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Citrus paradisi and *Citrus sinensis* flavonoids: Their influence in the defence mechanism against *Penicillium digitatum*

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Abstract

Citrus peel is rich in flavanone glycosides and polymethoxyflavones. In view of their importance for industrial application as well as for their pharmacological properties, their content was analyzed in the mature fruits of several *Citrus paradisi* (grapefruit) and *Citrus sinensis* (orange) varieties, with a view to select the most interesting for isolation. The results shows that the Star Ruby grapefruit and the Sanguinelli orange stand out for their high contents of naringin and hesperidin, respectively. The presence of the polymethoxyflavones nobiletin, heptamethoxyflavone and tangeretin, could be ascertained in all the grapefruit varieties analysed. Higher polymethoxyflavone levels were recorded in orange, with Valencia Late showing the greatest nobiletin, sinensetin and tangeretin contents and Navelate the highest heptamethoxyflavone levels. An in vitro study revealed that these compounds acted as antifungal agents against *Penicillium digitatum*, the polymethoxyflavones being more active than the flavanones in this respect. The possible participation of these phenolic compounds in the defence mechanism of *Citrus* against *P. digitatum* is discussed.

Keywords: *Citrus*; Orange; Grapefruit; Flavanones; Polymethoxyflavones; *Penicillium digitatum*

1. Introduction

Green mold (caused by *Penicillium digitatum*) is one of the most important post- harvest diseases in *Citrus* fruits (Holmes & Eckert, 1995). To prevent the development of this pathogen and to limit losses in commercial fruit shipments, treatment with chemical fungicides is a widely used procedure. However, such treatment may produce serious problems, with residues on the fruit (Cabras, Schirras, Pirisi, Garau, & Angioni, 1999), appearance of fungicide-resistant strains of *P. digitatum* (Ben-Yehoshua, Goldschmidt, & Bar-Joseph, 1994), and their possible accumulation in human adipose tissue constituing an additional health threat (Suwalsky, Rodríguez, Villena, Aguilar, & Sotomayor, 1999). An alternative strategy in the battle against this pathogen would be to alter the plant natural defence mechanisms. In this respect, some, but very few, studies have looked at the possible role that phenolic compounds might play as phytoalexins in some *Citrus* species (Arcas, Botía, Ortuño, & Del Río, 2000; Ben-Aziz, 1967; Del Río, Arcas, Benavente-García, & Ortuño, 1998, 2004a; Ortuño et al., 1997a, Ortuño, Arcas, Botía, Fuster, & Del Río, 2002).

The peel of *Citrus* fruits is a rich source of flavanones and many polymethoxylated flavones, which are very rare in other plants (Horowitz, 1961). Naringin and hesperidin are the principal flavanones in *Citrus paradisi* (grapefruit) and *Citrus sinensis* (orange), respectively (Albach & Redman, 1969; Berhow & Vandercook, 1989, 1991; Del Río & Ortuño, 1994; Fuster, 1997; Jourdan, McIntosh, & Mansell, 1985; Ortuño et al., 1995).

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However, little is known about the occurrence of polymethoxyflavones in *C. paradisi*, while previous studies in *C. sinensis* have 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 (Del Río et al., 1998, 2004a; Ooghe, Ooghe, Detavernier, & Huyghebaert, 1994).

These compounds, not only play an important physiological and ecological role, but are also of commercial interest because of their multitude of applications in the food and pharmaceutical industries. For example, naringin may act as an antioxidant (Chen, Zheng, Jia, & Ju, 1990), protecting against lipid peroxidation (Salvayre, Negre, Affany, Lenoble, & Douste-Blazy, 1988; Guengerich & Kim, 1990), and as an antimutagenic agent (Francis, Shetty, & Bhattacharya, 1989). Furthermore, naringin can be used as an alternative to caffeine or quinine in tonic beverages and other non-alcoholic drinks and produces a distinct bitter taste at low concentration (Anon., 1982). Hesperidin influences vascular permeability, increases capillary resistance and exhibits analgesic and antiinflammatory properties (Gabor, 1988; Emim, Oliveira, & Lapa, 1994). It is also an effective antioxidant, since it is able to quench the oxygen free radicals wich are involved in cancer (Berkarda, Koyuncu, Soybir, & Baykut, 1998; Fujiki et al., 1986; Lonchampt et al., 1989). Naringin and hesperidin also have another important industrial application in that they can be chemically converted into their corresponding intensely sweet dihydrochalcones (Bär, Borrego, Benavente, Castillo, & Del Río, 1990; Horowitz & Gentili, 1963; Horowitz, 1986; Krbechek et al., 1968).

Polymethoxyflavones are also of interest for their pharmacological potential (Benavente-García, Castillo, Sabater, & Del Río, 1997; Middleton & Kandaswami, 1992; Robbins, 1980), the most important of which are their anticarcinogenic properties (Chen, Montanari, & Widmer, 1997) due to their ability to absorb UV light (Shimoi, Masuda, Furogori, Esaki, & Kinae, 1994), their antimutagenic and antiproliferative effects against tumours (Bracke et al., 1994; Kandaswami, Perkins, Soloniuk, Drzewiecki, & Middleton, 1991; Stapleton & Walbot, 1994), their anti-inflammatory activities (Midleton & Drzewicki, 1982; Midleton, 1986), and their anti-allergic and analgesic properties (Gabor, 1986).

The objective of this work was to investigate the principal flavanones and polymethoxyflavones present in the mature fruit of *C. paradisi* and *C. sinensis* in order to identify those of most interest for isolation and industrial use. In addition, an attempt was made to assess the possible physiological role that these compounds may play in the defence mechanisms against *P. digitatum*.

2. Materials and methods

2.1. Plant material

Mature fruits of different *C. paradisi* L. and *C. sinensis* L. Osbeck varieties were used in the different experiments.

Varieties of *C. paradisi* L. used were Star Ruby, Marsh, Shambar and Red Blush, grafted onto Cleopatra mandarin orange, from a commercial plantation located in La Palma (Murcia, Spain).

Varieties of *C. sinensis* (L.) Osbeck used were Valencia Late and Summer Navel from a commercial plantation located in Alhama (Murcia, Spain); Navelate, from a commercial plantation located in Aguilas (Murcia, Spain), and Sanguinelli from the experimental plantation of the Instituto Murciano de Investigación y Desarrollo Agrario y Alimentario (IMIDA) in La Alberca (Murcia, Spain), respectively.

2.2. Extraction and measurement of flavonoids

Flavonoids were extracted and measured in whole fruit, which were dried at 50 °C to constant weight immediately after harvest. The dried fruits were ground and extracted with dimethylsulphoxide (DMSO) (Castillo, Benavente-García, & Del Río, 1992) for 2 h in a ratio of 40 mg of dry weight/ml in the case of the polymethoxyflavones extraction and 6 mg of dry weight/ml for the flavanone glycosides.

The resulting extracts were filtered through a 0.45-μm nylon membrane before analysis in a Hewlett–Packard liquid Chromatograph, model HP1050 (USA) coupled to a quaternary pump and automatic injector with a diode array detector (range scanned: 220–500 nm). The stationary phase was a Hewlett–Packard C_{18} (LiChroCART®, USA) analytical column with an average particle size of 5 μm (250 × 4-mm i.d.) at 30 °C. For the isocratic separation of flavanone glycosides, a mixture of water:methanol:acetonitrile:acetic acid (15:2:2:1) was used as solvent (Castillo et al., 1992). For polymethoxyflavones, the stationary phase was the same, but the solvent consisted of a tetrahydrofurane (A): water (B): acetonitrile (C) (Ooghe et al., 1994) 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 (Del Río et al., 1998). 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 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.

For the isolation of these compounds, a Hewlett–Packard C_{18} (LiChroCART®, USA) semipreparative column with an average particle size of 5 μm (250 × 10-mm i.d.) at 30 °C was used and, as solvent, the same as described above for flavones and flavanone glycosides. 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 in a Hewlett–Packard mass spectrometer (model 5989). For this, the flavanone glycosides were previously hydrolyzed with 2 N H_2SO_4 and purified according to previous papers (Castillo et al., 1992).

2.3. Fungal cultures and "in vitro" study of the antifungal activity of grapefruit flavonoid compounds

An isolate of the fungus *P. digitatum* obtained from the Spanish Collection of Type Culture (Valencia, Spain) (CECT 2954) was cultured on potato dextrose agar (PDA) medium at 25 °C to serve as inoculum.

For the measurement of the antifungal activity of the polymethoxyflavones (nobiletin, sinensetin, heptamethoxyflavone and tangeretin) and flavanones (naringin and hesperidin) isolated from the plants materials described above, a 5-mm diameter disk of culture medium containing mycelium of this fungus was placed in PDA culture medium (control) or in the same PDA culture medium to which a known concentration of the secondary compound described above had previously been added. In each case, fungus growth was analyzed at different times after inoculation by measuring the corresponding mycelial radius (centimetres). The effect of the phenolic compounds added to the media on the fungal growth was studied by the corresponding transmission electron microscopy, as described in previous papers (Del Río et al., 2001). In these media, the sporulation of the fungi was also studied at the macroscopic level.

2.4. Chemicals

Sinensetin and tangeretin were purchased from Extrasynthèse S.A. (Genay, France). Heptamethoxyflavone and nobiletin were isolated by semipreparative HPLC and identified by MS (Del Río et al., 1998). Hesperidin and naringin were obtained from the Sigma Chemical Co. (USA).

3. Results and discussion

3.1. Polymethoxyflavone and flavanone levels in C. paradisi L. fruits

The HPLC study on the flavanone glycoside extracts of different varieties of *C. paradisi* revealed the presence of one principal compound ($Rt = 11.7$ min). The absorption spectrum of this compound, obtained by means of a UV/Vis diode array detector, was in accordance with that obtained for compounds with a flavanone skeleton (maxima at 283 and 326 nm) identical to naringin (Fig. 1). This compound was isolated by the procedure described in Section 2. The MS of this compound was identical to that obtained for the naringin standard.

On the other hand, the HPLC analysis of the polymethoxyflavone extracts from these plant materials showed the

Flavonoids	R8	R7	R6	R5	R2-R3	R3	R4'	R3'	Name
Flavanones	H	α1-6 Rham-Glc	H	OH	Single bond	H	OCH_3	OH	HESPERIDIN
	H	α1-2 Rham-Glc	H	OH	Single bond	H	OH	H	NARINGIN
Polymethoxyflavones	H	OCH_3	OCH_3	OCH_3	Double bond	H	OCH_3	OCH_3	SINENSETIN
	OCH_3	OCH_3	OCH_3	OCH_3	Double bond	H	OCH_3	H	TANGERETIN
	OCH_3	OCH_3	OCH_3	OCH_3	Double bond	H	OCH_3	OCH_3	NOBILETIN
	OCH_3	OCH_3	OCH_3	OCH_3	Double bond	OCH_3	OCH_3	OCH_3	HEPTAMETHOXYFLAVONE

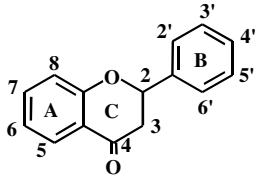

Fig. 1. Chemical structures of the flavanones, naringin and hesperidin, and the polymethoxyflavones sinensetin, tangeretin, heptametoxyflavone and nobiletin.

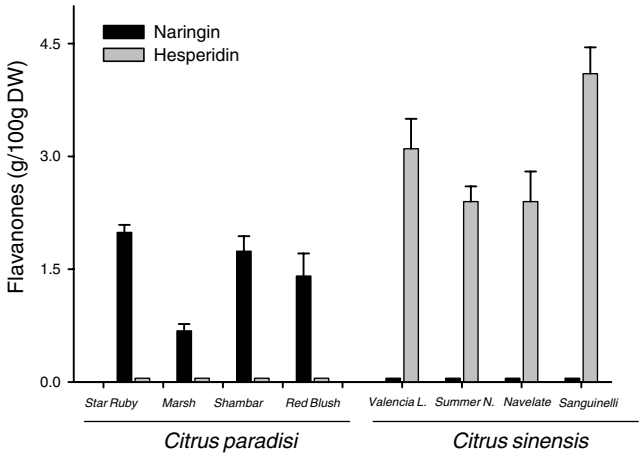

Fig. 2. Levels of the flavanones, naringin and hesperidin, in mature fruits of different varieties of *Citrus paradisi* (grapefruit) and *Citrus sinensis* (orange). The data represent mean values ± SD ($n = 3$) of these secondary metabolites (g/100 g DW).

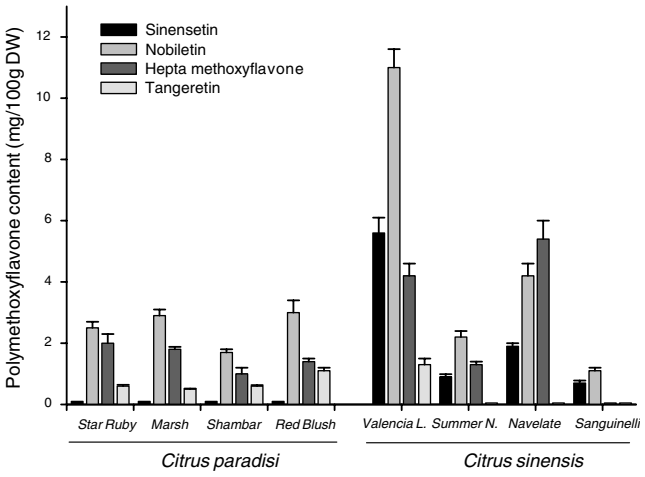

Fig. 3. Levels of the polymethoxyflavones, sinensetin, nobiletin, heptamethoxyflavone and tangeretin, in mature fruits of different varieties of *Citrus paradisi* (grapefruit) and *Citrus sinensis* (orange). The data represent mean values ± SD ($n = 3$) of these secondary metabolites (mg/100 g DW).

presence of three compounds with retention times which coincided with those the polymethoxyflavone, nobiletin (compound 1, $Rt = 16.6$ min), heptamethoxyflavone (compound 2, $Rt = 17.2$ min) and tangeretin (compound 3, $Rt = 25.5$ min). The absorption spectra of these compounds obtained by means of a UV/Vis diode array detector showed three maxima, at 245, 271 and 331 nm for compound 1, at 253, 268 and 340 for compound 2, and two maxima, at 271 and 324 nm for compound 3. The MS spectra of these compounds were identical to those obtained for nobiletin, heptamethoxyflavone and tangeretin (Fig. 1) in previous papers (Del Río et al., 1998).

Fig. 2 shows the levels of the flavanone naringin found in the mature fruit of the different varieties of grapefruit studied. The highest levels of naringin were detected in Star Ruby (2 g/100 g DW), followed by Shambar (1.7 g/100 g DW), Red Blush (1.4 g/100 g DW) and Marsh (0.7 g/100 g DW). These results were in agreement with previous results obtained for this and other *Citrus* species, in which flavanone levels were reported to be dependent on the variety (Del Río et al., 2004b; Ortuño et al., 1995, 1997b). However, this is the first time that polymethoxyflavones have been described in the above varieties of *C. paradisi*. Fig. 3 shows that the highest levels of nobiletin were detected in Red Blush and Marsh (around 3 mg/100 g DW), followed by Star Ruby (2.5 mg/100 g DW), and Shambar (1.7 mg/100 g DW). The highest heptamethoxyflavone levels were detected in Star Ruby (2.0 mg/100 g DW), followed by Marsh (1.8 mg/100 g DW), Red Blush (1.4 mg/100 g DW) and Shambar (1.0 mg/100 g DW), respectively. The highest tangeretin levels were detected in Red Blush (1.1 mg/100 g DW), while the other varieties showed similar levels of around 0.6 mg/100 g DW.

The results show that polymethoxyflavones are minority compounds in *C. paradisi* (Fig. 3) and occur in concentrations much below those of the flavanones (Fig. 2). These findings are in agreement with those obtained for other species and *Citrus* hybrids (Del Río et al., 2004a, 1995; Ortuño et al., 2002).

3.2. Polymethoxyflavone and flavanone levels in C. sinensis (L.) Osbeck fruits

The HPLC study of different extracts of *C. sinensis* revealed the presence of one principal compound with a retention time which coincided with that of the flavanone

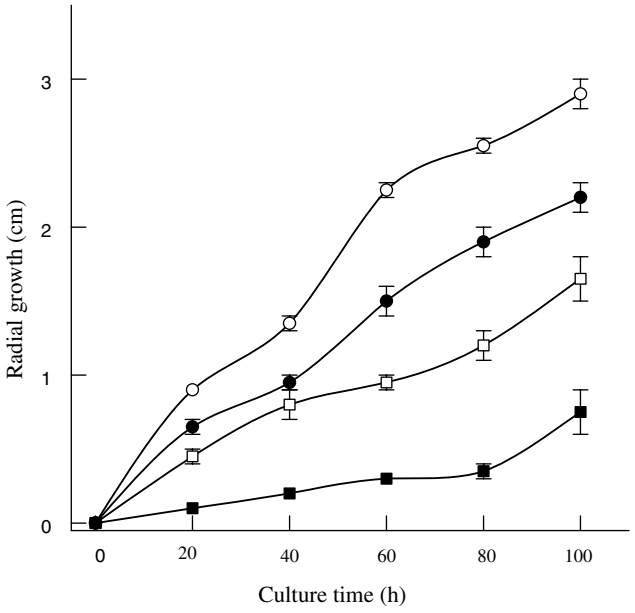

Fig. 4. Effects of naringin (•), hesperidin (□), and nobiletin (■) 8 mM on the growth of *P. digitatum*. The control is represented by (○). The data correspond to mean values of mycelial radius (cm). Verticals bars denote ±SD ($n = 3$) when larger than symbols.

rutinoside, hesperidin ($Rt = 15.5$ min). Both the absorption spectrum (UV/Vis diode array detector) and the MS analysis of this compound were identical to those obtained for hesperidin in previous studies (Del Río et al., 2004b).

The mature fruit of the pigmented orange variety, Sanguinelli, showed the highest hesperidin levels (4.1 g/100 g DW), similar to those found in previous studies involving tangelo Nova, a mandarin hybrid (*Citrus reticulata* B) × tangelo orlando (*Citrus reticulata* × *C. paradisi* Macf.) (Del Río et al., 1995). The other orange varieties analysed (Valencia Late, Summer Navel, Navelate) showed lower hesperidin levels (Fig. 2).

Fig. 3 shows the levels of polymethoxyflavones found in the different varieties of *C. sinensis*. Nobiletin and sinensetin (Fig. 1) were present in all the varieties analysed but in different concentrations, these being the only polymethoxyflavones found in the orange, Sanguinelli.

The highest concentration of nobiletin was detected in Valencia Late (11 mg/100 g DW), followed by Navelate (4.2 mg/100 g DW), Summer Navel (2.2 mg/100 g DW) and Sanguinelli (1.1 mg/100 g DW).

The highest concentration of sinensetin was detected in Valencia Late (5.6 mg/100 g DW), followed by Navelate (1.9 mg/100 g DW), Summer Navel (0.9 mg/100 g DW) and Sanguinelli (0.7 mg/100 g DW).

Heptamethoxyflavone, on the other hand, was at its highest level in Navelate (5.4 mg/100g DW), followed by Valencia Late (4.2 mg/100g DW). Summer Navel contained approximately 1.3 mg/100 g DW, while this polymethoxylflavone was not detected in the orange, Sanguinelli. Tangeretin was only detected in Valencia Late (1.3 mg/100 g DW) (Fig. 3).

Taking into consideration the potential for industrial and pharmacological applications, the results outlined above show Sanguinelli to be the most interesting orange variety for isolating hesperidin, while Valencia Late is the most promising variety for obtaining the polymethoxyflavones, nobiletin, sinensin and tangeretin, and Navelate for heptamethoxyflavone.

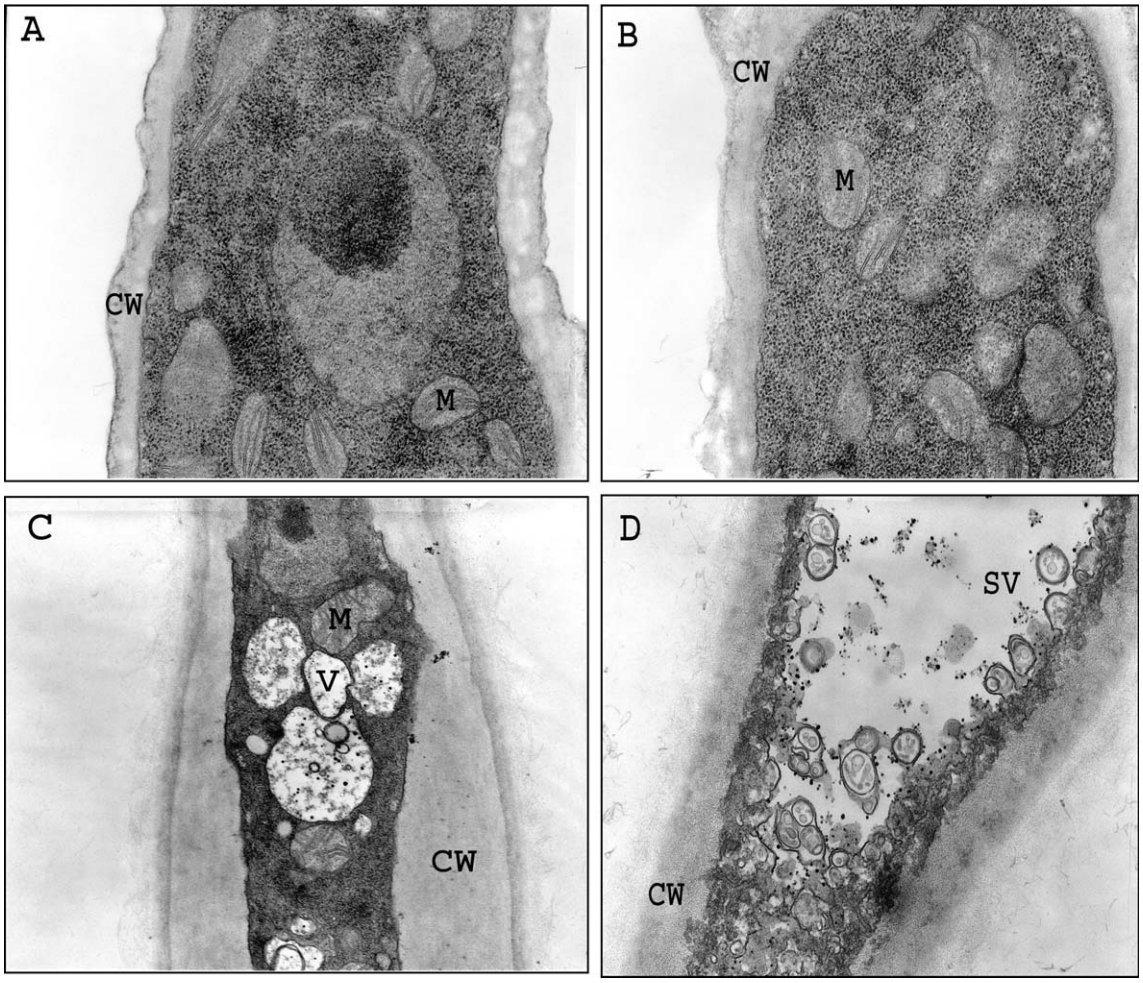

Fig. 5. Transmission electron micrographs of the hyphae of *P. digitatum* in control PDA culture medium (A and B) and in the same PDA culture medium to which nobiletin (8 mM) had been added (C and D). CW, cell wall. V, vacuoles. M, mitochondria. SV, secretory vesicles. 20,000×.

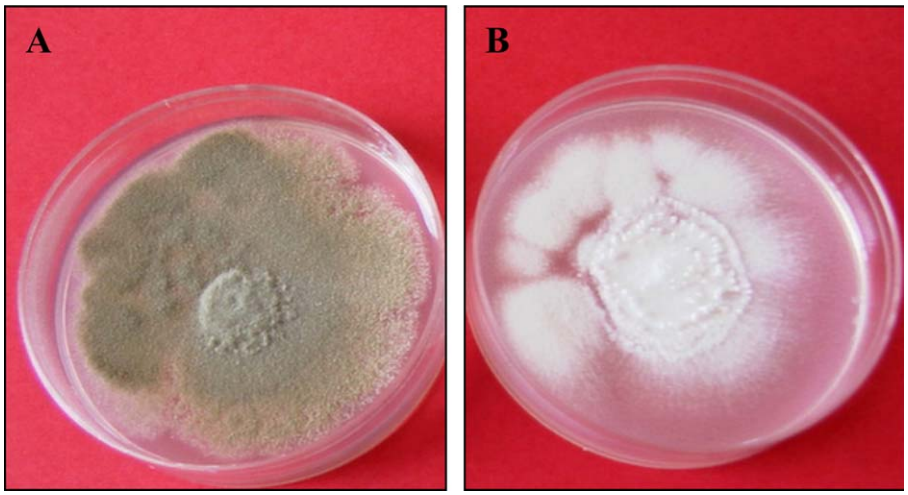

Fig. 6. *Penicillium digitatum* mycelial growth and sporulation inhibition by nobiletin (8 mM) added to the culture medium (B). Control (A).

3.3. Antifungal action of Citrus flavonoids against Penicillium digitatum

In vitro studies revealed that some flavonoids isolated from *C. paradisi* and *C. sinensis* fruits, when added to a PDA culture medium, reduce the radial growth of *P. digitatum* (Fig. 4). The polymethoxyflavone nobiletin was the most effective compound in this sense, followed by the flavanones hesperidin and naringin, the growth of the fungus one hundred hours after the start of the culture being inhibited by 75%, 38% and 25%, respectively, compared with the growths observed in the corresponding controls. This greater effectiveness of the polymethoxyflavone as fungitoxin, compared with the two flavanones, despite its occurrence in much lower concentrations, has been described in other species (Arcas et al., 2000).

Besides this inhibition of the radial growth of the fungus, different ultrastructural modifications in the hyphae were observed when *P. digitatum* was cultured in the presence of nobiletin (8mM) (Fig. 5) and the other flavanones assayed here (data not shown). The cell walls of the hyphae are nearly three times thicker, following exposure to the nobiletin, than those of untreated control. Furthermore, after nobiletin treatment, a smaller cytoplasmic density was observed, large vacuoles with an opaque content and many small droplets that are probably secretory vesicles bordering the plasma membrane when compared with untreated hyphae (see Fig. 5). These findings are in close agreement with those obtained by other authors for different phenolic compounds against pathogenic fungi (Amborabé, Fleurat-Lessard, Chollet, & Roblin, 2002; Rivera-Vargas, Schmitthenner, & Graham, 1993). In addition, an inhibition of spore production in the fungus was observed in the presence of nobiletin (Fig. 6) and of both naringin and hesperidin (data not shown).

The results obtained, and the fact that the compounds described are mainly located in the peel (the polymethoxyflavones in the flavedo and flavanones in the albedo), lends weight to the idea that they all play an active role in the protection of fruit against pathogen attack. Moreover, the application of extract enriched in these compounds to the citrus could be a natural way of sanitation.

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