Effect of Scoparone (6,7-Dimethoxycoumarin) Biosynthesis on the Resistance of Tangelo Nova, *Citrus paradisi*, and *Citrus aurantium* Fruits against *Phytophthora parasitica*

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The biosynthesis of scoparone (6,7-dimethoxycoumarin) was associated with resistance of mature fruits of tangelo Nova, *Citrus aurantium*, and *Citrus paradisi* to infection by *Phytophthora parasitica*. Levels of scoparone increased by treating fruit with Brotomax, which also enhanced the resistance of fruit to the fungus. Lesion development was reduced by as much as 40%. Scoparone is therefore proposed as a possible phytoalexin in fruits of these cultivars.

Keywords: *Citrus; coumarin; phytoalexin; scoparone*

INTRODUCTION

The genus *Citrus* produces coumarins, flavanones, flavones, and flavonols, which occur in the free form and/or as glycosides (Maier and Metzler, 1967; Horowitz and Gentile, 1977). Their expression varies from species to species (Albach et al., 1969; Castillo et al., 1992, 1993; Benavente-García, 1993; Del Río and Ortuño, 1994; Del Río et al., 1992, 1995, 1997; Horowitz and Gentile, 1977; Jourdan et al., 1985; Ortuño et al., 1995, 1997). The possibility of modulating the processes of synthesis and/or accumulation of some of these compounds (flavanones) has been described in previous papers, using different phytoregulators such as benzylaminopurine (Del Río et al., 1995), ethylene (García Puig et al., 1995), and Brotomax (Fuster et al., 1995), a phytosanitary product.

Coumarins can act as phytoalexins in resistance mechanisms of *Citrus* to pathogens. Some species of *Citrus* when infected by phytopathogenic fungi have been shown to accumulate certain coumarins such as xanthyletin, seselin, and scoparone (Afek and Sztejnberg, 1988, 1993; Afek et al., 1986; Arimoto et al., 1986; Arimoto and Homma, 1988; De Lange et al., 1976; Khan et al., 1985; Stange et al., 1993; Sulistyowati et al., 1990; Vernenghi et al., 1987), although the nature of the coumarin biosynthesized in this process varies within a species according to the pathogen in question. For example, *Citrus limon* accumulated scoparone after inoculation with *Penicillium digitatum* (Kim et al., 1991); however, there was no significant accumulation of any antifungal compounds in the tissues of lemons inoculated with *Geotrichum candidum* (Baudoin and Eckert, 1985).

We investigated the role of scoparone in possible defense mechanisms of the hybrid citrus tangelo Nova and the two species *Citrus paradisi* and *Citrus aurantium* to *Phytophthora parasitica*, the cause of brown rot, and determined the effect of stimulating scoparone accumulation on the defense of fruit against this fungus.

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MATERIALS AND METHODS

Plant Material and Brotomax Treatment. Sixty mature fruits of tangelo Nova, a mandarin hybrid [*Citrus reticulata* × tangelo Orlando (*C. reticulata* × *C. paradisi* Macf.)], *C. paradisi* (cv. Star Ruby), and *C. aurantium* (cv. Sevillano) were used in each experiment.

After harvesting, the fruit were washed in water before being separated into two lots of 30 fruits, one to be used as control and the other to be infected by the fungus *P. parasitica*.

To evaluate the effect of Brotomax (Agrometodos S.A., Madrid, Spain), another lot of 60 fruits was divided into two lots of 30 fruits each. In this case, the fruits were submerged in a 0.5% aqueous solution of Brotomax for 1 h. After drying, the fruits were stored for 8 days in a chamber at 15 °C before inoculation.

Fungal Cultures, Inoculation, and Evaluation of Fruit Resistance. An isolate of the fungus *P. parasitica* was selected from the collection of the Centro de Investigación y Desarrollo Agroalimentario (CIDA), Murcia, Spain, and was cultured on potato dextrose agar (PDA) medium, at 25 °C, to serve as inoculum.

Before inoculation, the fruits were sprayed with 96% ethanol and distributed on trays. Two 6 mm diameter sections of flavedo were removed from each fruit to be inoculated by means of a glass capillary and sterile scalpel. A disk of similar diameter of culture medium with mycelium of *P. parasitica* and (another) of fungus-free culture medium were then placed in each wound, representing infected and control fruit, respectively. Depending on the experiment, inoculation was carried out immediately or 7 days after wounding.

After inoculation of the fruit with the fungus, the inocula were sealed by adhesive plastic, which was done also for the control. The respective fruits were kept in a chamber at 20 °C and 85% relative humidity.

At different times after inoculation, the resistance of fruit to infection was determined by measuring the lesion diameter.

Extraction and Purification. Five fruits were used in each experiment. From these, 20 g (fresh weight) of flavedo tissue was taken from next to the wound area (control) or from the lesion of inoculated fruit. These samples were then sliced into 1 cm fragments for extraction by stirring with petroleum ether (1:3 w/v) for 24 h. The tissue was then homogenized and vacuum filtered. The extract was vacuum concentrated to approximately 2 mL. This was then resuspended in 3 mL of dichloromethane and dried under a stream of N_2. The crude extract obtained was frozen at −15 °C until use.

Scoparone was purified by dissolving the crude extract in 0.5 mL of dichloromethane. Components of these solutions (70 μL) were subjected to TLC using 0.2 mm thick silica gel

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Table 1. Scoparone Levels in Mature Fruits of Tangelo Nova[a]

	scoparone (μg/g FW)		
time (days)	A	B	C
0	ND	ND	ND
3	95 ± 2	14 ± 2	
7	274 ± 10	90 ± 3	
12	9 ± 0.4	24 ± 5	46 ± 2

[a] A, control fruits; B, fruits inoculated immediately after wounding; C, fruits inoculated 7 days after wounding. Time refers to the days elapsed since wounding. The scoparone levels in unwounded fruits are depicted at day 0. Data represent mean values ± SE (n = 3) of scoparone (μg/g fresh weight). ND, not detected.

Table 2. Effect of Brotomax on Scoparone Levels in Mature Fruits of Tangelo Nova[a]

	scoparone (μg/g FW)		
time (days)	A	B	C
0	ND	ND	ND
3	140 ± 11	60 ± 3	
7	1020 ± 162	187 ± 15	
12	78 ± 5	93 ± 4	428 ± 27

[a] A, control fruits; B, fruits inoculated immediately after wounding; C, fruits inoculated 7 days after wounding. Time refers to the days elapsed since wounding. The scoparone levels in unwounded fruits are depicted at day 0. Data represent mean values ± SE (n = 3) of scoparone (μg/g fresh weight). ND, not detected.

60 F_{254} (Macherey-Nagel), with toluene/ethyl acetate (4:1 v/v) as the solvent (Kim et al., 1991). The developed plates were dried and exposed to UV light for detection of the fluorescent compounds. In our analyses, scoparone was present at R_f of 0.3, similar to that of standard scoparone (Aldrich, Madrid, Spain).

Confirmation of Scoparone by HPLC. The R_f spots corresponding to scoparone were scraped from the chromatographic plates and redissolved with 2 mL of ethanol. The samples were centrifuged at 5000*g* for 10 min and concentrated under N_2 to 0.5 mL. These were then filtered through a 0.45 μm nylon mesh before analysis by HPLC with a Hewlett-Packard liquid chromatograph (Model HP 1050) with a diode array detector (range scanned = 220−500 nm). Reversed phase chromatographic separation was carried out on a μBondapak C_{18} (250 × 4 mm i.d.) analysis column (Waters Associates, Milford, MA). The particle size was 5 μm. Isocratic separation was performed according to the procedure described by Kim et al. (1991), using a mixture of methanol/water (4:1 v/v) at a flow rate of 0.5 mL/min at 35 °C. Changes in absorbance were recorded in the V/UV diode array detector at 340 nm. The scoparone was collected using a fraction collector at the exit of the column, and its identity was confirmed by reference to its nuclear magnetic resonance spectrum (^{1}H NMR) (360 MHz) (Bruker, Germany) in $CDCl_3$.

RESULTS AND DISCUSSION

Levels of Scoparone in Tangelo Nova Fruits Inoculated with *P. parasitica*. The presence of scoparone was confirmed by HPLC (R_t = 5.274 min). The absorption spectrum of this compound obtained by means of a V/UV diode array detector shows maxima at 292 and 341 nm. This compound was isolated at the exit of the HPLC column, and its identity was confirmed as 6,7-dimethoxycoumarin (scoparone) by reference to its ^{1}H NMR spectrum, which was identical to that obtained by Afek et al. (1986).

Table 1 shows the concentration of scoparone found in the control and infected mature fruit of tangelo Nova. Scoparone was not detected in the undamaged fruits but was clearly observable in all of the fruits that had been wounded, whether or not they were subsequently inoculated.

When this hybrid was inoculated with *P. parasitica* inmediately after wounding (Table 1), the levels of scoparone after 3 and 7 days were less than those observed in the control [14 and 90 compared with 95 and 274 μg/g fresh weight (FW), respectively], probably due to degradation of the scoparone itself during the fruit's defense against the fungus. After 12 days, however, scoparone levels in infected fruits exceeded those of the control (24 compared with 9 μg/g FW, respectively).

With regard to the time course trend of scoparone in wounded and infected fruit, levels increased toward the seventh day after wounding or infection but then fell.

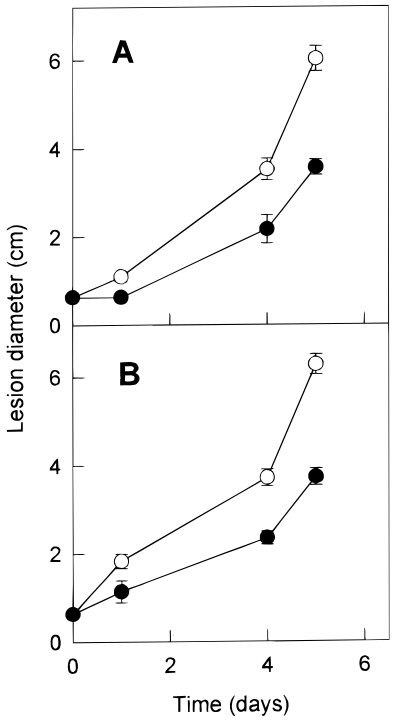

Figure 1. Lesion development during infection of mature fruit of tangelo Nova by *P. parasitica*: (●) fruit treated with Brotomax; (○) untreated fruit; (A) fruits inoculated 7 days after wounding; (B) fruits inoculated immediately after wounding. Data represent mean values of lesion diameter (cm) at different days after inoculation, and the vertical bars denote ± SE (n = 3), when larger than symbols.

Such an increase followed by degradation is typical of phytoalexin production in many species (Bayley and Mansfield, 1982).

With regard to the fruit infected 7 days after wounding, the level of scaparone 5 days after inoculation was far greater than that observed in the control fruits at the same age (46 compared with 9 μg/g FW) (Table 1).

These results suggest that scoparone synthesis in the plant material studied here may be involved in the defense mechanisms of these citrus against *P. parasitica*, in agreement with the results of others authors in other *Citrus* species infected by *Phytophthora citrophthora* (Afek and Carmely, 1986; Afek and Sztejnberg, 1988, 1993) or *Penicillium digitatum* (Kim et al., 1991). The findings also lend support to the idea proposed by some authors that phytoalexins are detected in mechanically injured fruit (Kim et al., 1991) or in response to microbial infection (Bailey and Mansfield, 1982).

Effect of Brotomax on the Scoparone Levels in Tangelo Nova, *C. aurantium*, and *C. paradisi* Fruits and on the Defense Mechanisms against *P.*

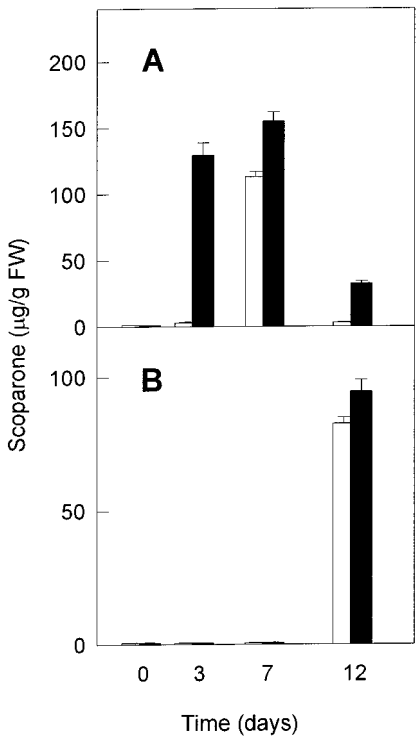

Figure 2. Effect of Brotomax on scoparone levels in mature fruits of *C. aurantium*: (□) untreated fruit; (■) treated fruit; (A) wounded fruits; (B) fruits inoculated 7 days after wounding. Time refers to the days elapsed since wounding. The scoparone levels in unwounded fruits are depicted at day 0. Data represent mean values ± SE ($n = 3$) of scoparone (μg/g FW).

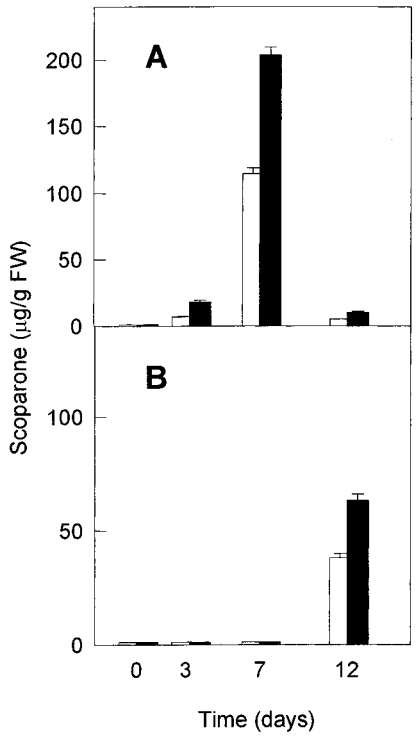

Figure 3. Effect of Brotomax on scoparone levels in mature fruits of *C. paradisi*: (□) untreated fruit; (■) treated fruit; (A) wounded fruits; (B) fruits inoculated 7 days after wounding. Time refers to the days elapsed since wounding. The scoparone levels in unwounded fruits are depicted at day 0. Data represent mean values ± SE ($n = 3$) of scoparone (μg/g FW).

parasitica. Table 2 shows concentrations of scoparone in the fruits of tangelo Nova after treatment with 0.5% Brotomax. Considerably higher levels of scoparone were present in the control fruits than in control fruits not treated with Brotomax (Table 1). Similarly, infected tissue taken from fruit treated with Brotomax contained greater concentrations of scoparone than infected tissue from fruits that had not been treated with Brotomax (93 compared with 24 μg/g for fruit infected immediately after wounding; 428 compared with 46 μg/g for fruit infected 7 days after wounding) (see Tables 1 and 2).

In previous studies we have shown how Brotomax can enhance the synthesis and/or accumulation of flavanone in *Citrus* (Fuster et al., 1995) at both leaf and fruit levels without producing any appreciable change in the growth of these organs. We concluded that the effect on flavanone expression may be due to the processes by which these secondary compounds are synthesized or accumulated. Similar metabolic processes altered by Brotomax may also account for the accumulation of phenolic compounds such as scoparone in *Citrus*.

When we measured brown rot lesions formed on Brotomax-treated fruit of tangelo Nova infected 7 days after wounding, development was inhibited approximately by 40% at 1, 4, and 5 days after inoculation (Figure 1A) compared with the untreated controls. Similar inhibition by Brotomax was observed when the fruits were infected immediately after wounding, although more severe brown rot lesions were observed in both control and brotomax-treated fruit (Figure 1B) than in fruit inoculated 7 days after wounding, probably because the initial levels of scoparone were lower in the first case (see Tables 1 and 2).

Similar to the findings for tangelo Nova fruits, scoparone was not detected in the undamaged fruits of *C. aurantium* or *C. paradisi*, although this secondary compound was observed in these fruits when they had been wounded or inoculated with *P. parasitica* (Figures 2 and 3, repectively) but at lower concentrations than in tangelo Nova (compare Figures 2 and 3 with Table 1) .

C. aurantium and *C. paradisi* fruits treated with 0.5% Brotomax showed higher levels of scoparone than the control fruits, which had not been treated with Brotomax, both in wounded (Figures 2A and 3A, respectively) and in infected fruit (Figures 2B and 3B, respectively). The treated fruit of both species studied also showed greater resistance to attack by *P. parasitica* (data not shown).

On the basis of these results we suggest that Brotomax induces resistance in *Citrus* by increasing the concentration of scoparone in tangelo Nova, *C. aurantium*, and *C. paradisi*. Similar results have been obtained using Fosetyl-Al and phosphorus acid with other *Citrus* species, in which the scoparone levels also increased to produce very effective defense mechanisms against *P. citrophthora* (Afek and Sztejnberg, 1989).

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