0·44		0·58		
Unknown I	0·34	0·40	0·34		0·60	*F. excelsior*
Unknown II	0·34	0·40	0·47	0·64	0·68	

PC: paper chromatography; IAW: Isopropanol/ammonia/water (10:1:1); 2% HAc: 2 per cent acetic acid; BA: butanol saturated with 1 per cent ammonia; EAW: ethanol/ammonia/water (35:2:13); BW: butanol saturated with water.

DISCUSSION

The presence of scopoletin in *Quercus robur*[7] and *Fraxinus excelsior*, two difficult-to-root plants, could well explain the low rooting capacity of these plants.

It is worth noting that *p*-coumaric acid is present in all six easy-to-root plants and completely lacking in all four difficult-to-root woody plants studied by us. *p*-Coumaric acid, a strong inhibitor of cell elongation, promoted adventitious root formation in the mung bean test, both alone and in combination with IAA.[9]

Although chlorogenic acid was not detected in the unhydrolysed extracts, caffeic acid is probably derived from that ester which is sparingly soluble in ethyl ether. The role of caffeic acid or chlorogenic acid in the low rooting ability of *F. excelsior* is not clear, due to the questionable properties of these compounds as rooting stimulators. Hess,[13] contrary to Fernqvist,[9] has found caffeic and chlorogenic acids inactive, but the differences might depend upon the use of completely etiolated or light-grown mung beans.

Syringic, vanillic and *p*-hydroxybenzoic acids belong to the "woody" pattern of phenolic constituents noted by Bate-Smith.[12] Nevertheless, the latter acid proved to be a growth-promoting compound,[1] and similar effects on the *Avena* coleoptile test were observed for the two other substances, which suggests that all these acids are perhaps more than building units in lignin.

It is rather difficult to explain the presence of *trans*-cinnamic acid, a strong competitive inhibitor for indoles in growth promotion,[8] in an easy-to-root plant, although there is growing evidence for a synergism between auxin and phenolic compounds, and *trans*-cinnamic acid is a precursor of the phenolic intermediates in lignin biosynthesis.

EXPERIMENTAL

Extraction, Separation and Chromatographic Analysis

3 kg of fresh woody cuttings of both *Alnus glutinosa* and *Fraxinus excelsior*, previously frozen at −25°, were sliced and extracted with methanol at 0–2° for 24 hr. Extraction, concentration and fractionation were performed as reported earlier.[1]

Acidic and phenolic substances were separated from ether solution by successive extraction with 0·5 N Na_2CO_3 and 0·5 N NaOH.

Whatman No. 3MM paper was used for all separation and purification, and Whatman No. 1 for R_f data; descending solvents were: 2 per cent acetic acid, isopropanol/ammonia/water (10:1:1) (IAW), butanol saturated with 1 per cent ammonia, and ethanol/ammonia/water (35:2:13). TLC was on 0·02 mm silica gel plates with butanol saturated with water.

The reagents used were a 0·1 per cent ethanolic 2,6-dichloroquinonechlorimide solution (DQC) with an overspray of aqueous saturated borax solution, diazotized *p*-nitroaniline (DPNA) or diazotized sulphanilic acid (DSA), both with aqueous 20 per cent Na_2CO_3 overspray.

Hydrolysis of the Aqueous Residue

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

Bioassay

The straight growth of *Avena* coleoptile sections was used with segments cut from the chromatograms. 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.[14] This formula is different from those used previously.[1-3,5]

Ultraviolet spectra

Spectra were determined with a manual Zeiss PMQ II spectrophotometer in methanol and the shifts were obtained by adding two drops of 5 per cent KOH.

[13] C. E. Hess, *Plant Physiol.* **40** (Suppl.), 45 (1965).
[14] P. E. Pilet and J. Dubouchet, *Rev. Gen. Botan.* **69**, 545 (1962).