# Accumulation of Scoparone, a Phytoalexin Associated with Resistance of Citrus to *Phytophthora citrophthora*

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**ABSTRACT**

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Citrus species resistant and susceptible to *Phytophthora citrophthora* were compared for production of scoparone in the bark 1–8 days after inoculation with the pathogen. Concentrations of scoparone were higher (maximum 440 $\mu$g/g fr wt after 4 days) and increased more rapidly in the resistant species within 24 hr after inoculation. In the susceptible species the maximum concentration was 41.6 $\mu$g/g fr wt. The lesion length caused by *P. citrophthora* in citrus bark 4 days after inoculation was 2.5–5.0 mm in the resistant, compared with more than 11 mm in the susceptible. Scoparone inhibited mycelial growth of *P. citrophthora* and spore germination of six other phytopathogenic fungi in vitro. Radioactivity was incorporated into scoparone in infected citrus bark that had been treated with $^{14}$C-phenylalanine. Resistant citrus species, treated with the inhibitor aminooxyacetic acid (AOA) before inoculation, became susceptible to *P. citrophthora*.

Phytoalexins are produced by plants as a defense mechanism in response to microbial infection (4,14). They are also produced after chemical (15,19,20) and physical (23) treatments. The role of phytoalexins in the response of citrus species to infection by *Phytophthora citrophthora*, the causal agent of collar rot and root rot, is unclear. Two compounds were found by Hartmann and Nienhaus (12) in bark of *Citrus limon* after inoculation with *P. citrophthora*. One substance was isolated and identified as xanthoxylin; however, there was no correlation between the intensity of resistance by the trees to *Phytophthora* and the accumulation of xanthoxylin. Citrus roots infected by *P. citrophthora* and *P. parasitica* accumulate seselin. This compound inhibits mycelial growth of *P. citrophthora* and *P. parasitica* in vitro (26).

Another substance that is induced in citrus tissue after infection with *P. citrophthora* is scoparone (6,7-dimethoxycoumarin). This compound is produced in the bark of resistant and susceptible citrus species, but the concentration 96 hr after inoculation is higher in the resistant. In vitro, scoparone inhibits the growth of several phytopathogenic fungi (1). Small amounts of scoparone have been isolated consistently in uninoculated citrus fruit peels (21,24).

An understanding of the biosynthesis of scoparone should facilitate attempts to inhibit synthesis and observe possible changes in resistance. Phenylpropanoid metabolism leads to the formation of activated cinnamic acid, which is derived biosynthetically from phenylalanine (3,10,11). Phenylalanine is deaminated to trans-cinnamic acid by the enzyme phenylalanine ammonia-lyase (PAL) (18). It is known that aminooxyacetic acid (AOA) is a competitive inhibitor of PAL (2,8,13,16,25). The precursor of coumarin and 7-methoxycoumarin is cinnamic acid (6).

We report herein that scoparone is a phytoalexin associated with the resistance of citrus against *P. citrophthora*, that phenylalanine is the precursor of scoparone, and that AOA inhibits the production of scoparone in the biosynthetic pathway in citrus.

## MATERIALS AND METHODS

**Plant material.** The following 3-yr-old citrus seedlings from Kibutz Netzer Syreni Nursery, Israel, were grown outdoors and at 22–26 C in the greenhouse: *Citrus sinensis* (L.) Osbeck (shamouti); *C. aurantium* L. (sour orange); *Poncirus trifoliata* Raf. (trifoliate orange); *C. jambhiri* Lush. (rough lemon); *C. macrophylla* Webster (macrophylla); and *C. reticulata* Blanco × *C. sinensis* (niva).

**Fungal cultures.** The following phytopathogenic fungi, most of them pathogenic to citrus, were selected from the collection of the Faculty of Agriculture, Rehovot, Israel: *Phytophthora citrophthora* (Smith & Smith) Leonian (isolate C-16), *Verticillium dahliae* Kleb, *Hendersonula toruloidea* Nattras, *Botryodiplodia theobromae* (*Diplodia natalensis*) Pat, *Colletotrichum gloeosporioides* Penzig, *Penicillium digitatum* Sacc. and *P. italicum* Wehmer. These fungi were cultured on potato-dextrose agar (PDA) medium, at 25 C, to serve as inoculum. *P. citrophthora* C-16 was isolated from Lerrer grove, Rehovot, Israel, in January 1983. No differences in virulence were found between this isolate and 10 other isolates of *P. citrophthora* tested.

**Inoculation.** Three-millimeter incisions, 0.2–0.5 mm deep, were cut with a sterile scalpel in the bark of 3-mo-old citrus branches that were 25–30 cm long and 7–10 mm thick. A 3-mm-diameter disk cut from an actively growing PDA culture of *P. citrophthora* was placed over the incision, fungal side downward, and the inoculated branch sections were incubated in humid chambers at 20 C in darkness.

**Extraction and purification.** Slices of inoculated, necrotic bark cut from the outer edge of wounds were extracted with distilled water at 10 ml/g fr wt of tissue, for 2 hr at 40 C. After partition with ethyl acetate (EtOAc), the predominant antifungal component (it was bioassayed with *P. citrophthora*) extracted from the inoculated bark was concentrated by evaporating the solvent at 40 C in a Rotovac evaporator. The crude concentrate was chromatographed on a silica gel (70-230 mesh ASTM Art. 7734 Kieselgel 60) H-column, 20 × 150 mm, with increasing concentrations of EtOAc in petroleum ether. The active ingredient was eluted with EtOAc/petroleum ether 1:1 (v/v) and partitioned with $CHCl_3$, from which it crystallized upon evaporation, as colorless needles with m.p. 146–147 C. The substances eluted by each eluting mixture were analyzed by thin-layer chromatography (TLC) (0.5 mm Art. 7730 Kieselgel 60 GF 254). The developing solvent was a mixture of toluene/EtOAc, 1:1 (v/v). Developed chromatograms were dried and then examined under UV illumination.

**Bioassay.** Scoparone was assayed with various phytopathogenic fungi in vitro. The $ED_{50}$ value for *P. citrophthora* (mycelial growth) was determined by adding increasing concentrations of scoparone to cooled molten PDA immediately before pouring into 3-cm-diameter plastic petri plates. A 3-mm-diameter disk, taken

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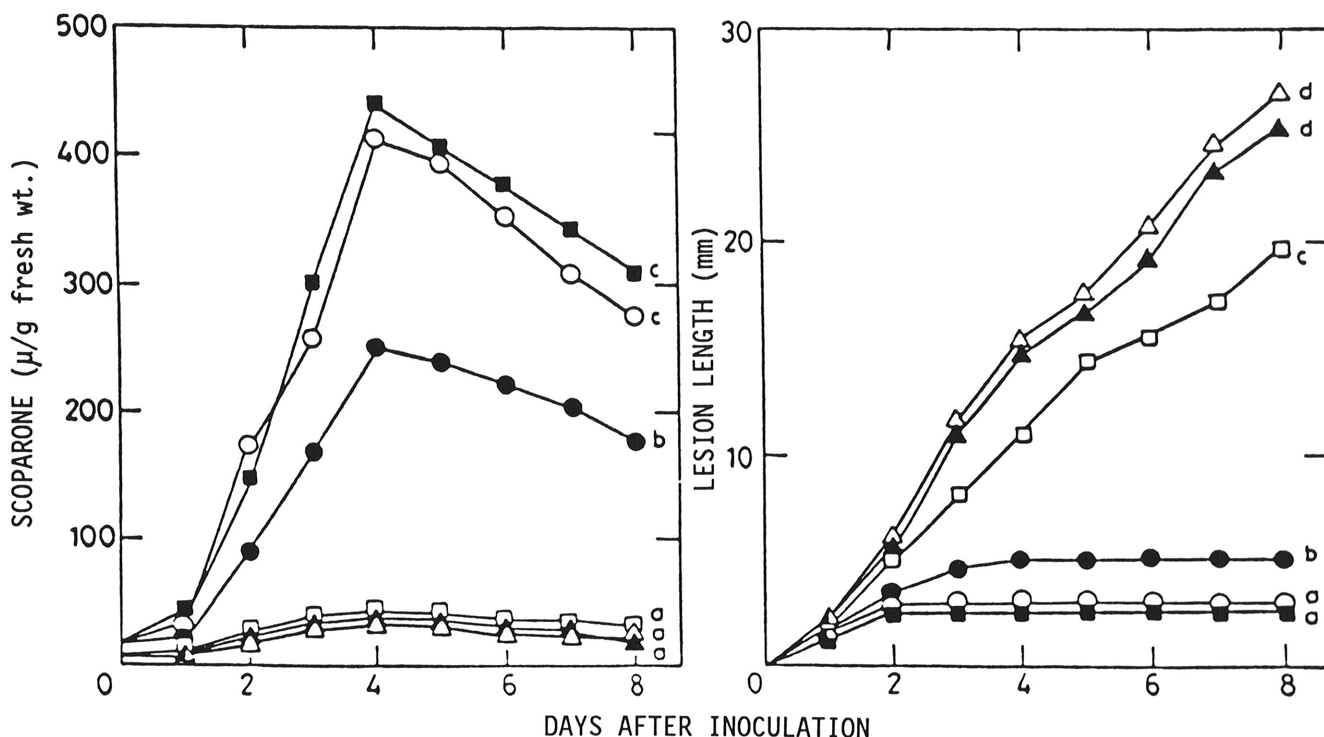


**Fig. 1.** Accumulation of scoparone and lesion length in citrus bark of the resistant species macrophylla (■), trifoliate orange (○), sour orange (●), and the susceptible species rough lemon (□), shamouti (▲), niva (△) after inoculation with *Phytophthora citrophthora*. Significant differences were indicated by different letters within each time period according to Duncan's multiple range test ($P = 0.05$). Statistical analysis of scoparone concentration was done starting on the second day and lesion length on the third day.

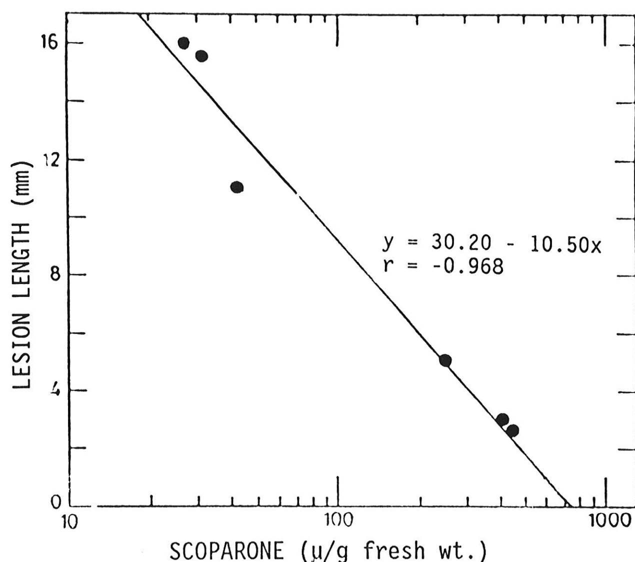


**Fig. 2.** The relation between the concentration of scoparone and lesion length in citrus bark 96 hr after inoculation with *Phytophthora citrophthora*.

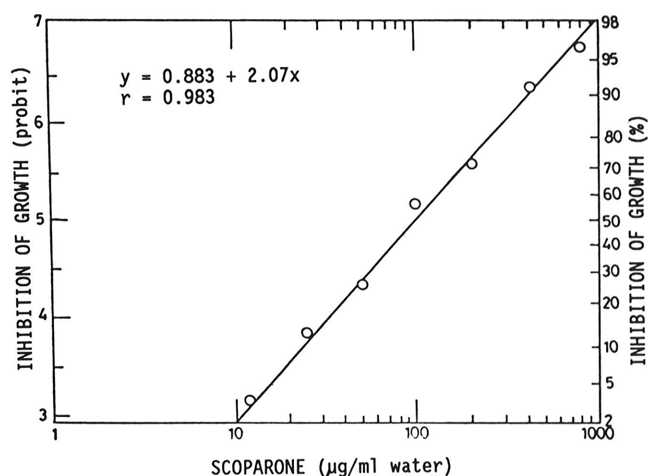


**Fig. 3.** Dosage-response of *Phytophthora citrophthora* mycelial growth to log concentration of scoparone, expressed as a linear regression.

from an actively growing colony on PDA, was placed fungal side downward in the center of each plate. Plates were incubated in the dark at 24 C for 7 days. The $ED_{50}$ value for spore germination was determined by adding increasing concentrations of scoparone to test tubes with a suspension of approximately $5 \times 10^5$ spores/ml of potato-dextrose broth (PDB). The test tubes were incubated in the dark at 24 C for 48 hr. $ED_{50}$ values were calculated from linear regression lines obtained by plotting the percent inhibition of mycelial growth and spore germination against the log concentration of scoparone. The standard error of $ED_{50}$ values was calculated from a linear regression analysis of the scoparone concentrations.

**Labeling.** Fifty microcuries (U-$^{14}$C)L-phenylalanine (specific activity 400–500 mCi/mmole) was diluted with sterile deionized water to 0.2 μCi/10 ml for use in the experiments. Excised branches (1 cm length and 0.5 cm diameter) from 3-mo-old citrus seedlings were immersed in the solution containing $^{14}$C-phenylalanine ($3.14 \times 10^5$ dpm) for 3 hr, inoculated with *P. citrophthora* and incubated at 24 C for 96 hr. Scoparone was extracted and purified as described earlier.

Radioactivity was measured with a Beckman LS 7800 liquid scintillation counter. The percent incorporation of $^{14}$C in the labeled scoparone was identified and compared to the healthy control (the amount of $^{14}$C-phenylalanine that had been incorporated into the bark which was extracted and measured immediately after a 3-hr soak in the radioactivity solution).

**Effect of AOA.** The effect of adding AOA to resistant and susceptible citrus inoculated with *P. citrophthora* was examined. Three-month-old resistant and susceptible citrus branches (5 cm

length, 0.5 cm diameter) were immersed in 10 mM AOA (in sterile deionized water) for 3 hr, inoculated with *P. citrophthora* and incubated at 24 C. The advance of the pathogen (lesion length) and concentration of scoparone were measured 96 hr later.

**Scoparone quantification.** Solutions of scoparone in distilled water (10–70 μg/ml) were used to prepare a standard curve to quantify concentrations of scoparone in inoculated and uninoculated citrus bark. Spectrofluorometry of these solutions revealed an excitation peak at 340 nm and an emission peak at 430 nm. Therefore, extracts of inoculated and uninoculated citrus bark, containing 1 g fr wt of tissue in 10 ml of distilled water were analyzed spectrofluorometrically with excitation at 340 nm and emission reading at 430 nm. Concentrations of scoparone in the tissue were calculated by a standard technique with respect to the standard curve.

Experiments were repeated three times and each treatment included five replicates.

## RESULTS

**Identification of scoparone.** Ultraviolet spectrophotometry infrared analysis, $^{1}$H-NMR and $^{13}$C-NMR spectra indicated that the active antifungal agent that was extracted and purified from citrus bark inoculated with *P. citrophthora* was 6,7-dimethoxycoumarin (scoparone) (1). Chromatograms developed with a mixture of toluene/EtOAc, 1:1 (v/v), and analyzed by TLC were examined under UV illumination and and gave a fluorescent spot ($R_f = 0.6$).

**Accumulation of scoparone.** The production of scoparone and lesion length in the bark of 3-mo-old citrus branches, resistant and susceptible to *P. citrophthora*, were measured 1–8 days after inoculation with this pathogen. Scoparone was induced in both groups of citrus, but the concentration was higher and increased more rapidly in the resistant species. Twenty-four hours after inoculation the concentration of scoparone in macrophylla,

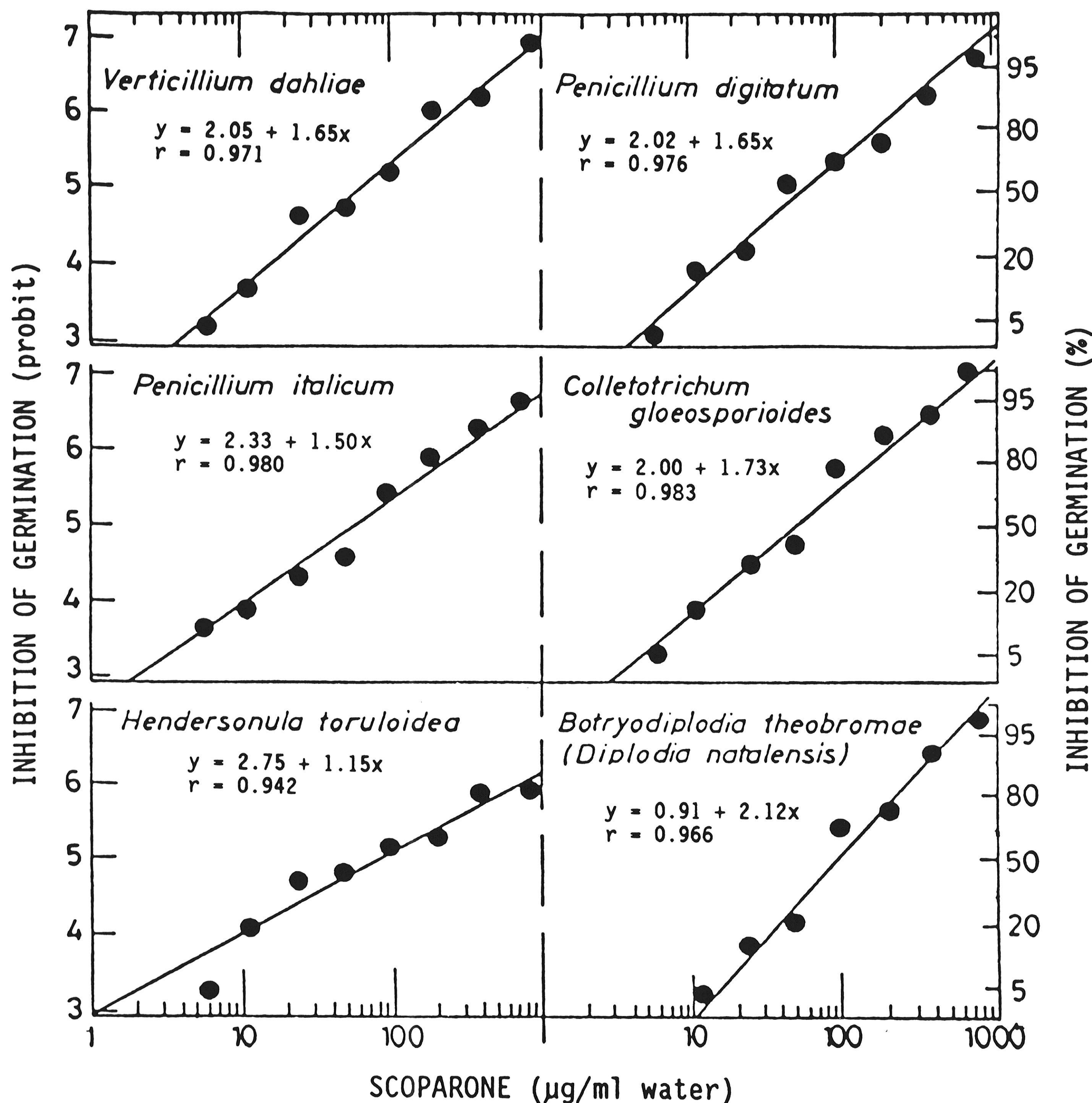


**Fig. 4.** Dosage-response of spore germination of six phytopathogenic fungi to log concentration of scoparone, expressed as a linear regression.

trifoliate orange and sour orange (resistant) reached 44.8, 32.0, and 28.8 μg/g fr wt as compared to 13.5, 12.9, and 12.3 μg/g fr wt in rough lemon, shamouti, and niva (susceptible), respectively. The maximum concentration in these species, resistant and susceptible, 4 days after inoculation, was 440, 415, and 250 μg/g ft wt and 41.6, 31.1, and 28.2 μg/g fr wt, respectively (Fig. 1). The advance of *P. citrophthora* (lesion length) in bark, 4 days after inoculation, was 2.5 mm in macrophylla, 3.2 mm in trifoliate orange, 5.0 mm in sour

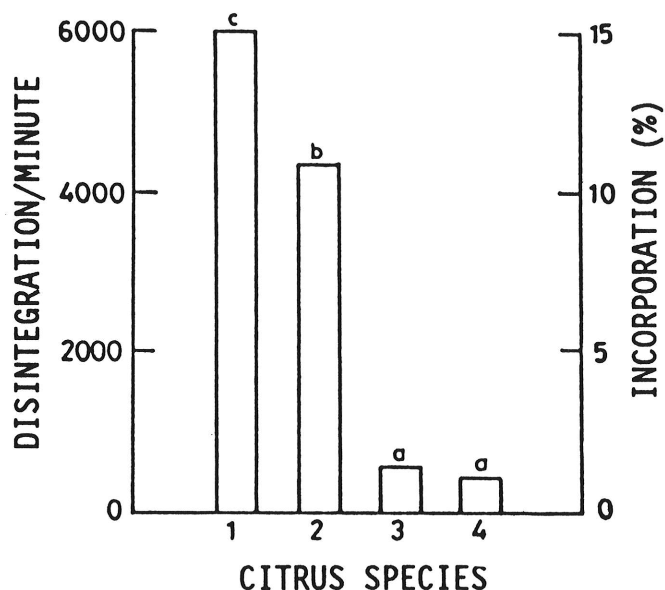


**Fig. 5.** Percentage incorporation of $^{14}$C-phenylalanine into scoparone extracted from infected bark 99 hr after application of the isotope (96 hr after inoculation with *Phytophthora citrophthora*) to the following citrus species: 1) macrophylla (resistant); 2) sour orange (resistant); 3) shamouti (susceptible); and 4) niva (susceptible). Temperature of incubation was 24 C. Different letters indicate significant differences according to Duncan's multiple range test ($P = 0.05$).

orange, 11.0 mm in rough lemon, 15.5 mm in shamouti, and 17.0 mm in niva (Fig. 1). In the control (uninoculated bark), the concentration of scoparone was 12–17.5 μg/g fr wt and wounding did not induce scoparone accumulation. The lesion length 4 days after inoculation with *P. citrophthora* was negatively correlated with increased accumulation of scoparone in vivo (Fig. 2).

**The $ED_{50}$ of scoparone.** Scoparone inhibited the growth of various phytopathogenic fungi in vitro. The $ED_{50}$ of scoparone for mycelial growth of *P. citrophthora* was 97 μg/ml. The $ED_{50}$ for spore germination of the following fungi was as follows: *Verticillium dahliae*, 61 μg/ml; *Penicillium digitatum*, 64 μg/ml; *P. italicum*, 60 μg/ml; *Colletotrichum gloeosporioides*, 54 μg/ml; *Hendersonula toruloidea*, 90 μg/ml; and *Botryodiplodia theobromae*, 85 μg/ml (Figs. 3 and 4).

**Scoparone radioactivity.** Measurement of the distribution of radioactive scoparone that was produced in the bark 99 hr after isotope application (96 hr after inoculation with *P. citrophthora*) showed that scoparone was labeled with $^{14}$C. Total incorporation was 11 and 15% in the resistant species sour orange and macrophylla, respectively, as compared to 1.5% in the susceptible species shamouti and niva (Fig. 5).

**The influence of aminooxyacetic acid (AOA).** Results with 3-mo-old resistant and susceptible citrus branches treated with 10 mM AOA and inoculated with *P. citrophthora* showed that concentrations of scoparone in the resistant species were 32.5–43.4 μg/g fr wt as compared to 295–472 μg/g fr wt in the control (inoculated and untreated branches). Lesion lengths in this group were 9.2–12.0 mm as compared to 2.8–5.1 mm in the control. In the susceptible species, concentrations of scoparone and lesion lengths treated and untreated with 10 mM AOA and inoculated with *P. citrophthora* were 21.4–46.4 μg/g fr wt and 11.0–12.9 mm (Fig. 6). No evidence of toxicity was found after treatment with 10 mM AOA.

## DISCUSSION

Two phenolic compounds were reported by Hartmann and Nienhaus (12) to be induced in citrus after infection with *P. citrophthora*. One of them was identified as xanthoxylin and the

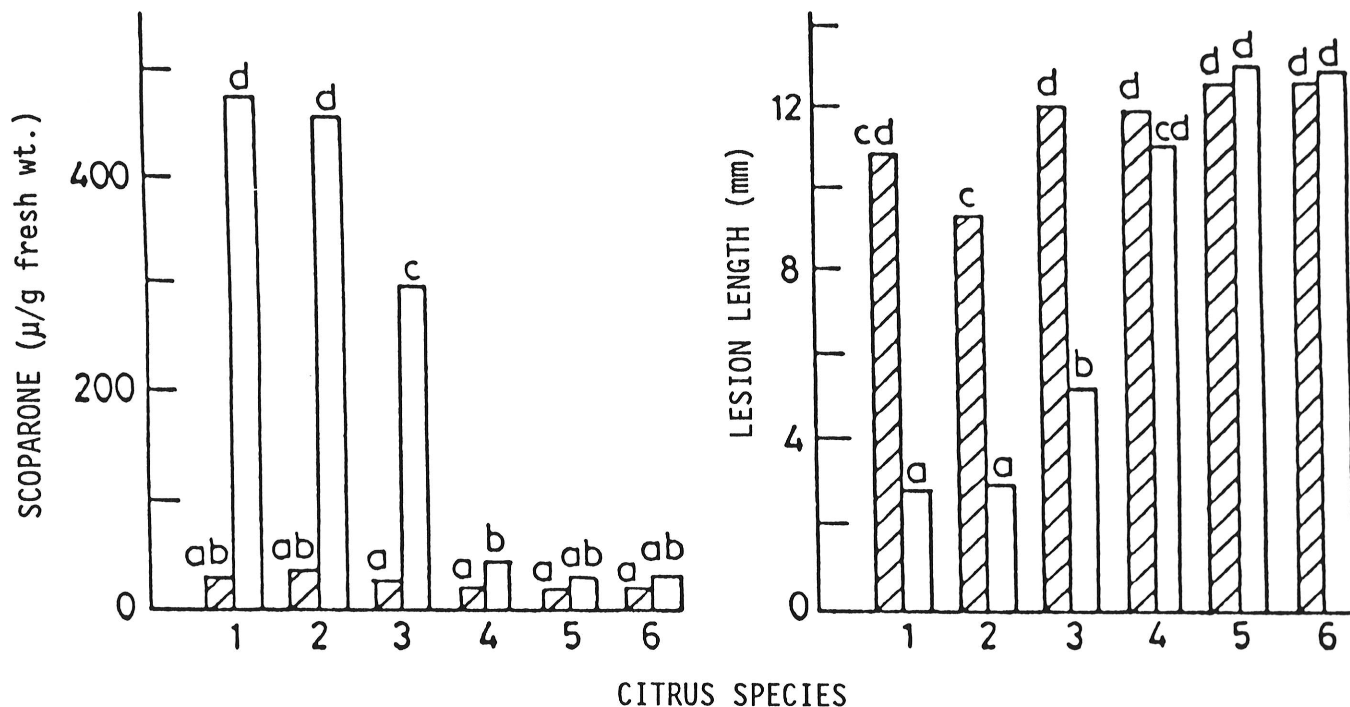


**Fig. 6.** The concentration of scoparone and lesion length in the bark of the following citrus species 96 hr after inoculation with *Phytophthora citrophthora*: 1) macrophylla (resistant); 2) trifoliate orange (resistant); 3) sour orange (resistant); 4) rough lemon (susceptible); 5) shamouti (susceptible); 6) niva (susceptible): treated (▨) and nontreated (□) with aminooxyacetic acid (AOA). Different letters indicate significant differences according to Duncan's multiple range test ($P = 0.05$).

other was not identified. There was no correlation between the intensity of resistance by the trees to *P. citrophthora* and the accumulation of xanthoxylin in the bark after infection with this pathogen. Accumulation appeared to be a result of the necrotic reaction and seemed to play a part in eliminating the pathogen after it had been inhibited by other defense mechanisms of the plant.

Broadbent (5) concluded that a morphological exclusion of the parasite in mature cells is not responsible for resistance to *P. citrophthora*, and biochemical or physiological differences must exist between resistant and susceptible species. Mature cells of resistant citrus may possess in vivo inhibitors, or hypersensitivity to infection may be induced by substances produced by the plant cells as a result of the infection process. Recently, it was reported that two coumarins, scoparone (1) and seseline (26), are induced in citrus tissue after inoculation with *Phytophthora* spp. and may be involved in the defense mechanisms of citrus against *Phytophthora*.

Results of this paper further suggest the involvement of scoparone in resistance of citrus against *P. citrophthora*. Scoparone is toxic to several fungi (Fig. 4) and its production in the resistant plants increases up to the fourth day and diminishes toward the eighth day after inoculation (Fig. 1). Such a pattern of accumulation and degradation is typical to phytoalexin production in many species (4). Scoparone is involved in resistance and not a result of resistance (necrosis). The lesion length caused by infection is inversely proportional to the increase in phytoalexin concentration (Fig. 1); as the lesion length (necrosis) increases, the concentration of scoparone decreases. Scoparone levels were highly correlated with resistance (Fig. 2).

Scoparone is inhibitory to *P. citrophthora* (Fig. 3) and its concentration in the susceptible/inoculated or resistant/uninoculated plants is very low, but rapidly increases after inoculation to fungitoxic levels only in the resistant species (Fig. 1). A comparison between concentrations of scoparone that inhibit the advance of *P. citrophthora* in vivo and its growth in vitro showed that the advance of the pathogen in macrophylla and trifolate orange terminates the second day after inoculation, when the concentration is above 150 $\mu$g/g fr wt. This concentration is equivalent to $ED_{65}$ for *P. citrophthora* in vitro.

Suppression of scoparone production by AOA was accompanied with nullification of resistance (Fig. 6). It is suggested, therefore, that scoparone is involved in resistance to *P. citrophthora* in three resistant citrus species. Other investigators have shown that AOA inhibited synthesis of phenylpropanoid in tomato (2,13) and of coumarins and phenolics in citrus (16).

Cinnamic acid was reported as a precursor of several coumarins in potato (7) and tobacco (9,22) plants. Other reports show that phenylalanine is the precursor of cinnamic acid in plants (3,10,11). Our results indicate that the precursor of scoparone in citrus is also phenylalanine as demonstrated by an accumulation of radioactive scoparone in infected citrus after treatment with $^{14}$C-phenylalanine (Fig. 5). The maximum incorporation of $^{14}$C from phenylalanine into scoparone fraction is 15% (Fig. 5), as compared to the maximum incorporation, 17%, of $^{14}$C from mevalonate into rishitin in potato tuber (17). Probably the incorporation rates of $^{14}$C in scoparone are not high, because phenylalanine is used as a precursor of other compounds in the plant (18), and it spreads among these compounds.

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