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Fungicidal potential of methoxylated flavones from citrus for *in vitro* control of *Colletotrichum gloeosporioides*, causal agent of anthracnose disease in tropical fruits

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Abstract: Four polymethoxylated flavones (3,5,6,7,3′,4′-hexamethoxyflavone, 3,5,6,7,8,3′,4′-heptamethoxyflavone, 5,6,7,8,4′-pentamethoxyflavone and 5,6,7,8,3′,4′-hexamethoxyflavone) were isolated and characterized from cold-pressed orange oil. Their antifungal activities were evaluated against *Colletotrichum gloeosporioides* (Penz) Penz & Sacc, a major plant pathogen of fruits that causes significant damage to crops in tropical, sub-tropical and temperate regions. Methoxylated flavones were effective in inhibiting mycelial growth of the fungus. As flavone concentration increased, mycelial growth decreased. 5,6,7,8,3′,4′-Hexamethoxyflavone completely inhibited the growth of *C gloeosporioides* at a concentration of 100 μg ml^{-1}.

Keywords: natural products; crop-protection agents; antifungal activity; *Colletotrichum gloeosporioides*; polymethoxylated flavones

1 INTRODUCTION

Colletotrichum gloeosporioides (Penz) Penz & Sacc in Penz, is the causal agent of a disease commonly known as anthracnose on many tropical, subtropical and temperate fruits.[1,2] Post-harvest problems caused by *C gloeosporioides* are particularly prevalent in the tropics, where they are often a significant factor in limiting export quality of produce and cause severe losses in fruit crops.[3] The economic cost of quiescent infections caused by *C gloeosporioides* is proportionally greater (about 25%) than that for field losses because the expenses of harvesting, transport, storage and packaging must be added to those of production.[4] The ability to survive as dormant appressoria on fruit surfaces and subsequently cause latent or quiescent infection, in which symptoms do not develop until the produce ripens, has grouped *C gloeosporioides* among the most important post-harvest pathogens.[5]

Where tropical fruits like mango (*Mangifera indica* L), avocado (*Persea americana* Mill) and papaya (*Carica papaya* L) are grown for export it is necessary to control anthracnose during transport and during the ripening period at the destination.[6–8] The use of hot water dips, either alone or incorporating a fungicide, kills many dormant appressoria and reduces disease incidence. Post-harvest fruit drenches with azole derivates, such as prochloraz, along with pre-harvest sprays of the benzimidazole, benomyl, are effective in reducing the disease.[7,9] Natural infection was shown to be reduced but not completely eliminated by washing, waxing and fungicide treatment in the packinghouse.[10] Benomyl in combination with multi-site non-mobile protectant fungicides, eg mancozeb or copper hydroxide, is currently one of the primary strategies used to control fruit anthracnose pre-harvest. Its use post-harvest is no longer permitted. Resistance of *C gloeosporioides* to benzimidazoles has been well documented, and opportunities exist to identify effective fungicide replacements.

In the present study, four methoxylated flavones were isolated from cold-pressed orange oil by high-performance liquid chromatography. The antifungal compounds were identified using spectral analyses. *In vitro* antifungal activity of methoxylated flavones against *C gloeosporioides* was evaluated by comparison with a commercial fungicide, benomyl.

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2 MATERIALS AND METHODS

2.1 Fungal cultures

Colletotrichum gloeosporioides cultures (CECT 2859) obtained from Colección Española de Cultivos Tipo (CECT) (Valencia, Spain), and originally isolated from *Citrus sinensis* fruits, were grown on potato dextrose agar (PDA, Difco, Laboratories, Detroit, MI) in the dark at 27 °C for 10 days and stored as stock cultures in sterile mineral oil at −20 °C.

2.2 Purification of fungicide compounds

Valencia late orange essential oil obtained by cold-pressing was distilled under vacuum until depletion; the non-volatile solids were called cold-pressed orange oil solids. A sample (10 g) of these solids was subjected to flash chromatography on 500 g of silica gel-60 (particle size 0.3–0.63 mm; Merck, Darmstadt, Germany). The fractions were eluted with 750 ml of each of the following six solvent systems, solvent polarity increasing in a linear gradient: A, hexane + dichloromethane (70 + 30 by volume); B, dichoromethane + ethyl acetate (95 + 5 by volume); C, dichoromethane + ethyl acetate (50 + 50 by volume); D, ethyl acetate (100); E, ethyl acetate + methanol (50 + 50 by volume); F, methanol (100). Twenty-seven fractions were eluted and tested for fungicidal activity as described in Section 2.4. Active fractions were selected and submitted to a subsequent fractionation by high-performance liquid chromatography (HPLC, Waters 600 E (Milford, MA, USA) with a photodiode array detector HP-1100 (Hewlett-Packard). The columns used in different fractionations were Licrosphere ODS-C_{18} (Merck, 125 × 4 mm, 5 μm) and Spherisorb-ODS-2 (Teknocroma, 250 × 7 mm, 5 μm). HPLC separations were carried out with the following solvent systems: G, methanol + water (90 + 10), H, methanol + water (85 + 15 by volume), and I, methanol + water (80 + 20 by volume). The pure flavones were collected using a Gilson fraction collector (Gilson Medical Electronics, Middleton, WI), and then concentrated under vacuum using a rotary evaporator (Labconco). The purity of isolated compounds was tested by HPLC coupled with a photodiode-array detector (Chemstation purity program). Mass spectrometry of the isolated compounds proved that the flavones were pure and contained no artifacts. The dried compounds were stored in a freezer at −20 °C for spectral analysis and biological assays.

2.3 Structure elucidation of the fungicides

Nuclear magnetic resonance (NMR) spectra of the pure flavones were recorded on a Varian Unity 400 MHz instrument (Varian Instruments, Germany). ^{1}H NMR and ^{13}C NMR spectra were recorded in deuterochloroform Chemical shifts are given in δ values (ppm) relative to the internal standard trimethylsilane. Mass spectral analyses were carried out on an HP-5988A quadrupole spectrometer with electron impact at 70 eV by direct introduction of a tiny amount of product laid in a capillary. Fourier-transform infrared spectra were obtained (potassium bromide pellet) on a Perkin-Elmer 781 spectrometer.

2.4 *In vitro* antifungal assay

The effects of collected fractions and pure compounds on colony growth of *C gloeosporioides* were evaluated according to the procedure described by March *et al*.[11] Collected fractions obtained from chromatographic separation and pure compounds were dissolved in 50 and 25 μl of acetone, respectively, and then incorporated into 5 ml of PDA at final concentrations of 500 μg ml^{-1} for collected fractions and 100, 50 and 25 μg ml^{-1} for pure compounds. Three plates of solid PDA media containing the fractions or pure compounds at the specified concentrations were centrally point-inoculated with spores from 7-day-old cultures of *C gloeosporioides*. Two control plates were included as a blank, which contained 25 or 50 μl of solvent without the compounds, and a positive control containing benomyl [methyl 1-(butylcarbamoyl)benzimidazol-2-ylcarbamate; Sigma–Aldrich, Milwaukee, USA) at 100, 50 and 25 μg ml^{-1}. Cultures were incubated in the dark at 27 °C. The colony diameter was measured every day. The percentage colony growth inhibition was determined by comparing the experimental with the control, which was considered to have 100% growth. The mean colony diameter and standard deviation (SD) of the mean were calculated. Each experiment was done in triplicate.

3 RESULTS

Cold-pressed oil, which passed through the silica column, was fractionated into 27 fractions. Fractions 1 to 16 showed no antifungal activity. Table 1 shows the effect of the remaining fractions on the growth of *C gloeosporioides* on PDA media. Fractions 17 through 26 eluted with solvents C, D, E and F showed antifungal activity. The more polar fractions (21 to 25) showed the greatest antifungal activity. Fractions 21 to 23 were selected for further study based on their antifungal activity and the weight of the fractions separated.

The analysis of fractions 21, 22 and 23 by preparative HPLC revealed four antifungal compounds, which were further purified following the HPLC separation. The pure isolated antifungal agents were eluted as a single peak when chromatographed on an analytical C18 column using various solvent systems (data not shown). They were analyzed by ^{1}H NMR, ^{13}C NMR, IR and mass spectrometry.

Methoxylation patterns of all of the flavones were determined from their ^{1}H NMR and ^{13}C NMR spectra. All flavones isolated had no A-ring aromatic proton resonance, indicating methoxylation at C-5, C-6, C-7 and C-8. It is well-known that resonance of methoxyl groups attached to di-ortho-substituted carbons occurs considerably downfield.[12,13] These characteristics provided a useful diagnostic tool for the structural analysis of polymethoxyflavones. Their

Table 1. Antifungal effect of fractions obtained of flash chromatography on silica gel-60 against colony growth of *Colletotrichum gloeosporioides*

Fraction (number)	Colony diameter[a] (cm)	Inhibition[b] (%)	Weight (mg)	Eluent[c]
17	1.27 (±0.06)	75.1	110.6	Solvent C
18	0.85 (±0.05)	83.4	113.8	Solvent C
19	0.75 (±0.09)	85.2	115.0	Solvent C
20	0.59 (±0.05)	88.4	186.2	Solvent D
21	0.40 (±0.04)	92.1	197.9	Solvent D
22	0.0	100.0	160.6	Solvent D
23	0.0	100.0	198.0	Solvent D
24	0.25 (±0.05)	95.0	191.0	Solvent E
25	0.43 (±0.07)	91.6	73.5	Solvent E
26	0.71 (±0.04)	86.0	31.0	Solvent F
Control	5.1 (±0.05)	0.0	—	—

[a] mean values of three replicates of colony diameter (±SD).
[b] Percentage of colony diameter inhibition respective to the control (100% of growth with no fraction added).
[c] See Section 2.2

spectra revealed a high degree of similarity to the structural features of polymethoxylated flavonoids.[14,15]

Flavone I was isolated from fraction F-21, and eluted to give with solvent G yellow needles. The compound was identified as 3,5,6,7,3′,4′-hexamethoxyflavone.[15]

Flavones II and III were isolated from fraction F-22; they were eluted with solvent H, and were identified respectively as 3,5,6,7,8,3′,4′-heptamethoxyflavone[16,17] and 5,6,7,8,4′-pentamethoxyflavone.[17]

Flavone IV was isolated from fraction F-23; it was eluted with solvent I, and was identified as 5,6,7,8,3′,4′-hexamethoxyflavone.[17,18]

The *in vitro* antifungal effect of the pure polymethoxylated flavonoids against *C gloeosporioides* is shown in Fig 1. As the concentration of methoxylated flavones increased, colony diameter decreased

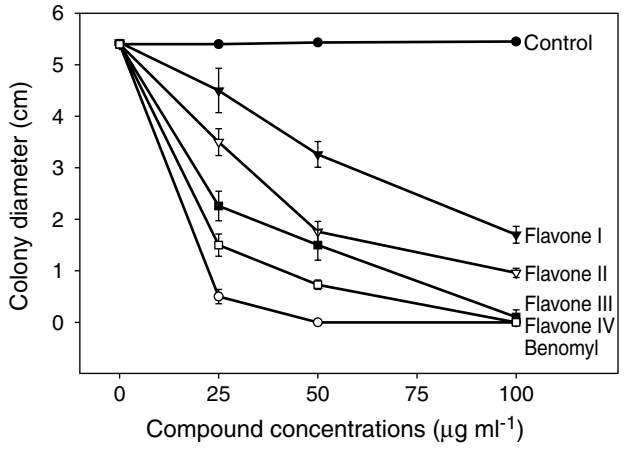

Figure 1. Colony diameter of *Colletotrichum gloeosporioides* after 6 days on potato dextrose agar amended with 0, 25, 50 or 100 μg ml^{-1} of methoxylated flavones and benomyl. (▲) flavone I, (△) flavone II, (■) flavone III (□) flavone IV, (○) benomyl, (●) control (without flavone added). Results are shown as mean values of three replicates of colony diameter; bar = ±SD.

for all compounds. At 100 μg ml^{-1} flavones III, IV and benomyl gave 98, 100 and 100% inhibition of growth, respectively. Above 100 μg ml^{-1} there was no detectable growth. According to the data obtained, the most promising antifungal agent for control of *C gloeosporioides*, as judged on its consistency in completely inhibiting growth of the fungus, was flavone IV. To determine the fungicidal sensitivity of flavone IV, this compound and the commercial fungicide benomyl were compared under the same assay conditions, over time at 50 and 25 μg ml^{-1} (Fig 2). Fungicide sensitivity has been used primarily to estimate the potential of compounds for chemical control.[19] Both concentrations of flavone IV caused a reduction in the colony diameter, 86.55% at 50 μg ml^{-1} and 72.37% at 25 μg ml^{-1}, while 50 μg ml^{-1} or more of benomyl was necessary to completely inhibit the growth of *C gloeosporioides* and 25 μg ml^{-1} reduced the colony diameter by 89%. The flavone IV and benomyl had the same level of activity over the initial 48 h of incubation, during which less of 50% the final growth of the control was reached. Therefore, 5,6,7,8,3′,4′-hexamethoxyflavone (flavone IV) was giving similar control to benomyl.

4 DISCUSSION

The higher incidence of *Colletotrichum gloeosporioides* in recent years in tropical, sub-tropical and temperate crops,[20] possibly caused by increased resistance of the pathogen to fungicides, prompted this investigation of alternative compounds for anthracnose control which are obtained from natural sources. Benomyl is currently one of the primary fungicides used to control certain fruit diseases, including anthracnose in pre-harvest situations. Reduction in field efficacy against certain *Colletotrichum* strains due to the occurrence of resistance in the pathogen has been well documented.[21] Thus, there is a significant need to identify new compounds for anthracnose control with an alternative mode of action. Efficacy trials with ethanolic leaf extracts of neem (*Azadirachta indica* Juss) conducted on anthracnose of mango,[22] demonstrated a similar level of inhibition against colony growth of *C gloeosporioides* to that which was obtained in this study with the flavones from cold-pressed orange oil. *Colletotrichum gloeosporioides* has differential sensitivity to methoxylated flavones, as demonstrated by the varying rates of inhibition of colony growth seen in this study. In fungicide disc assays, isolates of *C gloeosporioides* from citrus and papaya were highly sensitive to benomyl at 300 μg ml^{-1}.[20] 5,6,7,8,3′,4′-Hexamethoxyflavone in a similar bioassay showed inhibition at the concentration of 100 μg ml^{-1}. The antifungal properties of methoxylated flavonoids on *Cladiosporium cucumerinum* Ellis & Arth has been well documented by Tomas-Barberán *et al*[23] and on *Deuterophoma tracheiphila* (Petri) Kantachveli & Gikachvili by Piatelli and Impellizzeri.[24] The antifungal mechanism of methoxylated flavones has not

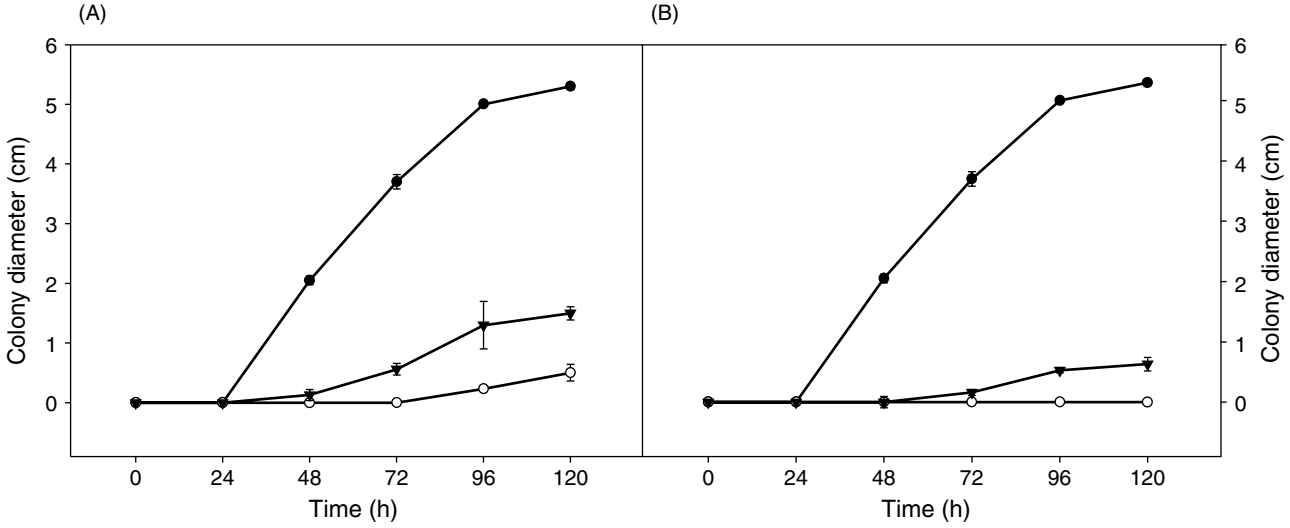

Figure 2. Antifungal effect of 5,6,7,8,3′,4′-hexamethoxyflavone and benomyl on *in vitro* growth of *Colletotrichum gloeosporioides* over time at (A) 25 μg ml^{-1}, (B) 50 μg ml^{-1}. (●) Control (without flavone added), (▲) 5,6,7,8,3′,4′-hexamethoxyflavone, (○) benomyl. Results are shown as mean values of three replicates of colony diameter; bar = ± SD.

been clearly demonstrated, however. Some authors believe that a biologically active compound like a fungicide must first diffuse from its site of application to its site of action. The rates of both these events will depend on the lipophilicity of the compound.[25] Lipophilic compounds tend to be efficiently transported to active sites within the target cell where they can exert their toxicity. This theory coincides with the finding of Harborne,[26] in which he showed that, in different fungal species, the antifungal activity of the fully methoxylated flavones decreased dramatically when the methyl group at position 5 was removed and a hydroxyl group was substituted. This difference in activity may be due to greater membrane uptake of the polymethoxylated flavones since methoxylation of the phenolic group decreases the hydrophilicity of the flavone.[27] If methoxylated flavonoids are shown to control effectively the growth of *C gloeosporioides*, they may have potential as crop-protection agents. The citrus methoxylated flavones exhibits a number of *in vitro* and *in vivo* anti-inflammatory and anticancer actions, show little effect on normal, healthy cells, and thus typically exhibit remarkably low toxicity in animals.[28] Currently we are testing polymethoxylated flavones on freshly harvested fruits.

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