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Role of *Citrus* Phenolic Compounds in the Resistance Mechanism against Pathogenic Fungi

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ABSTRACT

Fungi of the genera *Penicillium* and *Alternaria* are responsible for substantial post and pre-harvest losses in *Citrus*. To prevent their development and reduce commercial losses, chemical fungicides are used. However, such treatments can result in serious problems, such as residues on the fruit and the development of fungicide resistant strains. For this reason, it was of pressing interest to investigate alternatives to chemicals, such as flavonoids, that are believed to be involved in the defence mechanism of the genus, *Citrus*. The results obtained show that in the case of infection by *Penicillium digitatum*, certain citric flavonoids may well be involved in the defence mechanism, and may be considered as a phytoalexin against this fungus and to act as a chemical barrier. Although these secondary compounds exhibited phytotoxic activity against *Alternaria alternata* pv. citri in *in vitro* studies, the effect was not sufficient to slow down the development of the fungus. This fungus has developed a mechanism to metabolise these phenolic compounds which actually promotes the development of necrotic areas in the fruit, through which the fungus to spread more easily.

Keywords: *Alternaria alternata* pv. citri, *Citrus paradisi*, *Citrus sinensis*, defence mechanism, flavanones, 'Fortune', *Penicillium digitatum*, phytoalexins, polymethoxyflavones

INTRODUCTION

Fungi of the genera *Penicillium* and *Alternaria* are responsible for substantial losses in *Citrus* fruit, mainly postharvest in the case of the former, and pre-harvest in the case of the latter. However, susceptibility to *Penicillium digitatum* and *Alternaria alternata* pv. citri depends on the citrus species. Many studies have demonstrated that cultivars of *Citrus reticulata* and its hybrids, including tangelos 'Minneola' and 'Orlando', the tangor 'Murcott', and the hybrids 'Nova', 'Farchild', 'Lee', and 'Sunburst' are susceptible to *A. alternata* pv. citri. In contrast, the Satsumas (*Citrus unshiu* Mark. Marc.) and the Clementines (*Citrus clementina* Hort. ex Tan.) show a certain degree of resistance and other species, such as *Citrus sinensis*, *Citrus limon* (L.) Burm., and *Citrus margarita* (Lour.) Swing. are resistant to the pathogen (Pegg 1966; Gardner *et al*. 1986; Kohmoto *et al*. 1991; Vicent *et al*. 2004; Reis *et al*. 2007). In the case of *Penicillium digitatum*, similar studies have been carried out by our group and these will be discussed herein.

To prevent the development of these diseases, treatment with chemical fungicides is a widely used procedure. In some cases, such treatment may produce serious problems, including residues on the fruit (Cabras *et al*. 1999), which can accumulate in human adipose tissue eventually affecting to human health (Suwalsky *et al*. 1999) and the appearance of fungicide-resistant fungal strains (Ben-Yehoshua *et al*. 1994). In other cases, copper products used in the management of *Alternaria* (Timmer 2003) may cause stippling of the fruit when applied during periods of high temperatures and so must be used with caution.

An alternative strategy in the fight against these infections would be to modulate the natural defence mechanisms of the plant, in which certain phenolic compounds might be involved. For example, it is known that the peel of citrus fruit accumulates a series of species-specific flavanones and polymethoxyflavanones such as: hesperidin, naringin, sinensetin, tangeretin, heptamethoxyflavone and nobiletin (Albach and Redman 1969; Jourdan *et al*. 1985; Del Río and Ortuño 1994; Ooghe *et al*. 1994; Ortuño *et al*. 1995, 1997a; Del Río *et al*. 1998a, 2004a). A few studies also mention the role that some of these phenolic compounds may play as phytoalexins in some species of *Citrus* (Ben-Aziz 1967; Ortuño *et al*. 1997b; Del Río *et al*. 1998b; Arcas *et al*. 2000; Ortuño *et al*. 2002; Del Río *et al*. 2004b).

The objective of this work was to investigate the possible physiological role of the principal flavanones and polymethoxyflavones present in the *Citrus* sp. in the defence mechanisms against these fungi.

MATERIALS AND METHODS

Plant material

For the different assays we used mature fruits of 'Fortune' hybrid (*Citrus clementina* × *Citrus tangerina*), *Citrus limon* cv. 'Fino-49' and *Citrus paradisi* cv. 'Marsh'. For some of the analyses 'Fortune' fruit in different stages of development (120, 127, 134, 154, 168, 205, 219, 249 and 259 days after anthesis) were used. The 'Fortune' trees used were from a commercial orchard situated in Campo de Cartagena (Murcia, Spain), while *C. limon* cv. 'Fino-49' and *C. paradisi* cv. 'Marsh' trees grew in the IMIDA field station at La Alberca (Murcia, Spain).

Extraction and measurement of flavonoids

Whole fruits were used in each assay for the extraction and measurement of flavonoids. These fruits were dried at 50°C to constant weight immediately after collection. The dried fruits were ground to a powder and shaken with dimethylsulphoxide (DMSO) (Castillo *et al*. 1992) for 2 h in a proportion 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 HP 1050) (USA) coupled to a quaternary pump and automatic injector with a diode array detector (range scanned: 220-500 nm). The stationary phase was a C_{18} column (250 mm × 4

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mm i.d., particle size of 5 μm, thermostated 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, and as solvent we used 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*. 1998a). Eluent flow was 1 ml/min, in all cases.

The absorbance changes were recorded in a V/UV 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 C_{18} semipreparative column (250 mm × 10 mm i.d.) with a particle size of 5 μm thermostated at 30°C was used, and as solvent the same as described above for flavones and flavanone glycosides. Eluent flow was 3ml/min in all cases. The main flavonic compounds in these extracts were collected with a fraction collector (Gilson FC 203B) at the exit of the HPLC column for identification by mass spectrometry (Thermoquest TRACE/MS).

Fungal cultures, estimation of IC_{50}, fruit inoculation, and measurement of growth

An isolate of the fungus, *P. digitatum,* obtained from the Spanish Collection of Type Culture (Valencia, Spain) (CECT 2954) and *A. alternata* pv. citri (supplied by A. Lacasa IMIDA-Murcia-Spain) were cultured on potato dextrose agar (PDA) medium at 25°C for use as inoculum.

The antifungal activity of the polymethoxyflavones (nobiletin, sinensetin, heptamethoxyflavone and tangeretin), flavanones glycosides (naringin and hesperidin), and the corresponding aglycons (naringenin and hesperetin) isolated from *Citrus paradisi*, 'Fortune' and *Citrus limon* fruits were determined by *in vitro* assays with *P. digitatum* and *A. alternata* pv. citri following procedures described in previous papers (Del Río *et al*. 1998b; Arcas *et al*. 2000). 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, the fruits of the three citrus materials were sprayed with 90% ethanol and placed on trays to be inoculated. Twenty similar fruits were used in each of the inoculation assays described below, in which mycelium of *P. digitatum* or *A. alternata* pv. citri was deposited on unwounded fruit. The inoculated fruits were kept in a growth chamber at 20°C and 85% relative humidity and examined at different times post inoculation. Fungal growth was recorded as the diameter of the resulting lesion in mm.

Scaning electron microscopy

Sections (10 × 5 mm) of culture medium containing mycelium of fungus in PDA media culture medium (control) and in the same PDA culture medium to which phenolic compounds had been added (hesperetin 1 mM) 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 were rinsed with distilled water until the rising solution was completely clear, they were subjected to stepwise dehydration using a graded acetone series (10 min per step). The samples were critical point dried using a Balzers liquid CO_2 CPD 020 (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, Japan) at an accelerating voltage of 15 Kv.

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*. 1998b). Hesperidin, hesperetin, naringin and naringenin were obtained from Sigma Chemical Co. (St. Louis, MO).

Statistical analysis

Values are given as means±SD. Data were analyzed by one-way analysis of variance (ANOVA) and significant differences among treatment groups were evaluated by Duncan's Multiple Range Test (DMRT). All statistical analyses were made using Statgraphies Plus 5.0 software.

RESULTS AND DISCUSSION

Evaluation of the susceptibility of citrus fruit to inoculation with *A. alternata* pv. citri and *P. digitatum*

The degree on fungal development following artificial inoculation of the citrus fruits with *A. alternata* pv. citri or *P. digitatum* depended on the *Citrus* species.

Fruits of the hybrid 'Fortune' were the only fruits to develop Alternaria brown spot symptoms (**Fig. 1**). On the contrary, fruits of lemon and grapefruit were less susceptible to this fungus (**Fig. 1**).

In addition, the results obtained in Fortune suggested that the young fruit (about 140 days after anthesis) were the most susceptible to *A. alternata* pv. citri, while susceptibility decreased as the fruits matured (**Fig. 2**). These findings of fruit tolerance to *A. alternata* pv. citri is associated with advancing maturity reflect those of other authors (Gardner *et al*. 1986; Pegg 1966; Vicent *et al*. 2004; Reis *et al*. 2006).

However, our results with mature grapefruits contrast with those obtained by other authors with assays using leaves of different varieties of grapefruit. In these assays, a high susceptibility to *A. alternata* pv. citri was obtained (Vicent *et al*. 2004). The different observations are presumably due to the fact that different plant organs were studied or to the fact that we used mature fruit since, as we described above, the stage of fruit development may affect its susceptibility to this pathogen.

As regards the susceptibility of the citrus fruits analysed here to *P. digitatum*, the results show that fruits of *C. paradisi* (cv. 'Marsh') were more susceptible to this fungus than the mature fruit of 'Fortune' and *C. limon* (cv. 'Fino-49') (**Fig. 3**). Furthermore, in contrast to the response of 'Fortune' fruit to *A. alternata* pv. citri, the susceptibility of 'For-

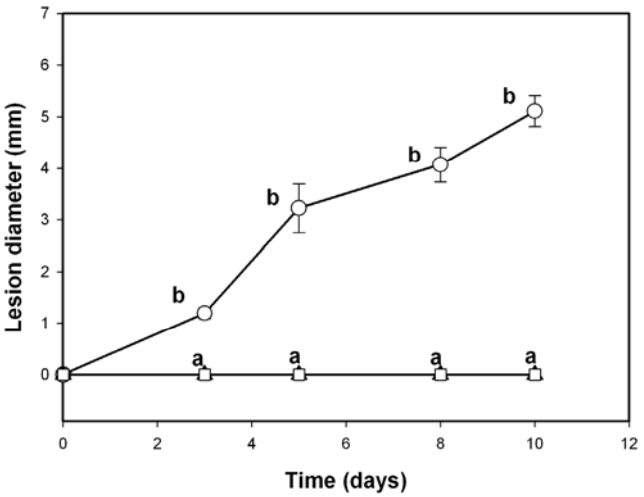


Fig. 1 Lesion development after inoculation of mature fruit with *Alternaria alternata* pv. citri. Artificial inoculation of fruit without wounding: 'Fortune' (○); *Citrus limon* cv. 'Fino-49' (▲); *Citrus paradisi* cv. 'Marsh' (□). Data represent mean values of lesion diameter (mm) on different days post-inoculation, and the vertical bars denote ±SD when larger than symbols. $^{(a-b)}$ $P< 0.001$ the values not sharing a common superscript letter are significantly different.

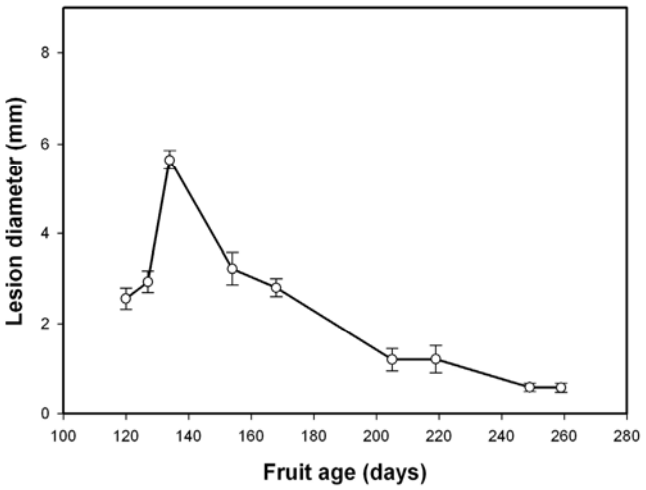


Fig. 2 Lesion diameter variation during the development of 'Fortune' fruit. Data represent mean values of lesion diameter (mm) in 'Fortune' fruit of different ages (days after anthesis) 3 days after inoculation with *Alternaria alternata* pv. citri: without wounding (○). The vertical bars denote ±SD when larger than symbols.

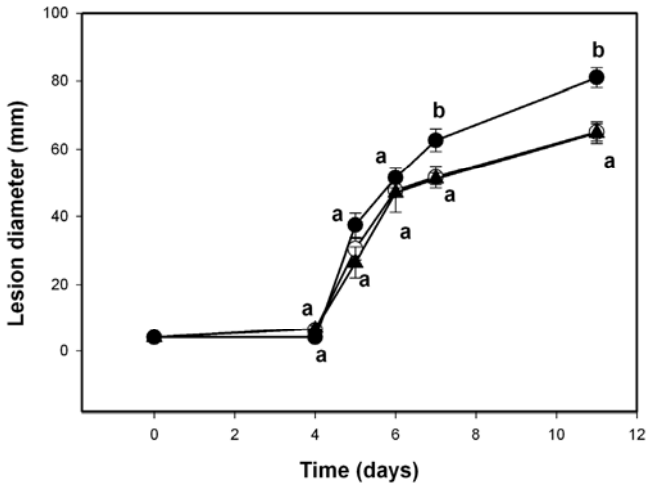


Fig. 3 Lesion development after inoculation of mature fruit by *Penicillium digitatum*. Artificial inoculation of fruit without wounding: 'Fortune' (○); *Citrus limon* cv. 'Fino-49' (▲); *Citrus paradisi* cv. 'Marsh' (●). Data represent mean values of lesion diameter (mm) at different days post-inoculation, and the vertical bars denote ±SD when larger than symbols. $^{(a-b)}$ $P< 0.05$ the values not sharing a common superscript letter are significantly different.

tune' fruit to *P. digitatum* increased with the advance toward maturity (data not shown). Similar results were described by Ben-Yehoshua *et al*. (2008) for *P. digitatum* and *C. limon* fruits.

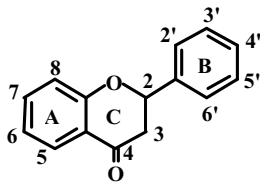
Changes in flavanones and polymethoxyflavones levels as a result of inoculation with *P. digitatum* and *A. alternata* pv. citri

HPLC study of the flavanone glycoside extracts of 'Fortune', *C. limon* (cv. 'Fino-49') and *C. paradisi* (cv. 'Marsh') revealed the presence of one principal compound with a retention time which coincided with that of the flavanone rutinoside, hesperidin (Rt = 15.5 min), in 'Fortune' and *C. limon*, and of the flavanone neohesperidoside, naringin (Rt = 11.7 min), in *C. paradisi* (**Fig. 4**). Both the absorption spectrum (obtained with V/UV diode array detector) and the MS analysis of this compound were identical to those obtained for hesperidin and naringin in previous studies (Del Río *et al*. 2004a; Ortuño *et al*. 2006).

On the other hand, the HPLC analysis of the polymethoxyflavone extracts from 'Fortune' fruits showed the presence of the three compounds with retention times coinciding with that the polymethoxyflavone, sinensetin (compound 1, Rt = 12.9 min), nobiletin (compound 2, Rt = 16.6 min) and tangeretin (compound 3, Rt = 25.5 min). The fruits of *C. paradisi* contained nobiletin, tangeretin and heptamethoxyflavone (compound 4, Rt = 17.2 min). In the case of *C. limon* fruit, polymethoxyflavones were hardly detectable. The absorption spectra of these compounds obtained by means of a V/UV diode array detector showed three maxima, at 265, 273 and 329 nm for compound 1, at 245, 271 and 331 nm for compound 2, and at 253, 268 and 340 for compound 4; and two maxima, at 271 and 324 nm for compound 3. The MS spectra of these compounds were identical to those obtained for sinensetin, nobiletin, tangeretin and heptamethoxyflavone (**Fig. 4**) in previous papers (Del Río *et al*. 1998b).

When fruits of the hybrid 'Fortune' were inoculated with *P. digitatum*, substantial changes in the flavanone glycoside (hesperidin) were observed. For example, five days after inoculation, the hesperidin levels observed were significantly lower in inoculated fruits (around 7.8% decrease) than in uninoculated fruits. Associated with this fall in the flavanone glycoside concentration in inoculated fruits was an increase in the concentrations of the corresponding aglycon hesperetin (7.2% increase). These changes in the levels of flavanone glycoside and its aglycon are due, at least partially, to the hydrolyzing action of *P. digitatum*. Similar results were obtained for hesperidin and naringin in fruits of *C. limon* and *C. paradisi*, following inoculation with *P. digitatum* (data not shown). On the other hand, in the fruit studied here, which do express detectable levels of polymethoxyflavones ('Fortune' and *C. paradisi*), the levels of some of these phenolic compounds were significantly higher in fruits five days after inoculation with *P. digitatum* than in uninoculated fruits (about 6% for nobiletin in 'Fortune' and 9% for nobiletin in *C. paradisi*).

In the case of *C. limon* fruits, the production of coumarins was particularly noteworthy after inoculation with the fungus. These secondary compounds may be involved as phytoalexins in the defence mechanisms of this species



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. 4 Chemical structures of the flavanones, naringin and hesperidin, and the polymethoxylated flavones sinensetin, tangeretein and nobiletin.

against *P. digitatum* (study in progress), as has been suggested by other authors (Ben-Yehoshua *et al*. 2008).

These results are in agreement with observations made previously for the defence mechanism of *C. aurantium* fruits against *P. digitatum* and *C. sinensis* fruits against *Phytophthora citrophthora*, when the levels of the major flavanone and the polymethoxiflavones were seen to decrease and increase, respectively (Arcas *et al*. 2000; Del Río *et al*. 2004b).

On the basis of the above results, we suggest that these constitutive secondary metabolites of the *Citrus* fruits studied (flavanones and flavones) may act as phytoalexins in the resistance mechanism against *P. digitatum* attack, acting as first and second defence barriers, respectively, since polymethoxyflavones are mainly localised in the outermost tissue of the fruit, the flavedo, while flavanones are located in the albedo, which lies immediately below the flavedo (Kanes *et al*. 1992; Ortuño *et al*. 1999).

A different type of response to that described above for *P. digitatum* was observed after the inoculation of 'Fortune' fruit with *A. alternata* pv. citri. In this case, there was as generalised decrease in flavanones glycoside and polymethoxyflavones. However, the decrease in flavanones glycoside was not due to the hydrolysing effect of the fungus since no increase in the corresponding aglycons was observed; rather, metabolic processes of transformation must have ocurred towards other derivates. That is, compounds that may be related to the development of the necrotic area associated with the infection. Presumably the higher the levels of flavonoids in fruit, the greater the development of the necrotic lesion caused by *A. alternata* pv. citri (study in progress).

This might explain the increased tolerance towards *Alternaria alternata* pv. citri observed in older fruit (see **Fig. 2**), bearing in mind that the highest levels of flavonoids are detected in the juvenile stage of fruit development, after which the levels decrease (Castillo *et al*. 1992; Benavente-García *et al*. 1993; Castillo *et al*. 1993; Del Río and Ortuño 1994; Ortuño *et al*. 1995).

Antifungal action of citrus flavonoids against *P. digitatum* and *A. alternata* pv. citri

In vitro studies show that, when added to PDA medium, some of the flavonoids isolated from citrus fruit reduce the radial growth of *P. digitatum* and *A. alternata* pv. citri. As with other plant species, the flavanones were less active in this respect than the polymethoxyflavones (**Table 1**) (Arcas *et al*. 2000; Del Río *et al*. 2004b). However, the higher levels of flavanones than of polymethoxyflavones in citrus fruit suggest that a possible physiological role of the former in the defence mechanism of citrus species should also be taken into account.

A comparison of the IC_{50} values shows that the flavanone, hesperidin, and the polymethoxylated flavones, tangeretin, heptamethoxyflavone and nobiletin, are more active against *A. alternata* pv. citri than against *P. digitatum*, while sinensetin is lightly more active against *P. digitatum* (**Table 1**).

Table 1 IC_{50} values of the flavanones and polymethoxyflavones of mature Fortune fruit for the fungi *Alternaria alternata* pv. citri and *Penicillium digitatum.* Data are presented as the means ±SD of each group. (a-f) P< 0.05 the values not sharing a common letter are significantly different.

Compounds		IC_{50} (mM)	
		Alternaria	*Penicillium*
Flavanones	Hesperidin	4.02 ± 0.2 a	7.86 ± 0.3 a
	Hesperetin	1.00 ± 0.3 b	0.12 ± 0.01 b
	Naringin	25 ± 1.2 c	17.9 ± 2.3 c
	Naringenin	4.2 ± 0.3 a	0.32 ± 0.02 d
Polymethoxyflavones	Tangeretin	0.16 ± 0.01 d	6.45 ± 0.5 a
	Heptamethoxy	0.7 ± 0.04 e	4.06 ± 0.2 e
	Nobiletin	0.28 ± 0.02 f	1.62 ± 0.3 f
	Sinensetin	0.35 ± 0.05 f	0.26 ± 0.01 d

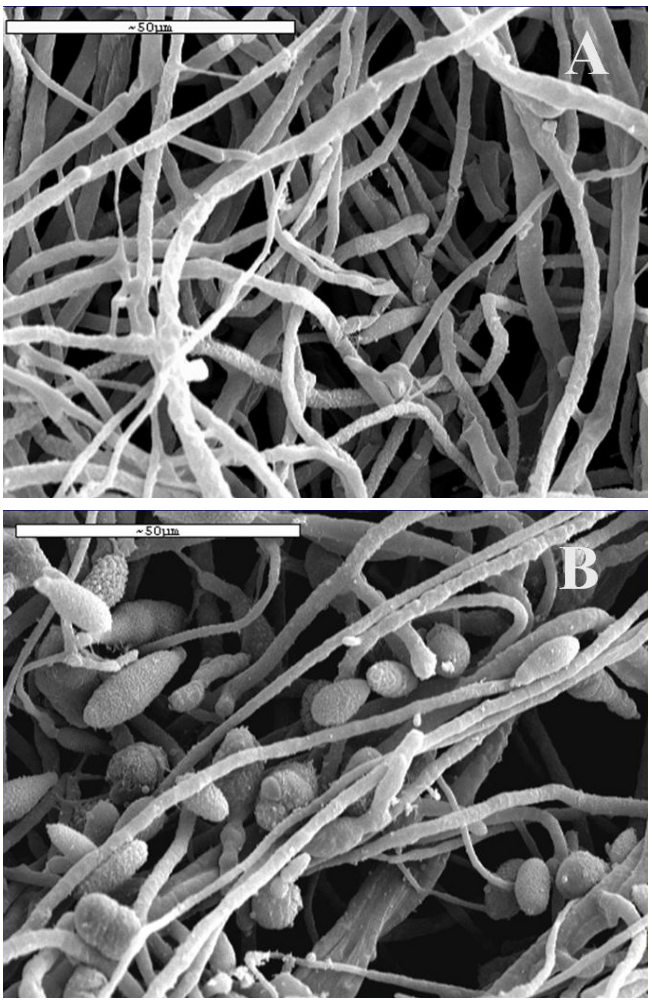


Fig. 5 SEM image of the changes in hyphal morphology and growth of *Alternaria alternata* pv. citri mycelium cultured in control PDA culture medium (A) and in the same PDA culture medium to which hesperetin (1 mM) (B) has been added. Bar = 50 µm.

Furthermore, as was mentioned above, the glycosylated flavanones are partially hydrolyzed by *P. digitatum* and the corresponding aglycons generated, such as hesperetin and naringenin, are more active as fungistatic agents against the two fungi studied at the concentration assayed, than their corresponding glycosylated flavanones (**Table 1**).

Besides the inhibition of radial growth observed, ultrastructural modifications of the hyphae were also seen when *P. digitatum* or *A. alternata* pv. citri were cultivated in the presence of the different flavanones and polymethoxyflavones described (data not shown). Thus, treatment with these phenolic compounds led to the swelling of the hyphal walls and reduced the cytoplamatic density, while large vacuoles with an opaque content and small secretory vesicles surrounding the plasmatic membrane appeared (Ortuño *et al*. 2006). These observations are in agreement with those made by other authors on different phenolic compounds against pathogenic fungi (Amborabé *et al*. 2002; Rivera-Vargas *et al*. 1993).

Moreover, in the case of *P. digitatum*, some of these phenolic compounds may inhibit spore production (Ortuño *et al*. 2006), while in the case of *A. alternata* pv. citri spore production increased (**Fig. 5**).

These results show that in *P. digitatum* infections, some citric flavonoids may be involved in the defence mechanism of the *Citrus* spp., and may be considered phytoalexins against the fungus, the polymethoxyflavones acting as a chemical barrier at the flavedo level and the flavanones at albedo level. In contrast, in the case of infection by *A. alter-*

nata pv. citri, although these secondary compounds have a fungitoxic effect, as demonstrated by the *in vitro* study, this is not sufficient to inhibit fungal development; indeed, quite the opposite occurs, since the fungus has developed a mechanism to metabolise these phenolic compounds, which helps the necrotic lesion to develop and through which the fungus continues its inexorable advance.

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