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Changes in the Levels of Polymethoxyflavones and Flavanones as Part of the Defense Mechanism of *Citrus sinensis* (Cv. Valencia Late) Fruits against *Phytophthora citrophthora*

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Phytophthora citrophthora causes serious losses in *Citrus* fruits through brown rot lesion. The effect of infection with *P. citrophthora* on *Citrus sinensis* (cv. Valencia Late) fruits was studied, with particular reference to the levels of the flavanones hesperidin and isonaringin and the polymethoxyflavones sinensetin, nobiletin, tangeretin, and heptamethoxyflavone, because flavonoids are most probably involved as natural defense or resistance mechanisms in this genus. Changes in the levels of these flavonoids were detected after infection. The hesperidin and isonaringin contents fell by 13 and 67%, respectively, whereas the contents of their corresponding aglycons, hesperetin and naringenin, increased, suggesting the hydrolyzing effect of this fungus on the glycosylated flavanones. The heptamethoxyflavone, nobiletin, sinensetin, and tangeretin levels increased by 48, 28, 26, and 24%, respectively. The in vitro study revealed that these compounds acted as antifungal agents, the most active being the aglycons (naringenin and hesperetin), followed by the polymethoxyflavones and flavanone glycosides. The participation of these flavonoids in the defense mechanism of this *Citrus* species is discussed.

KEYWORDS: ***Phytophthora citrophthora*; *Citrus sinensis*; polymethoxyflavones; flavanones**

INTRODUCTION

Previous studies in *Citrus sinensis* revealed the presence of the polymethoxyflavones sinensetin (5,6,7,3′,4′-pentamethoxyflavone), nobiletin (5,6,7,8,3′,4′-hexamethoxyflavone), tangeretin (5,6,7,8,4′-pentamethoxyflavone), and 3,5,6,7,8,3′,4′-heptamethoxyflavone and the flavanones hesperidin and isonaringin (*1*, *2*). The highest levels of these secondary compounds were found in young developing fruits (*3*−*7*), although it has been suggested that some polymethoxyflavones might be related to the maturation phase of fruit in some *Citrus* species (*7*).

These compounds are mainly located in the peel, the polymethoxyflavones in the flavedo and the flavanones in the albedo (*7*−*10*), suggesting that they may act in protecting the fruit from pathogenic attack (*11*−*14*). Polymethoxyflavones are more biologically active than flavanones, although they occur in much lower concentrations (*11*, *15*).

Phytophthora citrophthora causes severe losses in *Citrus* fruits as a result of brown rot lesion. Synthetic fungicides are usually used to control this fungus and other pathogens. However, such products cause serious problems due to the residues that remain in the different tissues of the fruit (*16*) and the evolution of fungal strains resistant to these fungicides (*17*). For this reason, increasing the natural defense or resistance mechanisms of plants may represent an alternative strategy for preventing this disease.

The objective of this study was to analyze any metabolic changes (synthesis and degradation) of flavones and flavanone glycosides in the fruit of *C. sinensis* (cv. Valencia Late) associated with infection by *P. citrophthora* and to establish the possible involvement of these secondary compounds in the defense mechanisms of this *Citrus* species.

MATERIALS AND METHODS

Plant Material. The plant materials used in the different experiments were mature *C. sinensis* (cv. Valencia Late) fruit obtained from an experimental plantation belonging to the University of Murcia (Murcia, Spain).

After harvesting, the fruits were washed with water and divided into three lots consisting of 10 fruits each. These were then stored in a chamber at 15 °C for 7 days before inoculation with the fungus.

Fungal Cultures, Estimation of IC_{50}, Inoculation of Fruit with *P. citrophthora*, and Measurement of Growth. An isolate of the fungus *P. citrophthora*, obtained from the Spanish Collection of Type Culture (Valencia, Spain) (CECT 2353) and maintained on potato dextrose agar (PDA) medium at 25 °C, was used as inoculum.

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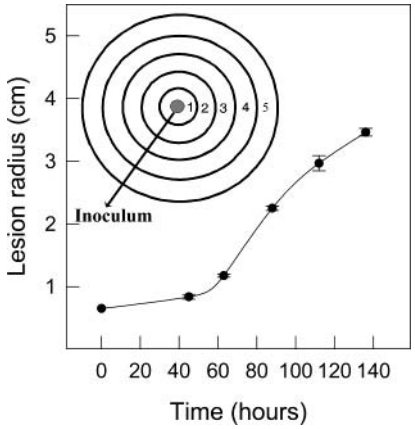

Figure 1. Lesion development during infection of mature fruit of *C. sinensis* (cv. Valencia Late) by *P. citrophthora*. Data represent mean values of lesion radius (cm) at different times (h) after inoculation, and the vertical bars denote ± SE ($n = 3$) when larger than symbols. (Inset) Concentric sampling zones of 1 cm radius. The central zone corresponds to the inoculum, whereas zones 1–4 correspond to zones of decreasing infection, the further they are from the inoculum site. Zone 5 corresponds to a healthy part of the fruit (136 h after infection by the fungus).

The antifungal activity of the polymethoxyflavones (nobiletin, sinensetin, heptamethoxyflavone, and tangeretin), flavanone glycosides (hesperidin and isonaringin), and their corresponding aglycons (hesperetin and naringenin) isolated from *C. sinensis* (cv. Valencia Late) fruits was determined by in vitro assays with *P. citrophthora* following procedures described in previous papers (*1*, *11*). The inhibition index (IC_{50}) was expressed as the concentration (mM) of these compounds required to provide 50% inhibition of radial growth. The IC_{50} was determined by linear regression.

To study the in vivo growth of the fungus in the fruit, mature fruits were sprayed with 96% ethanol and placed on trays before being inoculated. Two 6 mm diameter sections of flavedo (from opposite points in the equatorial zone) were removed from each fruit by means of a hollow glass tube. A disk of similar diameter of PDA culture medium with mycelium of *P. citrophthora* was then placed in each wound. Inoculation was carried out immediately after wounding, and the wounds were sealed by adhesive plastic strip (Sellotape). The infected fruits were kept in a growth chamber at 20 °C and 85% relative humidity. Fungal development in the fruit was analyzed at different times (45, 63, 88, 112, and 136 h) postinoculation by measuring the diameter of the mycelium.

Flavonoid Extraction and Chromatographic Analysis. One hundred and thirty-six hours postinoculation, concentric circles of 1 cm radius starting at the inoculation site were separated from the fruit of each lot. Each zone showed a decreasing degree of infection the further it was from the inoculation site (**Figure 1**; inset: zones 1−4 infected, zone 5 healthy). After these sections had been obtained for each lot of samples, they were mixed according to the zone from which they were taken and dried at 50 °C to constant weight. The dried peel was ground and shaken with dimethyl sulfoxide (DMSO) (*3*) for 2 h in a proportion of 40 mg of dry weight/mL in the case of the polymethoxyflavone extraction and 6 mg of dry weight/mL for the flavanone glycosides and their aglycons. The resulting extracts were filtered through a 0.45 μm nylon membrane before analysis by HPLC using a Hewlett-Packard liquid chromatograph (model HP 1050) (Hewlett-Packard Co., Palo Alto, CA), equipped with a diode array detector (range scanned = 220−500 nm). The stationary phase was a C_{18} column (250 mm × 4 mm i.d., particle size of 5 μm, thermostated at 30 °C). For the isocratic separation of flavanone glycosides and their aglycons, a mixture of water/methanol/acetonitrile/acetic acid (15:2:2:1) was used as solvent (*3*). For polymethoxyflavones, the stationary phase was the same, and as solvent we used a tetrahydrofuran (A)/water (B)/acetonitrile (C) (*18*) mixture, which was optimized for our particular work conditions with a gradient profile of 12% (A), 68% (B), and 20% (C) in 20 min and then 18% (B) and 70% (C) in 20 min. At 45 min, the mixture began to change to its initial composition, a process that lasted 15 min (*1*). Eluent flow was 1 mL/min in all cases.

The absorbance changes were recorded in a UV−vis diode array detector at 280 nm for the flavanone glycosides and their aglycons and at 340 nm for the flavones. The quantities of flavonoids were determined from the area given by the integrator using the response factor of the corresponding standards.

A C_{18} semipreparative column (250 mm × 10 mm i.d.) with a particle size of 5 μm thermostated at 30 °C was used for the isolation of these compounds, and the same solvent as described above for the flavones, flavanone glycosides, and their aglycons. Eluent flow was 3 mL/min in all cases. The fractions were collected with a Pharmacia FRAC 100 fraction collector (Pharmacia LKB Biotechnology, Uppsala, Sweden) at the exit of the HPLC column. Identification of these compounds was carried out using a Hewlett-Packard mass spectrometer (model 5989).

Scanning Electron Microscopy. Sections (10 × 5 mm) of culture medium containing mycelium of fungus in PDA culture medium (control) and in the same PDA culture medium to which hesperidin (19 mM), hesperetin (0.21 mM), or sinensetin (0.88 mM**)** had been added were fixed with glutaraldehyde (3%) for 4 h at room temperature. The samples were rinsed with buffer and then postfixed with OsO_4 (1%) at 0 °C for 2.5 h.

After the samples had been rinsed with distilled water until the rinsing solution was completely clear, they were dehydrated stepwise using an acetone series consisting of 30, 50, 70, 90, and 100% (10 min per step). The samples were critical point dried using a Balzers liquid CO_2 CPD 020 (Vaduz, Liechtenstein) for 2 h and then mounted onto aluminum stubs using double-sided adhesive tape. Finally, the samples were coated with 20 nm of gold for subsequent observation using a JEOL scanning electron microscope (JSM-6100, Tokyo, Japan) at an accelerating voltage of 15 keV.

Chemicals. Sinensetin, tangeretin, and isonaringin were purchased from Extrasynthèse S.A. (Genay, France). Heptamethoxyflavone and nobiletin were isolated by semipreparative HPLC and identified by MS (*1*). Hesperidin, hesperetin, and naringenin were obtained from Sigma Chemical Co. (St. Louis, MO).

RESULTS AND DISCUSSION

Polymethoxyflavone Levels in *C. sinensis* Fruits after Infection. HPLC analysis of healthy peel extracts (zone 5, see **Figure 1**) from mature *C. sinensis* (cv. Valencia Late) fruit revealed the presence of nobiletin, sinensetin, heptamethoxyflavone, and tangeretin as the principal polymethoxyflavones, as was found in the essential oil of sweet orange (*1*). In this zone, the highest levels corresponded to nobiletin [9 mg/100 g of dry weight (DW)], followed by heptamethoxyflavone (6 mg/100 g of DW), sinensetin (5 mg/100 g of DW), and tangeretin (1 mg/100 g of DW) (**Figure 2**).

Changes in the levels of these polymethoxyflavones were observed in vivo when these fruits were infected by *P. citrophthora.* In general, the different zones of the fruit infected by the fungus showed higher polymethoxyflavone levels than the healthy zone (**Figure 2**). This was particularly evident in zone 2, where the greatest mycelial growth observed was accompanied by heptamethoxyflavone, nobiletin, sinensetin, and tangeretin concentrations 48, 28, 26, and 24%, respectively, higher than in the healthy zone 5 (**Figure 2**). These increases continued to be evident, but to a less pronounced degree, as the level of infection decreased (zones 3 and 4). The behavior of the fungus in contact with the fruit suggests that the pathogen is taking advantage of, and altering, the metabolism of the host. Conversely, it cannot be ruled out that the levels depicted in **Figure 2** may be due to a balance between the synthesis of these compounds by the fruit as a defense mechanism against fungal attack and their consumption by the metabolism of the fungus itself. These findings support the idea that some of these

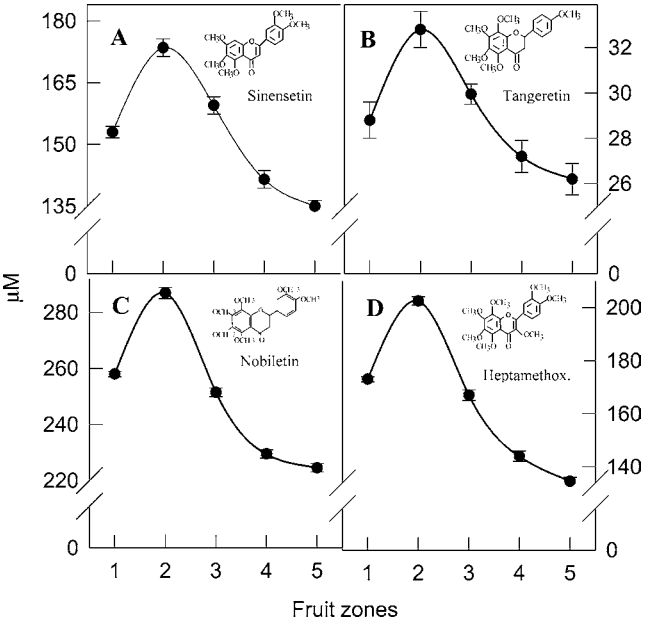

Figure 2. Effect of *P. citrophthora* on the polymethoxyflavone levels in mature fruits of *C. sinensis* (cv. Valencia Late). Data represent the mean values of sinensetin (**A**), tangeretin (**B**), nobiletin (**C**), and heptamethoxyflavone (**D**) (μM) in zones with different degrees of infection by *P. citrophthora* (136 h after infection by the fungus, zones 1–5, see inset of **Figure 1**). Vertical bars denote ± SE ($n = 3$) when larger than symbols.

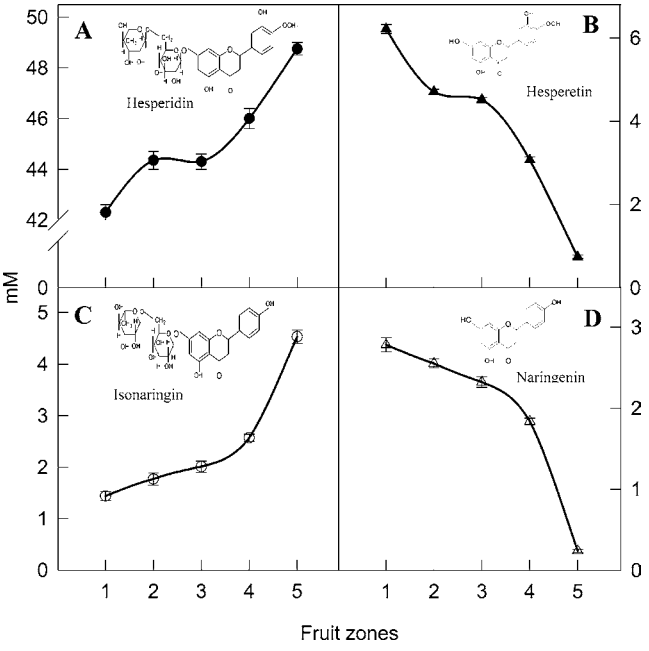

Figure 3. Effect of *P. citrophthora* on the metabolism of flavanone glycoside in mature fruits of *C. sinensis* (cv. Valencia Late). Data represent the mean values of hesperidin (**A**) and isonaringin (**C**) (mM) and of hesperetin (**B**) and naringenin (**D**) (mM) in zones with different degrees of infection by *P. citrophthora* (136 h after infection by the fungus, zones 1–5, see inset of **Figure 1**). Vertical bars denote ± SE ($n = 3$) when larger than symbols.

compounds may act as phytoalexins, although it cannot be discounted that other phenolic compounds may be involved in the defense mechanisms of this plant material, as has been described in other *Citrus* species (*19, 20*).

Metabolism of Flavanone Glycosides in Infected Fruits. Hesperidin was the principal flavanone in *C. sinensis* fruits with levels of 3 g/100 g of DW being measured in the healthy zone;

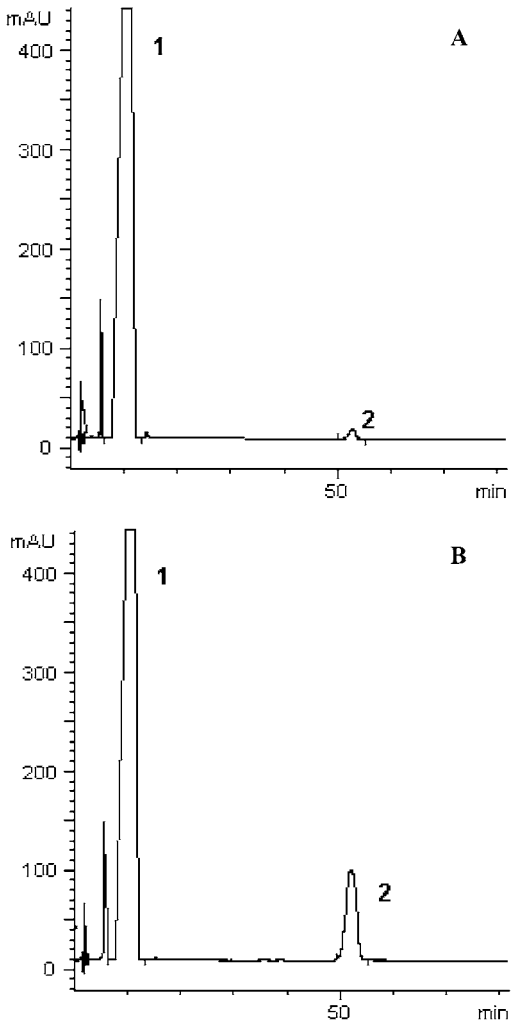

Figure 4. Amplified HPLC elution profile of a dimethyl sulfoxide extract of PDA culture medium in the presence of hesperidin (10 g/L) corresponding to a zone uninfected (**A**) and infected (**B**) by *P. citrophthora*. The column was eluted with water/methanol/acetonitrile/acetic acid (15:2:2:1 by volume) with a flow rate of 1 mL/min at 30 °C. Eluent was monitored at 280 nm. Identification: 1, hesperidin; 2, hesperetin.

isonaringin reached ~0.3 g/100 g of DW in the same zone (panels **A** and **C**, respectively, of **Figure 3**). The infected zones (zones 1−4) of the fruit had lower levels of both flavanone glycosides than the healthy zone (zone 5). These levels fell in all four zones, with the most pronounced decrease occurring in zones 1 and 2 (13% decrease in hesperidin and 67% decrease in isonaringin) (see **Figure 3A**,**C**).

A rise in the corresponding concentrations of aglycons was associated with this fall in flavanone glycoside concentration. The highest final aglycon concentrations were detected in zones showing the highest degree of infection, whereas they were practically undetectable in the healthy zone of the fruit (zone 5) (**Figure 3B**,**D**). For all of these reasons, it seems reasonable to think that the fall in the levels of these flavanone glycosides is due, at least partially, to the hydrolyzing action of the fungus, which breaks down the hesperidin and isonaringin molecules into hesperetin and naringenin, respectively, rhamnose, and glucose, in both cases. These results are in agreement with observations made previously for the defense mechanism of *Citrus aurantium* fruits against *Penicillium digitatum*, when the levels of naringin also fell (*11*).

This hydrolyzing action of *P. citrophthora* on the flavanone glycosides hesperidin and isonaringin is also evident from the

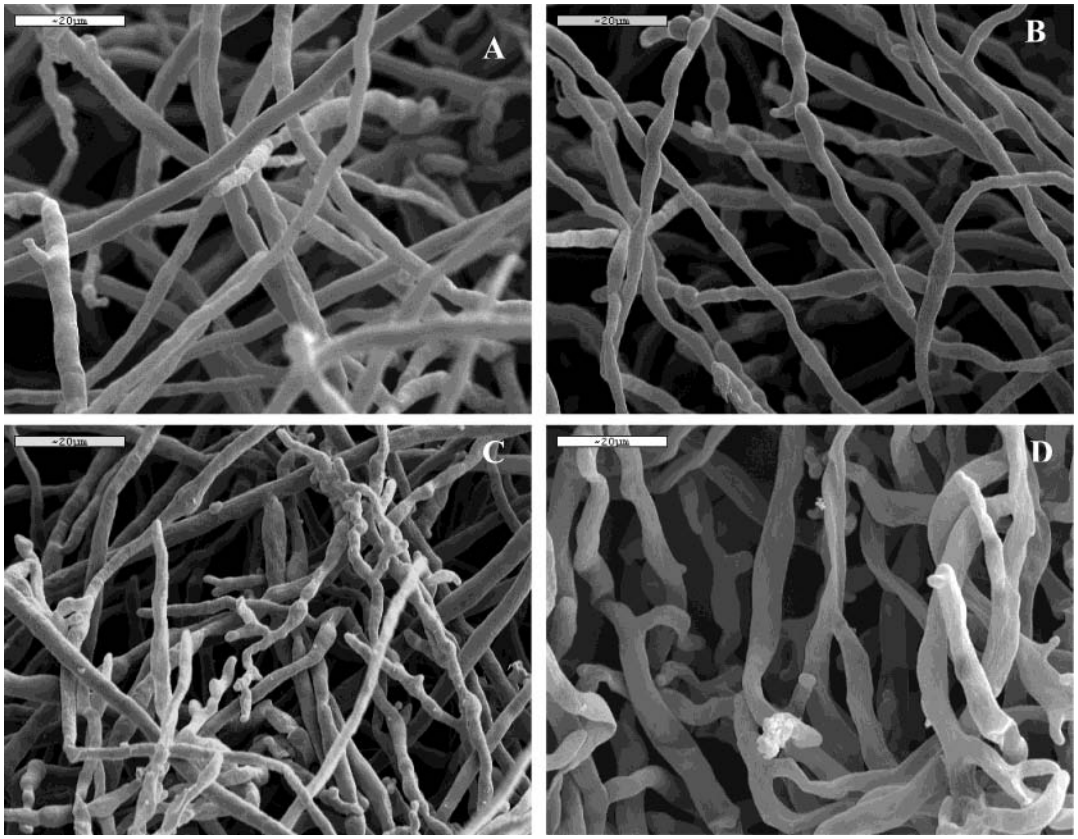

Figure 5. SEM image of the changes in hyphal morphology and growth of *P. citrophthora* mycelium cultured in control PDA culture medium (**A**) and in the same PDA culture medium to which hesperidin (19 mM) (**B**), hesperetin (0.21 mM) (**C**), or sinensetin (0.88 mM) (**D**) had been added.

in vitro experiments carried out after the incorporation of hesperidin and isonaringin isolated from *C. sinensis* (cv. Valencia Late) (10 g/L) in the corresponding PDA culture medium before inoculation with fungus. HPLC analysis of the extracts obtained in DMSO from the above culture media points to clear differences depending on whether they are from zones where the fungus is absent (PDA medium without mycelium) or present (PDA medium with mycelium) (panels **A** and **B**, respectively, of **Figure 4**). In the extracts from PDA medium without mycelium**,** hesperidin (compound **1**, $t_R = 12.52$ min, **Figure 4A**) and isonaringin ($t_R = 9.62$ min, data not shown) were the major compounds detected, whereas their corresponding aglycons were present at only trace levels (compound **2**, hesperetin, $t_R = 52.04$ min, **Figure 4B**; naringenin, $t_R = 41.33$ min, data not shown). However, in the extracts from PDA medium with mycelium, there was an increase in the area of compound **2** corresponding to hesperetin ($t_R = 52.04$ min, **Figure 4B**) or naringenin ($t_R = 41.33$ min, data not shown). Quantification of these compounds in the corresponding extracts showed that in these in vitro culture conditions, the quantity of hydrolyzed hesperidin and isonaringin after fungal development was ~4% in both cases.

Antifungal Potential of Compounds against *P. citrophthora*. The IC_{50} values obtained by in vitro study of the polymethoxyflavones isolated from this plant material revealed that nobiletin (0.62 ± 0.02 mM) is more active than sinensetin, heptamethoxyflavone, and tangeretin, which presented IC_{50} values of 0.88 ± 0.03, 1.06 ± 0.12, and 1.68 ± 0.09 mM, respectively. A comparison of the IC_{50} value for tangeretin against *P. citrophthora* and that obtained against *Penicillium digitatum* ($IC_{50} = 6.45 \pm 0.53$ mM) (*11*) shows that this polymethoxyflavone is more effective against *Phytophthora*.

With respect to the glycosylated flavanones, hesperidin ($IC_{50} = 19 \pm 2.3$ mM) is more active than isonaringin ($IC_{50} = 25 \pm 1.2$ mM) in inhibiting the growth of this fungus. These results reveal that in this plant material the glycosylated flavanones are less active than the polymethoxyflavones, as has been revealed in other plant materials (*11*). However, given that flavanones are present in higher levels than polymethoxyflavones (compare **Figures 3** and **2**) and that in certain *Citrus* species the flavanones may reach levels of around and even above 40% dry weight in the mature fruit (*6*, *21*, *22*), the possible physiological role of the flavanones in the defense mechanism of this species must be taken into account. Furthermore, as was mentioned above, it must be remembered that glycosylated flavanones are partially hydrolyzed by the fungus and that the corresponding aglycons generated, such as hesperetin and naringenin, are more active against *P. citrophthora* than their corresponding glycosylated flavanones and even the above-mentioned polymethoxyflavoness; they show IC_{50} values of 0.21 ± 0.01 and 0.19 ± 0.02 mM, respectively.

All of these flavonoids acted as fungistatic agents at the concentrations assayed, causing mycelial growth inhibition and marked abnormalities in hyphal size and morphology (compare panels **A**−**D** of **Figure 5**). The findings are in agreement with those obtained by other authors for different flavonic compounds against pathogenic fungi (*23*−*26*).

On the basis of the above results, we suggest that the constitutive secondary metabolites of *C. sinensis* studied (flavones and flavanones) may act as fungitoxins in the resistance mechanism against fungal attack, acting as first and second defense barriers, respectively, because polymethoxyflavones are mainly localized in the outermost tissue of the fruit, the flavedo (*8*), whereas flavanones (heperidin and isonaringin) are located

in the albedo, which is immediately below the flavedo (*8*). However, this finding does not discount the fact that other secondary compounds induced after infection, such as coumarins, may also act in the defense mechanism of this plant material, as has been described in other *Citrus* species (*19*, *20*).

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