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GROWTH SUBSTANCES ISOLATED FROM WOODY CUTTINGS OF *SALIX VIMINALIS* L. AND *FICUS CARICA* L.*

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Abstract—A study of growth substances in cuttings of *Salix viminalis* L. and *Ficus carica* L. has been made by extraction with methanol, paper and thin layer chromatography, ultraviolet, infrared and fluoresence spectroscopy and *Avena* coleoptile straight growth test. From *S. viminalis*, *p*-hydroxybenzoic, protocatechuic, two hydroxycinnamic acids and catechol were isolated and their growth properties studied. The growth stimulating zone in the acidic biohistogram may be explained by the presence of the first two acids. In addition to the compounds characterized, syringic, vanillic and *p*-coumaric acids were isolated from the hydrolysate. From *F. carica*, syringic, vanillic, *p*-coumaric acids and umbelliferone have been isolated and identified. The relevant growth-promoting activity in the acidic biohistogram seems not to be completely due to the presence of the acids mentioned, but no IAA has been found.

INTRODUCTION

IN A systematic study on possible chemical factors involved in rooting, we are carrying out some research with cuttings of woody plants. Our results on *Ribes rubrum*,[1] *Salix atrocinerea*,[2] *Platanus orientalis*,[3] *Castanea sativa*,[4] *Quercus robur* and *Juglans regia*,[5] have been published. This paper reports on compounds in two easy to root plants: *Salix viminalis* and *Ficus carica*.

RESULTS OF UNHYDROLYSED FRACTIONS

Compounds in the Acidic Fraction of Salix viminalis

One zone of growth promoting activity (R_f 0·00–0·45) and another of erratic activity (R_f 0·45–1·00) were found in the biohistogram of the acidic fraction (Fig. 1-A).

Attempts to detect IAA in the first zone by chemical tests, u.v. absorption, fluorescence spectra and by the synergistic action of phloroglucinol, which considerably enhances the effect of IAA in the *Avena* coleoptile test, gave negative results. Protocatechuic and *p*-hydroxybenzoic (PHB) (4 mg/kg plant) acids were detected in this zone at R_f values 0·00–0·08 and 0·08–0·21 respectively, and have been isolated and purified by rechromatography

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on paper with 2 per cent acetic acid (R_f 0·39 and 0·48 respectively). Moreover, two fluorescent hydroxycinnamic acids have been detected in chromatograms developed with 2 per cent acetic acid; one (R_f 0·20) similar to isoferulic acid and another at R_f 0·80.

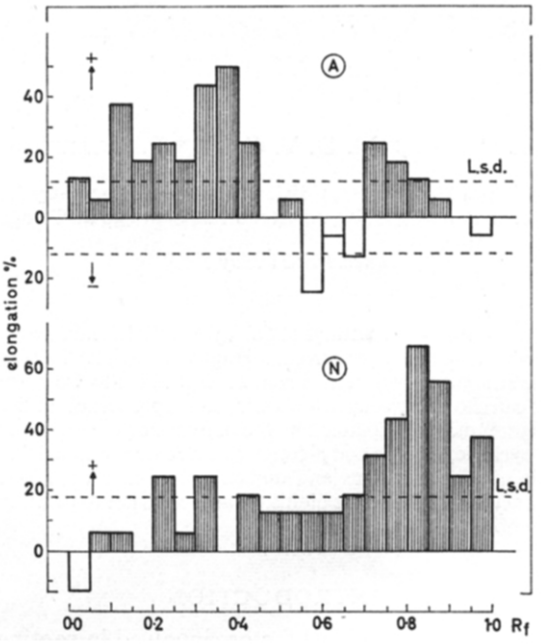

FIG. 1. BIOHISTOGRAMS (*Avena* COLEOPTILE SECTIONS TEST) OF THE ACIDIC (A) AND NEUTRAL (N) FRACTIONS OF UNHYDROLYSED EXTRACTS OF *S. viminalis*. DOTTED LINE: L.S.D. AT LEVEL 5 PER CENT.

Catechol was isolated from the second zone. The compounds were identified by chromatography, colour reactions, fluorescence and u.v. spectra.[4, 5] (Tables 1–3). Additionally, catechol, *p*-coumaric, and protocatechuic acids were methylated and the R_f values of their methyl derivatives were undistinguishable from those of authentic samples. Unknown gives green colour as catechol does when treated with solid phloroglucinol and the i.r. spectrum of an unknown was identical to that of authentic umbelliferone.

Compounds in the Neutral Fraction of S. viminalis

The neutral fraction showed a zone of a great growth-promoting activity (R_f 0·65–1·00) (Fig. 1-N). Indole compounds were not detected in this zone using chromatography or by reaction with $LiAlH_4$, alkaline (5 per cent alcoholic NaOH) or acidic (6 N HCl) hydrolysis, using Ehrlich's reagent. The active compound appears to be an ester or a nitrile, because its activity disappeared both by reduction and hydrolysis. No positive reaction was observed with 2,4-dinitrophenylhydrazine.

Compounds in the Acidic Fraction of F. carica

The biohistogram of the acid fraction (Fig. 2) showed a zone of noticeable growth promoting activity (R_f 0·25–0·45) and a zone of weak growth inhibition (R_f 0·55–0·70). From the latter, umbelliferone (6mg/kg plant) and an unidentified phenolic acid were isolated.

TABLE 1. R_f VALUES OF AUTHENTIC SAMPLES AND *S. viminalis* ELUATES IN SEVERAL SOLVENTS

Solvent	Eluate	PHB	Eluate	PCA	Eluate	Catechol	Eluate	Protocatechuic acid
Paper chromatography								
Isopropanol/ammonia/water (10:1:1)	0·19	0·19	0·24	0·24	0·71	0·71	—	—
2 per cent acetic acid	0·48	0·48	0·37	0·37	—	—	0·39	0·41
Butanol/acetic acid/water (4:1:5)	—	—	—	—	0·88	0·88	—	—
Butanol saturated with 1 per cent ammonia	0·14	0·13	0·20	0·19	0·83	0·83	—	—
Butanol saturated with water	0·52	0·51	0·52	0·51	0·85	0·85	—	—
TLC								
Butanol saturated with water	0·48	0·48	0·42	0·42	0·78	0·79	0·0–0·33	0·0–0·32
Isopropanol/ammonia/water (10:1:1)	0·40	0·42	—	—	0·67	0·67	—	—
Isobutanol/methanol/water (75:15:10)	0·48	0·52	—	—	0·88	0·87	0·44	0·44
Chloroform/acetic acid (95:5)	—	—	0·40	0·40	0·34	0·34	—	—
Acetic acid/HCl/water (30:3:10)	—	—	0·93	0·93	0·93	0·93	—	—
Benzene/methanol/acetic acid (45:8:4)	—	—	—	—	—	—	0·39	0·39

PCA = *p*-coumaric acid.

TABLE 2. R_f VALUES OF AUTHENTIC SAMPLES AND *F. carica* ELUATES IN SEVERAL SOLVENTS

Solvent	Eluate	Syringic acid	Eluate	Vanillic acid	Eluate	*p*-Coumaric acid
TLC						
Butanone/hexane (3:1)	—	—	0·44	0·41	0·52	0·48
Isobutanol/methanol/water (75:15:10)	0·49	0·55	0·53	0·52	0·51 0·58	0·50 0·59
Butanol saturated with water	0·41	0·38	0·46	0·49	—	—
Paper chromatography						
Butanol saturated with 1 per cent ammonia	0·11	0·11	0·10	0·10	0·20	0·20
2 per cent acetic acid	—	—	0·41	0·41	0·27 0·55	0·30 0·57
Isopropanol/ammonia/water (10:1:1)	—	—	0·15	0·15	0·26 0·35	0·25 0·33
Ethanol/ammonia/water (35:2:13)	0·51	0·51	0·51	0·51	—	—

TABLE 3. ULTRAVIOLET AND FLUORESCENCE SPECTRA OF PURE SPECIMENS AND COMPOUNDS ISOLATED FROM *S. viminalis* AND *F. carica* (IN METHANOL)

Substance	Fluorescence		Ultraviolet	
	Activation λ_{max} nm	Fluorescence λ_{max} nm	Neutral pH λ_{max} nm	Alkaline pH λ_{max} nm
Unknown	302	360	260; 295	275; 300
Protocatechuic acid	300	360	255; 296	276; 299
Unknown	285	315	278	240; 290
Catechol	284	315	279	239; 290
Unknown	338	395	324	231; 370
Umbelliferone	338	395	325	231; 370
Unknown	—	—	287	336
p-Coumaric acid	300	360	291	334–335

The former zone was rechromatographed in paper with 2 per cent acetic acid, yielding vanillic and syringic acids (R_f 0·10–0·22) and *p*-coumaric acid (R_f 0·25–0·30). Although the zone of highest growth activity appeared at R_f 0·50–0·65, where IAA would appear, chemical tests, synergistic bioassay with phloroglucinol, u.v. and fluorescence spectra failed to detect this compound.

RESULTS OF HYDROLYSATE FRACTIONS

Compounds in the Acidic Fraction from S. viminalis *Hydrolysate*

Although its biohistogram did not show high growth stimulation zones, bands on the chromatogram (corresponding to reactive zones of a narrow strip sprayed with DQC) were eluted with methanol, and the eluates rechromatographed. The following substances were separated and identified: PHB, protocatechuic, syringic, vanillic and *p*-coumaric (1·30 mg/kg plant) acids and catechol (10 mg/kg plant). A further product (R_f 0·38–0·43) was also isolated, which appears to be *m*-hydroxybenzoic acid (MHB).

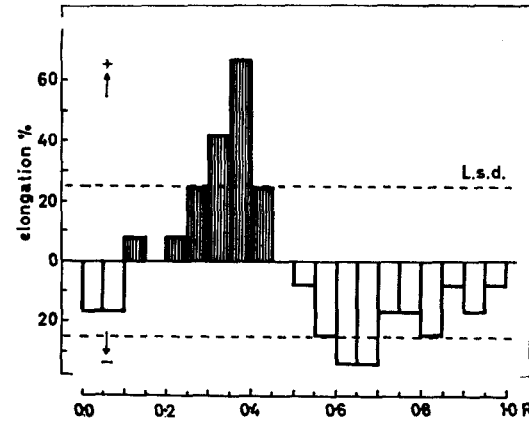

FIG. 2. BIOHISTOGRAM (*Avena* COLEOPTILE SECTIONS TEST) OF THE ACIDIC FRACTION OF *F. carica* EXTRACTS. DOTTED LINE: L.S.D. AT LEVEL 5 PER CENT.

Compounds in the Phenolic and Neutral Fractions from S. viminalis *Hydrolysate*

The biohistograms of these fractions were not of great interest. From the phenolic fraction catechol was separated and identified. From neutral fraction pink spots were obtained with Ehrlich's reagent at R_f 0·25, 0·51, 0·89 and 0·95. Although not characterized, they were shown not to be tryptamine, dimethyltryptamine, indoleacetonitrile, gramine and indolealdehyde.

Growth Activity of the Isolated Compounds

Growth stimulation and inhibition of the isolated substances was studied using either their eluate from the plant extract chromatograms or authentic compounds. The results obtained from both sources were in good agreement.

Protocatechuic acid. Tests have been carried out with concentrations from 0·1 to 200 μg/ml. Growth stimulation starts at 50 μg/ml, reaches a maximum at 150 μg/ml, and rapidly decreases to become toxic at 200 μg/ml (Fig. 3).

Mixture is of IAA and protocatechuic acid. Protocatechuic acid has also been tested in presence of IAA. Protocatechuic acid had a positive synergistic action from 10 to 100 μg/ml, with a maximum at about 25–50 μg/ml, in presence of 0·025 μg/ml IAA, but showed no synergism at 150 μg/ml. In presence of 0·1 μg/ml, a lower synergistic action was observed between 10 and 80 μg/ml, and an antagonism was shown at and above 100 μg/ml. In presence of 1 μg/ml of IAA, an antagonism which increased with the concentration was observed at and above 50 μg/ml.

Catechol. Experiments were carried out with concentrations from 0·1 to 1·500 μg/ml. Catechol had no action at concentrations lower than 500 μg/ml; but appeared toxic and a growth inhibitor at higher concentrations. The effect of IAA in combination with concentrations of catechol lower than 200 μg/ml remained unchanged. Toxic and inhibiting effects became apparent for the three IAA concentrations with higher amounts of catechol.

Umbelliferone. Neither elongation stimulation nor toxic effects were noted over the range 0·1 to 1500 μg/ml. At 50 and 100 μg/ml umbelliferone appeared to be a root inhibitor but at 25 μg/ml showed no effect on rooting tests with *Phaseolus aureus*. Neither synergism nor antagonism were observed on the elongation caused by 0·025 or 1 μg/ml IAA. In the rooting bean test, the rooting initiation provoked by 5 μg/ml IAA, was reduced by 100 μg/ml umbelliferone.

Other phenolic acids. Growth activity of other phenolic acids, like PHB, vanillic, syringic and *p*-coumaric acids have been reported earlier.[1–5]

DISCUSSION

As has been reported above, biohistograms of the acidic chromatograms of the unhydrolysed extract of both plants have shown a zone of growth stimulation. None of these however had the shape and intensity of the same zone of *Salix atrocinerea*.

No IAA was found in either of the plants studied. The presence of protocatechuic and PHB acids may allow for the growth promoting zones of *S. viminalis*. However the growth properties of syringic, vanillic and *p*-coumaric acids do not explain completely the growth promoting activity of *F. carica*, and its active zones in paper chromatography with 2 per cent acetic acid have shown a prominent intensity at the expected R_f value of IAA although this compound could not be detected.

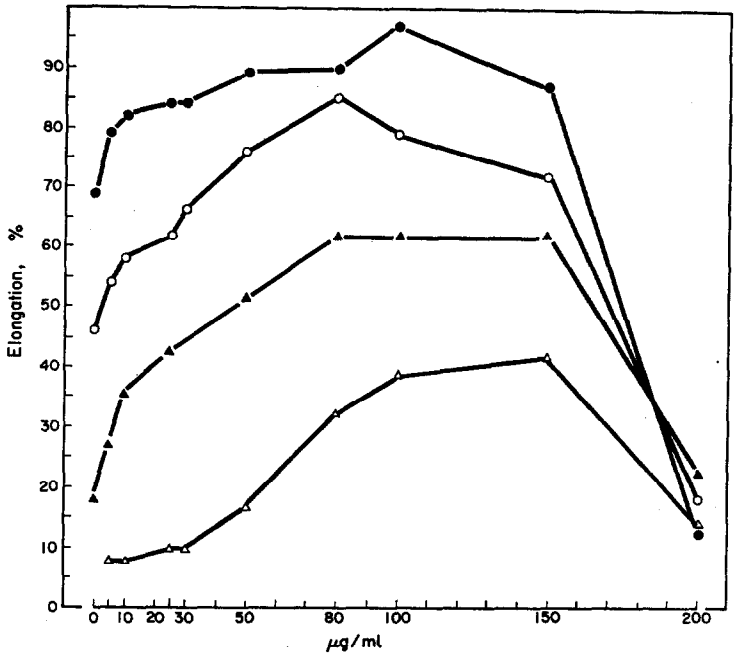

FIG. 3. BIOLOGICAL ACTIVITY (*Avena* COLEOPTILE SECTION TEST) OF PURE PROTOCATECHUIC ACID WITH AND WITHOUT IAA. L.S.D.: 15 PER CENT AT LEVEL 5 PER CENT. (△——△) NO IAA. (▲——▲) 0·025 μG/ML IAA. (○——○) 0·1 μG/ML IAA. (●——●) 1 μG/ML IAA.

Compounds isolated from the hydrolysates are consistent with the shape of the corresponding biological histograms and the bioassay tests of their eluates, but no explanation was found for the great growth promoting activity of neutral chromatogram of *S. viminalis*.

Salicylic acid was not detected in cuttings of *S. viminalis*, but special attention was given to searching for gentisic acid in the hydrolysate, since Zenk[6] mentioned that up to 30 per cent of salicylic acid fed to young leaves of *S. viminalis* was incorporated as gentisic acid glucosides. No positive results were however obtained.

Hess[7] found catechol as promoter of adventitious root formation in the 'light' mung bean test in presence of IAA, a result which was not confirmed by Fernqvist[8] with etiolated beans in an analogous test. However, the presence of catechol in the Salicaceae[9], might be connected with the rooting ability of *Salix* spp., although Vieitez *et al.*[2] did not detect catechol in *S. atrocinerea*, another very easy to root *Salix* sp. Contrary to the findings of Tomaszewski,[9] catechol was present in a combined form. However, Tomaszewski studied only one *Salix* species and the variation could be assigned to differences in species.

EXPERIMENTAL

Extraction, separation and chromatographic analysis. 1·6 kg of fresh *Ficus carica* L. woody cuttings and 4·0 kg of those of *Salix viminalis* L., previously frozen at −25°, 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]

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[7] C. E. HESS, *Proc. 16th Internat. Hort. Congr.* **4**, 382 (1964).
[8] I. FERNQVIST, *Lantbrukshögsk. Ann.* **32**, 109 (1966).
[9] M. TOMASZEWSKI, *Bull. Acad. Polon. Sci.* **8**, 61 (1960).

Acidic, phenolic and neutral substances were separated from ether solution by successive extraction with aqueous solutions of 0·5 N Na_2CO_3 and 0·5 N NaOH. Alkaline substances contained in the early methanolic aqueous extract, 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 the ether soluble compounds, promoted the growth of *Avena* coleoptiles and was hydrolysed with 0·5 N $Ba(OH)_2$ at 1 atm and the hydrolysate was fractionate as above.

Bioassay. The straight growth of *Avena* coleoptile sections was used. Percentages of elongation in the biohistograms were calculated as before.[4,5]

Paper chromatography. Unless otherwise specified, isopropanol:ammonia:water (10:1:1; v/v) (IAW) on Whatman paper No. 1 was used as descending solvent. The reagents used were a 0·1 per cent ethanolic 2,6-dichloroquinone chlorimide solution (DQC) with aqueous saturated borax solution overspray, and diazotized *p*-nitroaniline (DPNA) with aqueous 20 per cent Na_2CO_3 overspray for phenols. Indole compounds were detected with Ehrlich reagent.

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 5μg/ml or 1 μg/ml in neutral methanol.