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The phytotoxicity of some phenolic metabolic products of Ophiostoma ulmi to Ulmus sp.

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Abstract

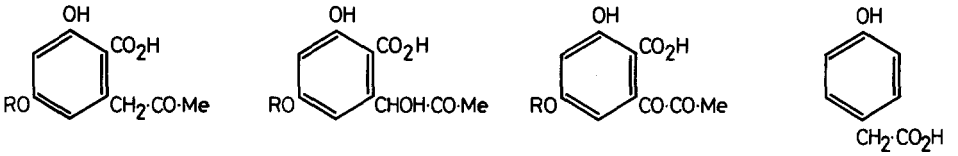
The c_{10} phenolic acids, 2,4-dihydroxy-6-acetonylbenzoic acid (1) and its 6-(1-hydroxyacetonyl) (2) and 6-pyruvyl (3) analogues, metabolic products of *O. ulmi* on a chemically defined medium are also produced on elm tissue medium but are rapidly metabolised once the carbon source is exhausted. The phytotoxicity to cut shoots of *U. glabra* and *U. hollandica* of these acids and their 4-*O*-methyl ethers (4–6 respectively) and of 4-hydroxyphenylacetic acid (7) has been investigated. None of the C_{10} acids consistently caused wilting or leaf necrosis in *U. glabra* but all three were toxic to *U. hollandica*. Diaporthic acid (4) caused severe leaf necrosis in both *Ulmus* sp. and the phenolic acid (7) caused wilting and necrosis of *U. glabra* shoots. Attempts to reproduce these effects in rooted *U. glabra* saplings were unsuccessful. Autoradiography of *U. glabra* shoots treated with [^{14}C]-labelled acids (2) and (4) showed a uniform distribution of radioactivity in the tissues, but the labelled acids could not be recovered. There is no evidence that the low molecular weight metabolic products of *O. ulmi* are toxic to rooted saplings or proof that they are formed in vivo.

Additional keywords: *Ulmus glabra*, *U. hollandica*, diaporthic acid, [^{14}C]-C_{10} phenolic acids, 4-hydroxyphenylacetic acid, phytotoxicity.

Introduction

Although wilting of the foliage, resulting from decreased water movement, is the principal symptom of Dutch elm disease, Dimond (1947) reported the occurrence of necrotic lesions of the leaf tissue of *Ulmus americana*. It is generally agreed that high molecular weight glycoproteins produced by the causative fungus *Ophiostoma ulmi* (Buisman) Nannf. (*Ceratocystis ulmi* (Buisman) C. Moreau) are able to produce wilting (Salemink et al., 1965; Rebel, 1969; Van Alfen and Turner, 1975; Strobel et al., 1978); but the glycoproteins do not cause leaf necrosis. Low molecular weight metabolites, the C_{10} phenolic acids (Fig. 1, 1–3), are also produced by *O. ulmi* (Claydon et al., 1974) and are structurally related to known phytotoxins (Hardegger et al., 1966) containing the isocoumarin ring system. The keto acid (1) is a metabolic product of some other plant pathogens and shows moderate phytotoxicity (Iwasaki et al., 1973; Kameda et al., 1973) in a number of bioassays. In the present study the toxicity of the C_{10} phenolic acids, and some analogues, towards cut shoots and rooted saplings of elm has been investigated. 4-Hydroxyphenylacetic acid (7), a metabolic

Fig. 1. Metabolic products of *O. ulmi*. Structures of the phenolic acids and some derivatives.



1; R=H 2; R=H 3; R=H 7

4; R=Me 5; R=Me 6; R=Me

Fig. 1. Metabolische produkten van O. ulmi. Structuren van methylfenolzuren en enige derivaten.

product of some strains of *O. ulmi* (Grove, unpublished) was also examined. This acid, produced by several plant pathogens, is known to be phytotoxic (Aoki et al., 1963).

Materials and methods

Plant material. Nursery-grown callus cuttings (Elgersma, 1969) of *U. hollandica* cl. Belgica were used in Holland and young shoots cut from *U. glabra* saplings in May-June were used in the UK.
O. ulmi culture. An aggressive strain, UICP 78, equivalent in its properties to strain 44 (Claydon et al., 1974) was used.

Chemical compounds. The C_{10} phenolic acids and their analogues were obtained by fermentation (Claydon et al., 1974) or by synthesis (Grove and Pople, 1979). 4-Hydroxyphenylacetic acid, m.p. 148 °C was purchased from Koch-Light Ltd. The crude glycoprotein fraction was prepared according to Salemink et al. (1965). The light petroleum had a b.p. of 60–80 °C.

Natural media. Leaves or defoliated twigs (100 g fresh wt. each) of *U. glabra* were macerated separately and the macerates were made up to 1 litre with water. Aliquots of 250 ml were transferred to conical flasks (1 litre) and heated in the autoclave for 10 min at 121 °C.

Fermentation and analysis for phenolic metabolic products. The flasks were inoculated with a spore suspension of the *O. ulmi* culture and incubated at 25 °C (Claydon et al., 1974). Aliquots of 10 ml of the culture filtrate were removed at intervals for the spectrophotometric and thin layer chromatography (t.l.c.) assays (see below). In this work the culture filtrate was first extracted with chloroform at the natural pH and then, after acidification to pH 2, with ethyl acetate. The yield of the C_{10} phenolic acids was calculated, as before, from the absorbance of the acid fraction at 302 nm under standard conditions. Thin layer chromatography of the acid fraction was carried out on silica gel in the diisopropyl ether-formic acid-water system (Claydon et al., 1974).

Phytotoxicity tests. A. *Cut shoots.* The method described by Elgersma and Overeem (1971) was used with *U. hollandica*. With *U. glabra*, shoots were added to tubes containing the test solution (10 ml) and placed close to a north-facing window at room temperature.

B. *Rooted saplings.* Four year old *U. glabra* saplings, 1.5–2.0 m in height and 2.5–5.0 cm diam., were treated with solutions of the compounds by the cone method (Elgersma and Overeem, 1971) or by pressure injection using a smaller version of the lance described by Clifford et al., (1976). The solution was injected at 345 newtons m^{-2} using two lances clamped on opposite sides of the sapling at ground level.

[^{14}C]-C_{10} *phenolic acids. O. ulmi* was grown in surface culture on a chemically defined medium (Claydon et al., 1974) and after 9 days diethyl [2-^{14}C]-malonate 100 μCi, 18.8 μCi/mg) in ethanol (1 ml) was distributed equally between four flasks. Periodic t.l.c. analysis, using a Berthold gas-flow plate scanner showed that radioactivity was accumulating in spots with R_f 0.52 and 0.33. After 20 days the fermentation was harvested yielding a brown gummy acid fraction (62 mg) which was purified by preparative t.l.c. Semi-solid material (3 mg) from a band with R_f 0.31 was diluted with unlabelled ketol-acid (2) (8 mg) and recrystallised from ethyl acetate to constant radioactivity (Packard 3375 liquid scintillation counter) giving the [^{14}C]-ketol-acid (8 mg). Likewise, semi-solid material (3 mg) from a band with R_f 0.50 was diluted with unlabelled ketone-acid (1) (10 mg) and recrystallised from ethyl acetate-light petroleum to constant radioactivity giving the [^{14}C]-ketone-acid (5 mg). Methylation with diazomethane followed by hydrolysis of the ester group gave [^{14}C]-diaporthic acid (4) (2.5 mg), R_f 0.55.

Autoradiography. Two *U. glabra* shoots were treated with a neutralized solution (5 ml) of the [^{14}C]-ketol-acid (8 mg) in water (10 ml) during 2 days. Uptakes were 3.7 and 3.0 ml compared with the water control (3.0 ml). The shoots were pressed between filter paper and dried for 24 h at 55 °C. They were then laid on Kodak DF 54 X-ray film (25 × 30 cm) and left in the dark for 10 weeks.

A similar experiment was carried out with a neutralized solution of [^{14}C]-diaporthic acid (4) (2.5 mg) in water (5 ml). Uptakes were 1.9 and 2.1 ml.

Attempted recovery of the [^{14}C]*-ketol-acid (2) and* [^{14}C]*-diaporthic acid (4) from leaf tissue.* The dried leaves from the autoradiography experiments were cut up and powdered in a mortar, and the powders were extracted in a Soxhlet apparatus with light petroleum followed by ethyl acetate. The residues were macerated in a top-drive homogenizer with sodium hydrogen carbonate (2.5 ml) and water (2.5 ml), filtered, and the filtrates were acidified to pH 3 with concentrated hydrochloric acid and extracted with ethyl acetate. Thin layer chromatography of the solvent extracts showed no material or radioactivity at R_f 0.31 or 0.55, respectively, and all the extractable radioactivity remained close to the origin (R_f 0.01).

Results and discussion

The purpose of this investigation was to examine the status of the C_{10} phenolic acids and possible vivotoxins. The C_{10} acids were isolated from *O. ulmi* growing on a chemically defined medium (Claydon et al., 1974). It was necessary, first, to show that these compounds could be produced on a natural medium, derived only from elm tissue, without added nutrients, trace elements or accessory factors. Both leaves and defoliated twigs were used (Table 1). Compared with the chemically defined medium, the characteristic C_{10} phenolic acid chromophore (λ max 265 300 nm), accompanied by the appropriate t.l.c. spots at R_f 0.67, 0.52 and 0.31 appeared more rapidly on the elm

Table 1. Course of *O. ulmi* fermentation on elm tissue media: change with time in the pH of the medium and the yield of C_{10} phenolic acids.

		Days			
		0	6	10	13
Leaf medium	pH	5.3	5.3	5.6	7.3
	C_{10} acids (mg/ml)	0	0	13.1	0
Twig medium	pH	5.7	7.5	7.5	7.3
	C_{10} acids (mg/ml)	0	12.4	3.2	0

Tabel 1. Verloop van de fermentaties door O. ulmi in media met iepeweefsel; verandering van de pH van het medium en de opbrengst van C_{10} fenolzuren in de tijd.

tissue media. However, the pH rose an indication that the carbon source was exhausted, the C_{10} acids were rapidly metabolized and disappeared altogether. This encouraging result, whilst not inconsistent with the production of the C_{10} acids in vivo, does not of course constitute a proof of their formation in infected trees.

The phenolic metabolites were tested as their sodium salts against *U. glabra* shoots in the UK. (Tables 2 and 3) and against *U. hollandica* sprouts in Holland (Tables 4 and 5). Initially (Tables 2 and 4) the major C_{10} metabolite, the ketol-acid (2), was compared with the crude glycoprotein fraction from the *O. ulmi* fermentation (Salemink et al., 1965). This fraction induced wilting in shoots of both elm species. Although the ketol-

Table 2. Toxicity to *U. glabra* shoots of the ketol-acid (2) and diaporthic acid (4), as their sodium salts, compared with the crude glycoprotein fraction (GlyP), all at 1000 ppm. Variation with time in the number of shoots showing symptoms (10 shoots used in each test). Symptoms were grouped into two categories: (N) necroses i.e. shoots with necrotic spots or necrotised leaves; and (W) wilted i.e. shoots showing incipient wilt or totally wilted leaves.

Compound	Time of treatment (hours)			
	16		42	
	N	W	N	W
2	0	0	2	2
4	8	0	8	2
GlyP	0	6	0	6

Tabel 2. Toxiciteit voor U. glabra spruiten van het ketolzuur (2) en diaporthinezuur (4) als natriumzouten, vergeleken met de ruwe glycoproteinfractie (GlyP), alle bij 1000 ppm. Verandering in het aantal spruiten met symptomen in de tijd (per toets werden 10 spruiten gebruikt). Symptomen werden gegroepeerd in twee categorieën: (N) necrosen, d.w.z. spruiten met necrotische vlekken of genecrotiseerde bladeren en (W) verwelkt, d.w.z. spruiten vertonen slap hangende of totaal verwelkte bladeren.

Table 3. Toxicity to *U. glabra* shoots of the keto-acid (1) compared with the ketol-acid (2), the diketo-acid (3), diaporthic acid (4) and 4-hydroxyphenylacetic acid (7), as sodium salts, and the crude glycoprotein fraction (GlyP), all at 1000 ppm. Variation with time in the number of shoots showing symptoms (10 shoots were used in each test). Symptoms are grouped as described for Table 2.

Compound	Time of treatment (hours)			
	42		66	
	N	W	N	W
1	0	0	0	0
2	0	0	0	0
3	0	0	0	0
4	5	4	6	5
7	1	7	2	8
Glyp	1	7	2	10

Tabel 3. Toxiciteit voor U. glabra spruiten van het ketozuur (1) vergeleken met het ketolzuur (2), het diketozuur (3), het diaporthinezuur (4) en 4-hydroxyphenylazijnzuur (7) als natriumzouten en de ruwe glycoproteine fractie (GlyP), alle bij 1000 ppm. Verandering in aantal spruiten met symptomen in de tijd (per toets werden 10 spruiten gebruikt). Symptomen werden gegroepeerd als beschreven voor Tabel 2.

acid had little effect on *U. glabra*, extensive leaf necrosis without wilting, was seen with *U. hollandica*. However, the 4-*O*-methyl ether of the keto-acid, diaporthic acid (4), a degradation product of the isocoumarin diaporthin, the phytotoxin of *Endothia parasitica* (Hardegger et al., 1966) caused both wilting and leaf necrosis of *U. glabra*.

These tests were therefore extended to include the minor C_{10} metabolites (1) and (3) (Tables 3 and 5) and their 4-*O*-methyl ethers (5) and (6) (Grove and Pople, 1979) (Table

Tabel 4. Toxicity to *U. hollandica* sprouts of the ketol-acid (2) as the sodium salt (1000 ppm) compared with the crude glycoprotein fraction (GlyP) (300 ppm). Variation with time in the number of shoots showing symptoms (20 shoots were used in each test). Symptoms are grouped as described for Table 2.

Compound	Time of treatment (hours)			
	26		42	
	N	W	N	W
2	0	0	13	0
GlyP	0	15	0	15

Tabel 4. Toxiciteit voor U. hollandica spruiten van het ketolzuur (2) als natriumzout (1000 ppm) vergeleken met de ruwe glycoproteine fractie (GlyP) (300 ppm). Verandering in het aantal spruiten met symptomen in de tijd (per toets werden 20 spruiten gebruikt). Symptomen werden gegroepeerd zoals beschreven in Tabel 2.

Table 5. Toxicity to *U. hollandica* sprouts of the keto-acid (1) compound with the ketol-acid (2), the diketo-acid (3) and diaporthic acid (4) (all at 500 and 1000 ppm as sodium salts). Variations with time in the number of shoots showing symptoms (10 shoots were used in each test). Symptoms are grouped as described for Table 2.

Compound	Time of treatment (hours)							
	24				41			
	500 ppm		1000 ppm		500 ppm		1000 ppm	
	N	W	N	W	N	W	N	W
1	0	4	2	6	2	8	6	5
2	0	1	0	0	4	0	10	2
3	8*	1	8*	0	7*	2	8*	4
4	9	0	10	0	10	0	10	0

*Instead of leaf necrosis, the diketo-acid (3) produced a dark discolouration of the stem and veins.

Tabel 5. Toxiciteit voor U. hollandica spruiten van het ketonzuur (1), het ketolzuur (2), het diketozuur (3) en diaporthinezuur (4) (alle als natriumzout getoets bij 500 en 1000 ppm). Verandering in het aantal spruiten met symptomen in de tijd (per toets werden 10 spruiten gebruikt). Symptomen werden gegroepeerd zoals beschreven in Tabel 2.

6). Whilst non of the C_{10} acids (1–3) was toxic to *U. glabra*, all affected *U. hollandica* which appeared to be more susceptible; the keto-acid (1) caused wilting as well as leaf necrosis and the diketo-acid (3) discolouration of the stems and veins. The earlier result with diaporthic acid (4) against *U. glabra* was confirmed, but this compound caused no wilting of *U. hollandica* sprouts, although extensive leaf necrosis was observed. The methyl ethers (5) and (6) were tested only on *U. glabra*, against which they were ineffective at a concentration where diaporthic acid (4) produced significant wilting and necrosis. The more polar and more chemically-reactive ethers (5) and (6) thus seem to be the least phytotoxic. Diaporthic acid (4) showed the greatest toxicity to shoots of *Ulmus* sp., but there is no evidence that this compound is a metabolic product of *O. ulmi*. 4-Hydroxyphenylacetic acid (7) was tested only on *U. glabra*, causing significant wilting and some necrosis. All efforts to reproduce these effects in rooted *U. glabra* saplings (Table 7), using both the cone method (Elgersma and Overeem, 1971) and pressure injection (Clifford et al., 1976), were unsuccessful. No wilting or leaf necrosis was observed, but staining of the sapwood in twigs well removed from the point of uptake was sometimes seen.

In an attempt to show whether the C_{10} acids were taken up by cut shoots and translocated to the leaves, unchanged, [^{14}C]-labelled specimens were prepared. By inspection, the C_{10} acids appear to be simple pentaketides and to arise by the acetate-polymalonate biosynthetic pathway (Fig. 2). Addition of diethyl [2-^{14}C]-malonate to an *O. ulmi* fermentation (Claydon et al., 1974) at the point of maximum rate of production of the C_{10} acids led to the isolation of [^{14}C]-ketol-acid (2) and [^{14}C]-keto-

Table 6. Toxicity to *U. glabra* shoots of diaporthic acid (4) compared with the ketol (5) and diketone (6) analogues, as sodium salts (all at 1000 ppm). Variation with time in the number of shoots showing symptoms (10 shoots were used in each test). Symptoms are grouped as described for Table 2.

Compound	Time of treatment (hours)			
	22		46	
	N	W	N	W
4	4	2	4	5
5	0	1	0	0
6	0	0	0	1

Tabel 6. Toxiciteit voor U. glabra spruiten van diaporthinezuur (4) vergeleken met het ketol (5) en diketon (6) analogen als natriumzout (alle bij 1000 ppm). Verandering in het aantal spruiten met symptomen in de tijd (per toets werden 10 spruiten gebruikt). Symptomen werden gegroepeerd zoals beschreven in Tabel 2.

Fig. 2. Theoretical labelling pattern of the pentaketide skeleton (1–3) by [2-^{14}C]-malonate. * Indicates labelled atom.

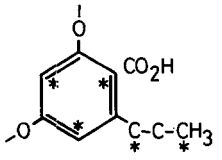


*Fig. 2. Theoretisch labelling patroon van het pentaketide skelet (1–3) door [2-^{14}C]-malonate. * Geeft gelabled atoom aan.*

Table 7. Toxicity to *U. glabra* saplings of *O. ulmi* phenolic metabolic products administered using the cone method (C) or by pressure injection (P).

Compound	Method	Uptake (mg)	Toxicity
1	C	25	–
2	P	400	–
3	C	50	–
4	P	150	–
7	P	133	–

Tabel 7. Toxiciteit voor U. glabra zaailingen van fenolachtige metabolische produkten gevormd door O. ulmi, toegediend via de trechtermethode (C) of de methode van injectie (P) d.m.v. druk.

acid (1). The latter was converted (Grove and Pople, 1979) to [^{14}C]-diaporthic acid (4). When *U. glabra* shoots were placed in solutions of the labelled compounds (2) and (4), autoradiography showed that radioactivity from (2) was distributed uniformally in both the stem and in the leaf tissue, but that the radioactivity from (4) was more concentrated in the stem. However, neither compound could be recovered by extraction of the dried leaf tissue and the radiolabel was concentrated in a more polar fraction. It seems probable therefore that both compounds were either bound or degraded in the shoot, and that the development of leaf necrosis in treated elm shoots is not necessarily due to the unchanged phenolic acid.

Although we have shown that the low molecular weight phenolic metabolic products of *O. ulmi* produce leaf necrosis in cut shoots of *Ulmus* sp. at high concentration, there is no evidence that they are toxic to rooted saplings or proof that they are formed in vivo. The effects observed by Dimond (1947) remain unexplained.

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Samenvatting

De fytotoxiciteit van enige fenolische metabolieten van Ophiostoma ulmi voor Ulmus sp.

De C_{10} fenolzuren, 2,4-dihydroxy-6-acetonylbenzoëzuur (1) en haar 6-(1-hydroxy acetonyl) (2) en 6-pyruvyl (3) analogen, metabolische produkten van *O. ulmi* in synthetisch medium, worden ook in medium bereid met iepeweefsel gevormd, maar worden snel omgezet als de koolstofbron uitgeput is. De fytotoxiciteit van deze zuren en hun 4-*O*-methylethers (4–6 respektievelijk) en van 4-hydroxyphenylazijnzuur (7) voor afgesneden scheuten van *U. glabra* en *U. hollandica* werd onderzocht. Geen van de C_{10} zuren veroorzaakten constant verwelking of bladnecrosen in *U. glabra*, maar alle drie waren toxisch voor *U. hollandica*. Diaporthinezuur (4) veroorzaakte ernstige bladnecrosen in beide *Ulmus* soorten en het fenolzuur (7) veroorzaakte in beide verwelking en necrosen van *U. glabra* scheuten. Pogingen om deze effecten in jonge *U. glabra* bomen te reproduceren mislukten. Autoradiografie van *U. glabra* scheuten behandeld met [^{14}C]-gemerkte zuren (2) en (4) vertoonde een uniforme verspreiding van radioactiviteit in de weefsels, maar de gemerkte zuren konden niet teruggewonnen worden.

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