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6,7-DIMETHOXYCOUMARIN, A CITRUS PHYTOALEXIN CONFERRING RESISTANCE AGAINST *PHYTOPHTHORA* GUMMOSIS

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Key Word Index—*Citrus* spp.; *Poncirus trifoliata*; Rutaceae; *Phytophthora citrophthora*; Pythiaceae; phytoalexin; resistance; coumarin; scoparone; esculetin dimethyl ether.

Abstract—6,7-Dimethoxycoumarin was isolated from the bark of citrus trunks, branches and fruit peels following inoculation with the fungus *Phytophthora citrophthora*. The compound inhibited growth *in vitro* of *Phytophthora citrophthora*, *Verticillium dahliae*, *Penicillium digitatum*, *P. italicum*, *Colletotrichum gloeosporioides*, *Diplodia natalensis* and *Hendersonula toruloidea*.

INTRODUCTION

Phytoalexins are produced by plants as a defense mechanism in response to microbial infection [1]. They are also produced following chemical (herbicide, fungicide) and physical (UV, heat, wounding) treatments [2, 3]. Two fungitoxic compounds were found by Hartmann and Nienhaus in the bark of *Citrus limon* after infection with *P. citrophthora*. One substance was isolated and identified as xanthoxylin. No xanthoxylin could be demonstrated in healthy bark. There was no correlation between the intensity of resistance of the trees for *Phytophthora* infection and accumulation of xanthoxylin [4]. Preliminary experiments indicated that a fluorescent fungitoxic compound was induced in citrus bark following inoculation with the fungus *Phytophthora citrophthora*, which causes *Phytophthora* gummosis (collar rot) and brown rot disease [5]. We report herein that 6,7-dimethoxycoumarin is the fluorescent compound (phytoalexin) responsible for the defense mechanism against *P. citrophthora* in citrus.

RESULTS AND DISCUSSION

Citrus species resistant and susceptible to *P. citrophthora* were compared for production of the fluorescent fungitoxic compound in the bark, following inoculation with this pathogen. This compound was induced in both groups of citrus, but the concentration was higher and increased more rapidly in the resistant species within 24 hr after inoculation. A maximum concentration of 440 μg/g fr. wt was reached after 4 days. In the susceptible species the maximum concentration was only 31.5 μg/g fr. wt, likewise reached 4 days after inoculation. *In vivo* the advance of the pathogen (lesion size) 4 days post-inoculation was 2–4 mm in the resistant species, as compared to over 10 mm in the susceptible ones (Table 1).

Table 1. Accumulation of 6,7-dimethoxycoumarin (μg/g fr. wt) and length of lesion (mm) in the bark of four citrus species, resistant and susceptible, following infection by *Phytophthora citrophthora* and incubation at 20°

Species \ Time	Control*	Day 1		Day 2		Day 3		Day 4	
	(μg/g fr. wt)	(μg/g fr. wt)	lesion length mm	(μg/g fr. wt)	lesion length (mm)	(μg/g fr. wt)	lesion length (mm)	(μg/g fr. wt)	lesion length (mm)
C. macrophylla (resistant)	17.5a†	44.8a	1.2a	148.2a	2.5a	300.0a	2.5a	440.0a	2.5a
C. aurantium (resistant)	17.2a	28.8b	1.4a	89.8b	3.5a	170.3b	4.5b	250.0b	5.0b
C. lemon (susceptible)	15.3a	13.5c	2.0a	25.0c	5.0b	31.5c	8.0c	41.6c	11.0c
C. sinensis (susceptible)	14.5a	12.9c	2.0a	22.3c	6.0b	26.9c	11.0d	31.1d	15.5d

*The control is healthy tissue, checked on days 1, 2, 3 and 4; five replicates each day. Mechanical injury had no effect on phytoalexin concentration.

†Each number is an average of five replicates. Figures followed by the same letter in each column are not significantly different ($p = 0.05$).

Bioassays with the compound inhibited the growth of various phytopathogenic fungi (see Experimental) *in vitro*. The ED_{50} of 6,7-dimethoxycoumarine for *Phytophthora citrophthora* was 97 ppm, as compared with those of the fungicides: fosetyl-Al (55 ppm), H_3PO_3 (7 ppm), metalaxyl (0.25 ppm). The ED_{50} of 6,7-dimethoxycoumarin for *P. citrophthora* and six additional species of phytopathogenic fungi is presented in Table 2.

Two other related compounds were detected by ^{1}H NMR and ^{13}C NMR: their toxicity was checked against *P. citrophthora* and found to be nil; their chemical structure remains unknown. 6,7-Dimethoxycoumarin was extracted from peels of sweet orange, grapefruit, sour orange, lemon, trifoliate orange and Troyer citrange fruit, following inoculation with the pathogen. UV spectrophotometry, IR analysis, ^{1}H NMR and ^{13}C NMR spectroscopy proved that the compound is 6,7-dimethoxycoumarin (scoparone). This compound had been extracted from grapefruit peels 7 days after gamma irradiation with 300 Krads [6], but nothing had been published about its possible antifungal activity. This is the first report showing that scoparone is a phytoalexin in citrus conferring resistance against *Phytophthora* gummosis disease.

Table 2. Effective dose of 6,7-dimethoxycoumarin for 50% inhibition (ED_{50}) of mycelial growth of *Phytophthora citrophthora* compared with conidial germination inhibition of six other phytopathogenic fungi, *in vitro*

Fungal species	ED_{50} of 6,7-dimethoxycoumarin (ppm)
Phytophthora citrophthora	97
Verticillium dahliae	61
Penicillium digitatum	64
Penicillium italicum	60
Colletotrichum gloeosporioides	54
Hendersonula toruloidea	90
Diplodia natalensis	85

EXPERIMENTAL

Plant material. Citrus seedlings: *Citrus sinensis*, sweet orange; *C. paradisi*, grapefruit; *C. aurantium*, sour orange; *C. limon*, lemon; *Poncirus trifoliata*, trifoliate orange; *C. macrophylla*, alemow and Troyer citrange; 3-year-old plants were grown in a greenhouse at 24° and outdoors. Fruits were collected from commercial groves. The following species of fungi: *Phytophthora citrophthora*, *Verticillium dahliae*, *Penicillium digitatum*, *P. italicum*, *Colletotrichum gloeosporioides*, *Hendersonula toruloidea* and *Diplodia natalensis* were cultured for inoculum preparation on potato dextrose agar (PDA) medium at 25°.

Extraction. Inoculated bark (80 g) was extracted with H_2O (800 ml) for 2 hr at 40°. Partition between the H_2O and EtOAc components showed that the antifungal component was in the EtOAc fraction. The eluted crude material (36 mg) following solvent evaporation was chromatographed on a silica gel-H column (2 × 15 cm) eluted with increasing percentage of EtOAc in petrol. The active compound, eluted with EtOAc–petrol (1:1, 13 mg), gave a single spot in TLC (silica, toluene–EtOAc, 1:1, UV detection) and crystallized from $CHCl_3$ as colourless needles, mp 146–147° [7]. CIMS *m/z* 207 $[M+1]^+$; UV λ_{max}^{MeOH} nm: 229, 292 and 341; IR ν_{max}^{KBr} cm^{-1}: 3400, 2970, 1725, 1630, 1560, 1500, 1420, 1280, 1140, 1000; ^{1}H NMR (360 MHz, $CDCl_3$): δ6.29 (*d*, J = 9.5 Hz, H-3), 7.62 (*d*, J = 9.5 Hz, H-4), 6.86 (*s*, H-5), 6.84 (*s*, H-8), 3.95 (*s*, 6-OMe), 3.92 (*s*, 7-OMe); ^{13}C NMR (90.5 MHz, $CDCl_3$): δ161.2(*s*, C-2), 108.4 (*d*, C-3), 141(*d*, C-4), 113.6 (*d*, C-5), 146.5 (*s*, C-6), 153.1 (*s*, C-7), 100.2 (*d*, C-8), 150.1 (*s* C-9), 111.5 (*s*, C-10) [8].

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