Citrus Polymethoxylated Flavones Can Confer Resistance against *Phytophthora citrophthora*, *Penicillium digitatum*, and *Geotrichum* Species

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The polymethoxylated flavones observed in the essential oils of the byproducts of various *Citrus* fruits after industrial processing were as follows: sinensetin, nobiletin, and heptamethoxyflavone in clementine; sinensetin, quercetogetin, nobiletin, heptamethoxyflavone, and tangeretin in sour orange and sweet orange. *Penicillium digitatum* was totally inhibited (100%) by application of the polymethoxylated flavones present in the extracts from the clementine and sweet orange oils and 77% inhibited by those extracted from the sour orange oil. *Phytophthora citrophthora* was 100% inhibited by sour orange oil, 72% by clementine oil, and only 14% by sweet orange oil. *Geotrichum* sp. was more sensitive to the sour orange extract, being inhibited by ~57%, whereas clementine and sweet orange produced inhibition rates of 47 and 34%, respectively.

Keywords: *Clementine; heptamethoxyflavone; nobiletin; quercetogetin; sinensetin; sour orange; sweet orange; tangeretin*

INTRODUCTION

Of the flavonoids present in genus *Citrus* (family Rutaceae) the glycoside flavanones are accumulated in greatest quantity, although the concentration of these compounds depends on the age of the plant. The highest concentrations occur in tissues showing pronounced cell divisions, in which they may represent 40−45% of the dry weight (Castillo et al., 1992, 1993; Benavente-García et al., 1993; Del Río et al., 1995, 1997; Ortuño et al., 1995, 1997a). In addition to their possible physiological role in plant growth (Harborne, 1967; Jacobs and Rubery, 1988; Lynn and Chang, 1990), these compounds are of considerable commercial interest for the agrofood and pharmaceutical industries (Horowitz, 1964; Gábor, 1988; Salvayre et al., 1988; Francis et al., 1989; Guengerich and Kim, 1990; Chen et al., 1990; Bär et al., 1990; Benavente-García et al., 1997).

Coumarin production has been studied in this genus because of their role as phytoalexins in the resistance mechanisms against pathogens (De Lange et al., 1976; Khan et al., 1985; Vernenghi et al., 1987; Afek and Sztejnberg, 1988; Kim et al., 1991; Ortuño et al., 1997b).

However, less is known about other structural groups of *Citrus* flavonoids, the flavones and flavonols, although they have been studied in greater depth in other plant families, such as Asteraceae, Fabaceae, Labiatae, and Primulaceae, in which they are more abundant than in Rutaeae (Wollenweber and Jay, 1988; Marín et al., 1998).

The flavones in *Citrus* are found in glycosylated and aglycon states, the latter showing a greater variety of compounds with their structure frequently multisubstituted by hydroxyl and/or methoxyl groups. Among these polymethoxylated flavones are scutellarein (5,6,7,4′-tetramethoxyflavone), sinensetin (5,6,7,3′,4′-pentamethoxyflavone), tangeretin (5,6,7,8,4′-pentamethoxyflavone), quercetogetin (3,5,6,7,3′,4′-hexamethoxyflavone), nobiletin (5,6,7,8,3′,4′-hexamethoxyflavone), 3,5,6,7,8,3′,4′-heptamethoxyflavone, 7-hydroxy-3,5,6,3′,4′-pentamethoxyflavone, and 7-hydroxy-3,5,6,8,3′,4′-hexamethoxyflavone (Horowitz and Gentili, 1977; Tatum and Berry, 1972; Bianchini and Gaydou, 1981; Gaydou et al., 1987; Ooghe et al., 1994; Manthey and Grohmann, 1996; Chen et al., 1997; Del Río et al., 1998).

Polymethoxyflavones, like other flavonoids, play an important role in plants, acting as antioxidants and inhibitors of numerous enzymes such as phenolases (Challice and Willians, 1970) and pectinmethyltransferases (De Swardt et al., 1967). Moreover, because they show a characteristic distribution pattern, they can be used for taxonomic purposes (Ooghe et al., 1994). Furthermore, they have numerous pharmacological applications due to their antithrombogenic properties, which regulate human blood erythrocyte concentration and aggregation (Robbins, 1976), and cardiotonic action (Itoigawa et al., 1994). They have also been shown to have a cytotoxic effect toward cancerous cell lines (Kupchan et al., 1965) and to act as antimutagenics (Francis et al., 1989). Despite this, little is known about the part these secondary metabolites may play in the defense mechanism of plants against pathogenic attack, although sinensetin, nobiletin, and tangeretin have been described as having an antiviral and antimicrobial capacity. These compounds, together with the other components of the essential oil, probably confer a certain degree of resistance against microbial infections in *Citrus* (Ben-Aziz, 1967; Huet, 1982).

The objective of this study was to isolate and identify the polymethoxyflavones present in the essential oils obtained from byproducts of the *Citrus* processing industry, to see whether such byproducts may be considered as a potential source of these secondary compounds. In addition, the antifungal potential of

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these compounds against *Phytophthora citrophthora*, *Penicillium digitatum*, and *Geotrichum* sp. was studied.

MATERIALS AND METHODS

Materials. The materials used for polymethoxyflavone extraction were essential oils from clementine, sour orange, and sweet orange, provided by Antonio Muñoz and Co., Murcia, Spain.

Polymethoxyflavone Extraction and Purification. Carotenoids and terpenes were first separated from the different essential oils following the procedure described by Tatum and Berry (1972) with slight modifications. For this 500 mL of the essential oil from the different species was placed in a decantation funnel, to which was added 100 mL of 2-propanol and 80 mL of distilled water. Three extractions were made with hexane, using 20 mL in each extraction. The hexane used for solubilizing the carotenoids and terpenes was then poured off, and the 2-propanol/water phase was concentrated in a vacuum rotavapor (Heidolph VV2000, Kelheim, Germany). One 100 mL aliquot of water was added, and a liquid−liquid extraction was carried out with benzene (100 mL × 4).

The organic phase that separated the flavonoids was separated and concentrated in a rotavapor to a volume of 4−5 mL. The water contained in the sample was eliminated by using anydrous sodium sulfate.

Separation by Thin-Layer Chromatography (TLC) and Spectral Study. The polymethoxyflavones extracted from the essential oils were separated by TLC after 200 μL of the concentrate in benzene was placed on a silica gel layer with glass support (20 × 20 cm, layer thickness = 0.5 mm) (Merck, Darmstadt, Germany) using benzene/acetone (3:1, v/v) as eluent. The different compounds separated during chromatographic development were localized by their fluorescence when illuminated with UV at 360 nm, and their R_f values were calculated.

Two millimeters of the central part of each band was removed by scraping and solubilized in methanol for spectroscopic study and analysis by HPLC and mass spectrometry (MS).

The spectral study of each band isolated by TLC was carried out in a Unicam (Cambridge, U.K.) UV−vis spectrophotometer. The absorbance spectra of the different compounds were obtained between 200 and 400 nm. A spectral study was also made by adding two drops of 5% $AlCl_3$ in methanol to the solution of each band to ascertain whether hydroxyl substituents existed in the ortho position in the molecules.

HPLC and MS Analysis of the Polymethoxyflavones. The equipment used was a Hewlett-Packard HP 1050 equipped with a diode array detector.

The stationary phase was a (250 mm × 4 mm i.d.) C_{18} Spherisorb ODS column with a particle size of 5 μm thermostated at 30 °C. As solvent we used tetrahydrofuran (A), water (B), and acetonitrile (C) (Ooghe et al., 1994), optimized for our particular work conditions with a gradient profile of 12% A, 68% B, and 20% C during 20 min, changed to 18% B and 70% C in 20 min. At 45 min it began to change to its initial composition, a process that lasted 15 min. Eluent flow was 1 mL/min. The absorbance changes were recorded in the UV−vis diode array detector at 340 nm. The amounts of the polymethoxyflavones were determined from the area given by the integrator using the response factor of the corresponding standards.

Identification of the compounds isolated by TLC was carried out in a Hewlett-Packard mass spectrometer (model 5989).

Fungal Cultures and Measurement of Growth. Isolates of the fungi *P. citrophthora*, *P. digitatum*, and *Geotrichum* sp. were selected from the Spanish Type Culture Collection (Valencia, Spain) and cultured on potato dextrose agar (PDA) medium, at 25 °C, to serve as inoculum.

A 5 mm diameter disk of culture medium containig mycelium of these fungi was then placed in PDA culture medium (control) and in the same PDA culture medium to which a polymethoxyflavone extract (obtained from 500 mL of each essential oil concentrated to dryness and redissolved in 1 mL of methanol) had been added at a final concentration of 1%.

In the different assays, fungus growth was analyzed at different times after inoculation by measuring the corresponding mycelial diameters (millimeters).

Chemicals. Sinensetin and tangeretin were purchased from Extrasynthèse S.A. (Genay, France). Quercetogetin, heptamethoxyflavone, and nobiletin were obtained by TLC and identified by MS.

Table 1. Spot at 360 nm and R_f Values of the Compounds Separated by TLC from the Essential Oils of Clementine, Sour Orange, and Sweet Orange

oil	compd	spot (360 nm)	R_f
clementine	**1**	bright blue	0.31
	2	gray	0.51
	3	grayish brown	0.58
sour orange	**1**	bright blue	0.31
	2	pale blue	0.48
	3	gray	0.51
	4	bright blue	0.55
	5	grayish brown	0.58
sweet orange	**1**	bright blue	0.31
	2	pale blue	0.48
	3	gray	0.51
	4	bright blue	0.55
	5	grayish brown	0.58

RESULTS AND DISCUSSION

Chromatographic Characteristics of the Polymethoxyflavones Extracted. The different bands obtained by TLC separation of each of the essential oils are shown in Table 1. The bands were visualized under UV light (360 nm) and numbered in ascending order of their R_f values.

The bands obtained previously by TLC and solubilized in methanol were analyzed by HPLC to ascertain their purity and retention times. In each case the chromatograms showed only one peak, the purity of which was determined by superimposing the spectrum on that of the corresponding standard, both obtained with the diode array detector. The peaks showed a correlation coefficient of >0.99, so they were considered pure. The retention times for each of the components, corresponding to the different essential oils studied, are shown in Table 2.

Spectroscopic Characteristics of the Polymethoxyflavones Extracted. The spectrophotometric study of all the compounds separated by TLC and HPLC in all of the oils studied, for which R_f and t_R values are shown in Tables 1 and 2, pointed in all cases to a flavone skeleton with two absorbance maxima, one at 310−350 nm and a secondary one at 250−280 nm (Table 2).

The addition of $AlCl_3$ showed no bathochromic shift in any of the bands, meaning that these compounds cannot have a free hydroxyl group in position 5 or orthodiphenols in the ring preceding the shikimic acid pathway.

Identification and Quantification of Polymethoxyflavones Isolated from *Citrus* Essential Oils. The results obtained from MS analysis of the TLC isolated bands in the different essential oils, which were identified as pure substances by HPLC, reveal that the clementine, sweet orange, and sour orange oils showed the presence of a compound having a mass spectrum identical to that of the standard, sinensetin (see Figure 1) with peaks at *m*/*z* (relative intensity) 373 (44), 357 (100), 329 (5), 314 (6), 165 (9), and 162 (2) (see Figure

Table 2. Wavelength Maxima of the Compounds Separated by TLC, Retention Times after HPLC Analysis, and Identification by Mass Spectrometry

oil	compd	t_R	λ_{max}	identification	g/L[a]
clementine	**1**	12.5	265, 273, 329	sinensetin	0.9 ± 0.06
	2	16.6	245, 271, 331	nobiletin	0.04 ± 0.002
	3	17.2	253, 268, 340	heptamethoxyflavone	1.3 ± 0.3
sour orange	**1**	12.9	265, 273, 329	sinensetin	0.02 ± 0.003
	2	16.1	266, 334	quercetogetin	1.6 ± 0.1
	3	16.6	245, 271, 331	nobiletin	5.4 ± 0.2
	4	25.5	271, 324	tangeretin	1.4 ± 0.3
	5	17.2	253, 268, 340	heptamethoxyflavone	0.5 ± 0.07
sweet orange	**1**	12.9	265, 273, 329	sinensetin	0.1 ± 0.02
	2	16.1	266, 334	quercetogetin	0.1 ± 0.03
	3	16.6	245, 271, 331	nobiletin	1.0 ± 0.1
	4	25.5	271, 324	tangeretin	0.5 ± 0.03
	5	17.2	253, 268, 340	heptamethoxyflavone	2.5 ± 0.4

[a] Data of the polymethoxyflavone content (g/L) are means of three independent samples for each essential oil ± SE. Compounds **1**−**5** correspond to those mentioned in Table 1.

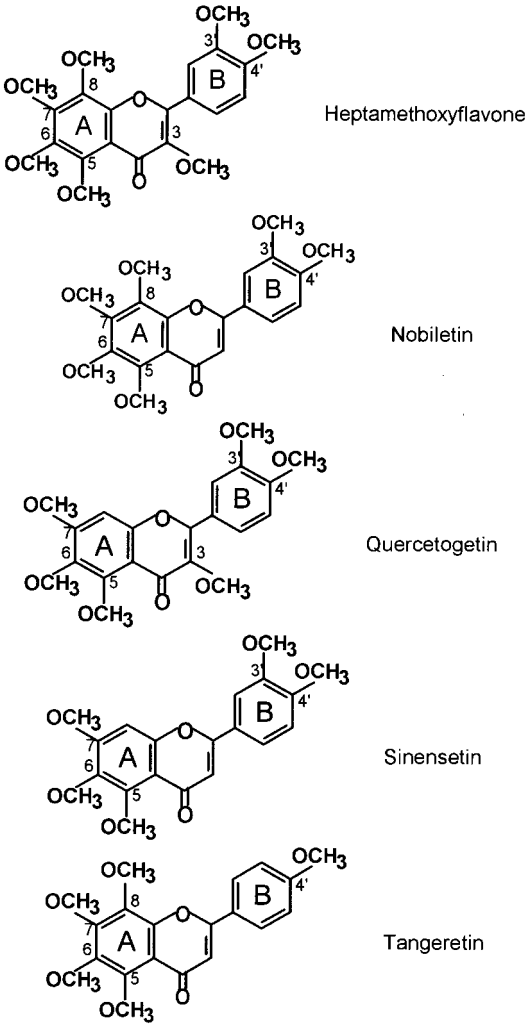

Figure 1. Chemical stuctures of heptamethoxyflavone, nobiletin, quercetogetin, sinensetin, and tangeretin.

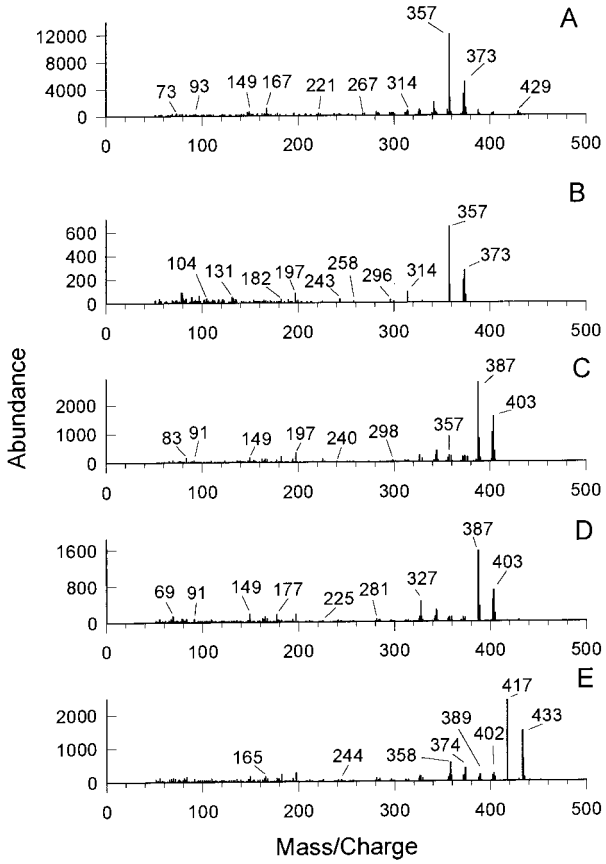

Figure 2. Mass spectra of polymethoxylated flavones isolated from *Citrus* essential oils: (A) sinensentin; (B) tangeretin; (C) nobiletin; (D) quercetogetin; (E) heptamethoxyflavone. The bars express the absolute abundance of the different ions.

2A). These corresponded, respectively, to the adduct formed by the molecular ion of the substance plus one proton $(M + H)^+$, to the fragment after loss of a methyl (M − 15) and characteristic of flavones with a methoxyl group in the 6 or 8 carbon positions of the A ring of its structure (Mabry and Markham, 1982), to the remaining fragment after loss of the carbonyl in C4 (M − 43), to the fragment resulting from loss of a second methyl (M − 58), and the fragments resulting from the molecule's partition and corresponding to two different ions of the B ring of the flavone structure, $B_1^{\bullet+}$ and $B_2^{\bullet+}$ (Mabry and Markham, 1982). The *m*/*z* values of 162 and 165 for the $B_1^{\bullet+}$ and $B_2^{\bullet+}$ fragments, respectively, point to the existence of two methoxyl groups in this B ring. On the other hand, no fragments corresponding to the A ring of the $(A_1 + H)^+$ molecule were found (Mabry and Markham, 1982).

The oils from sour orange and sweet orange showed the presence of a compound having a spectrum similar to the above with peaks at *m*/*z* (relative intensity) 373 (42), 357 (100), 329 (2), 314 (14), 243 (5), 135 (5), and 131 (8). The peaks corresponding to the $(M + H)^+$ fragments, M − 15, M − 43, and M − 58, coincided with those of sinensetin. However, there were differences in

the peaks corresponding to the $B_1^{\bullet+}$ and $B_2^{\bullet+}$ fragments, the *m/z* values of which at 131 and 135, respectively, revealed the presence of only one methoxyl in the B ring of the flavone structure, and to the $(A_1 + H)^+$ fragment, which did not appear in the case of sinensetin and which revealed the presence of four methoxyl groups in the A ring of the structure of this substance, which was identified therefore as tangeretin (see Figure 1), because its mass spectrum (Figure 2B) coincided with a standard supplied by Extrasynthèse. The identities of these two substances were additionally confirmed by their UV spectra, in which the absorbance maxima (λ_{max}) (Table 2) for sinensetin in clementine, sweet orange, and sour orange and for tangeretin in sour orange and sweet orange coincided with those quoted in the bibliography for these substances (Tatum and Berry, 1972; Machida and Osawa, 1989).

Another substance with a mass spectrum differing from that of tangeretin was identified in the sour orange, sweet orange, and clementine oils. This substance showed peaks at *m/z* (relative intensity) 403 (57), 387 (100), 359 (7), 344 (14), 165 (4), and 162 (4). The first peak, as in the above cases, corresponded to the $(M + H)^+$ fragment, indicating the presence in this compound's structure of one methoxyl more than in the case of sinensetin and tangeretin, meaning that it must be a hexamethoxyflavone. Peaks corresponding to the M − 15, M − 43, and M − 58 fragments were also visible, although the $(M + H)^+$ and M − 43 fragments were more abundant than in the above-mentioned pentamethoxyflavone. With regard to the peaks corresponding to the $(A_1 + H)^+$ and $B_2^{\bullet+}$ fragments, the spectrum of this compound showed the same signals as sinensetin, with no peak corresponding to the $(A_1 + H)^+$ fragment and the presence at *m/z* 162 and 165 of the peaks corresponding to fragments $B_1^{\bullet+}$ and $B_2^{\bullet+}$, respectively. These two fragments showed approximately the same relative presence as in the case of sinensetin. All of these data indicate that this substance isolated from the essential oils of sour orange, sweet orange, and clementine was the hexamethoxyflavone nobiletin (see Figures 1 and 2C). This was confirmed by its spectral characteristics (Table 2), which coincided with those of the bibliography (Tatum and Berry, 1972; Machida and Osawa, 1989).

Another substance identified in the sour orange and sweet orange oils showed a mass spectrum with peaks at *m/z* (relative intensity) 403 (44), 387 (100), 359 (7), 344 (15), 327 (29), 165 (8), and 162 (5). The fragments $(M + H)^+$, M − 15, M − 43, and M − 58 were identical to those observed for nobiletin, their proportions even being similar, which clearly indicated that it was a hexamethoxyflavone. As in the case of nobiletin, this compound showed no fragment identifiable as $(A_1 + H)^+$, whereas the fragments $B_1^{\bullet+}$ and $B_2^{\bullet+}$ at *m/z* 162 and 165, respectively, were present in greater abundance than in nobiletin.

The peak that differentiated this substance from nobiletin corresponds to the fragment at *m/z* 327 resulting from the loss of a hydroxyl from M − 58. This fragment was relatively abundant and typical of flavones substituted in C3 (Mabry and Markham, 1975). Although this substance had a molecular weight identical to that of the substance identified as nobiletin, both its mass spectrum and UV spectrum (Table 2) differed from those of nobiletin, for which reason it was identified as quercetogetin (see Figures 1 and 2D).

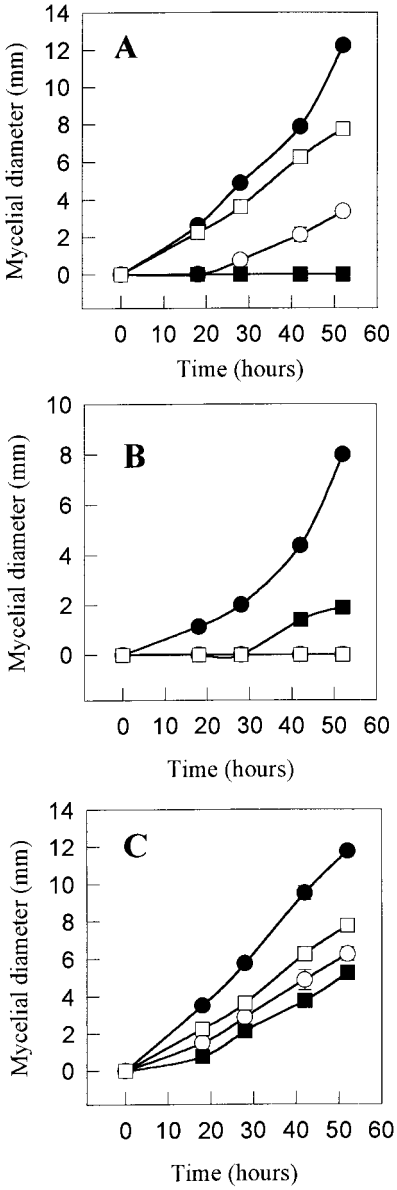

Figure 3. Effect of the polymethoxylated flavones present in the essential oils of *Citrus* sp. on the growth of *P. citrophthora* (A), *P. digitatum* (B), and *Geotrichum* sp. (C): clementine oil (○); sour orange oil (■); sweet orange (□) oil; control (●). Experiments were repeated three times. The data correspond to mean values of mycelial diameters (mm). Vertical bars denote ± SE when larger than symbols.

Furthermore, the oils from clementine, sour orange, and sweet orange showed the presence of a compound that has a mass spectrum with peaks at *m/z* (relative intensity) 433 (62), 417 (100), 389 (8), 374 (16), 358 (23), 244 (2), 165 (7), and 162 (2), which correspond to fragments $(M + H)^+$, M − 15, M − 43, M − 58, M − 75, $(A_1 + H)^+$, $B_1^{\bullet+}$, and $B_2^{\bullet+}$, respectively. The mass of the $(M + H)^+$ fragment showed this substance to be a heptamethoxyflavone, and the M − 75 showed that it was substituted in C3, whereas the $(A_1 + H)^+$ fragment indicated the presence of four methoxyl groups in the A ring of the flavone structure, confirming the substance's identity as a heptamethoxyflavone (see Figures 1 and 2E). This was further substantiated by the UV spectrum (Table 2) (Tatum and Berry, 1972; Machida et al., 1989).

On the basis of the results obtained (Table 2), we can say that sinensetin is more abundant in clementine (0.9

g/L) than in sweet orange and sour orange (0.1 and 0.02 g/L, respectively) essential oils. Heptamethoxyflavone is found in greater quantities in the oil from sweet orange (2.5 g/L) than in clementine (1.3 g/L) and sour orange (0.5 g/L) essential oils. Nobiletin is more abundant in sour orange essential oil (5.4 g/L) than in sweet orange and clementine (1 and 0.04 g/L, respectively) essential oils. Quercetogetin is more abundant in sour orange (1.6 g/L) than in sweet orange (0.1 g/L) essential oils. Tangeretin is found in greater quantities in the oil from sour orange (1.4 g/L) than in sweet orange (0.5 g/L).

Antifungal Action of Polymethoxylated Flavones from *Citrus* against *P. citrophthora*, *P. digitatum*, and *Geotrichum* Species. Figure 3 shows the effect of the polymethoxyflavone extracts isolated from *Citrus* essential oils on *P. citrophthora* (Figure 3A), *P. digitatum* (Figure 3B), and *Geotrichum* sp. (Figure 3C).

P. citrophthora was 100% inhibited by the "sour" extract (sour orange), 72% by clementine oil, and only 14% by sweet orange oil (Figure 3A) with respect to its growth in the respective control assays.

P. digitatum was totally inhibited (100%) by application of the extracts from the so-called "sweet" *Citrus* species (clementine, sweet orange) and 77% inhibited by those extracted from the "sour" species (sour orange) (Figure 3B) with respect to its growth in the respective control assays.

Geotrichum sp. was more sensitive to the "sour" extract, with an inhibition of ~57%, whereas clementine and sweet orange led to inhibition rates of 47 and 34%, respectively (Figure 3C), with respect to control assays.

Bearing in mind that these secondary compounds are found only in the peel (Cheng et al., 1985; Machida and Osawa, 1989; Manthey and Grohmann, 1996; Del Río et al., 1998), the idea proposed by Wollenweber (1994) that they may play a protective role against pathogenic attack is supported by our findings. The above-described strong antifungal effect of the sour extract against *Phytophthora* might partly explain the natural high degree of resistance of *Citrus aurantium* (sour orange) against this fungus.

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