JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Antifungal Synergistic Effect of Scopoletin, a Hydroxycoumarin Isolated from *Melia azedarach* L. Fruits

MARÍA C. CARPINELLA, CARLOS G. FERRAYOLI, AND SARA M. PALACIOS*

CEPROCOR, Agencia Córdoba Ciencia S.E., Alvarez de Arenales 230, Barrio Juniors, X5004AAP Córdoba, Argentina

In the continuous search for antifungal compounds from plants, the hydroxycoumarin scopoletin (**1**) was isolated from seed kernels of *Melia azedarach* L. from which three other compounds, vanillin (**2**), 4-hydroxy-3-methoxycinnamaldehyde (**3**), and (±) pinoresinol (**4**), have also been isolated. Guided fractionation through autobiography on TLC using *Fusarium verticillioides* (Saccardo) Nirenberg as test organism led to the isolation of **1**, which exhibited a minimum inhibitory concentration (MIC) of 1.50 mg/mL in the microbroth dilution method. Despite its own weak activity, when the coumarin was combined with the above-mentioned compounds, a strong enhancement of the antifungal effect was observed, even showing a complete inhibition in the growth of the pathogen when **1** was added at a concentration of up to 5% of its MIC value. The same level of effectiveness was observed when the synthetic antifungal agents Mancozeb and Carboxin were each combined with compounds **1**−**4**, in which cases it became possible to decrease the effective concentrations of these commercial compounds by up to 2.5 and 3%, respectively.

KEYWORDS: Antifungal activity; *Melia azedarach*; *Fusarium verticillioides*; scopoletin; synergism; Mancozeb; Carboxin

INTRODUCTION

Members of the *Fusarium* genus are widely distributed soil fungi and among those most commonly isolated by plant pathologists (*1*). Certain *Fusarium* fungi, particularly *Fusarium verticillioides*, are capable of causing a variety of diseases in corn, including seedling disease and stalk and ear rots (*2*, *3*). The *Fusarium* stalk and ear rot complex constitutes a widespread disease problem in maize (*Zea mays*) that can be found in nearly every maize field at harvest (*2*). Yield losses occur as a result of premature plant death and lodging, with a reduction in the quantity or quality of the crop. *F. verticillioides* can infect kernels without causing visible symptoms (*3*, *4*) but still affecting grain quality by producing mycotoxins, particularly fumonisins (*4*, *5*). Fumonisins cause, in humans and animals, liver and kidney toxicity and carcinogenicity, neurotoxicity, pulmonary edema, immunosuppression or -stimulation, and disruption of sphingolipid biosynthesis through inhibition of ceramide synthase (*6*−*8*). The presence of the fungus or its toxins is particularly disadvantageous in developing countries, where maize and maize-based products are the staple food for large populations.

Nowadays *F. verticillioides* infection is controlled by many synthetic products (*9*), which are also, however, highly toxic to human and animal life (*9*, *10*) and responsible, among other inconveniences, for the generation of toxic residues and their negative impact on the environment (*11*) and for the development of resistance in pathogens (*12*, *13*). There is thus an urgent need to be able to control this and other pathogenic fungi and their mycotoxin production by negligibly toxic and environmentally friendly methods.

In the search for new sources of products suitable for safe and sustainable agriculture management, bioactive substances obtained from plants are a promising way of controlling this kind of fungus. Plants have an almost limitless ability to synthesize secondary metabolites, most of which are phenols or their oxygen-substituted derivatives (*14*). In many cases these substances provide the plant with a resistance mechanism against disease or insect attack (*15*, *16*).

Our research group has long been studying the chemistry of a highly active Meliaceae, *Melia azedarach* L. (*17*−*19*), looking for active principles that can be used as safe biopesticides. This tree, commonly named paraiso, grows easily in temperate and cold-temperate zones of Argentina, where it is widespread. Extracts from different plant structures of *M. azedarach* have already been studied and show important antifungal activity, especially the seed kernel extract (SKE) (*20*, *21*), from which antifungal compounds such as vanillin (**2**), 4-hydroxy-3-methoxycinnamaldehyde (**3**), and (±) pinoresinol (**4**) have been isolated (*21*). We have now investigated the antifungal effect exhibited by scopoletin alone and in combination with the three previously isolated antifungal compounds (*21*). The synergistic effect developed after the combination of Mancozeb or Carboxin with compounds **1**−**4** was also studied.

* Author to whom correspondence should be addressed [telephone (03541) 489650/53; fax 03541-488181; e-mail sarapalacios@uolsinectis.com.ar].

10.1021/jf0482461 CCC: $30.25
Published on Web 03/25/2005

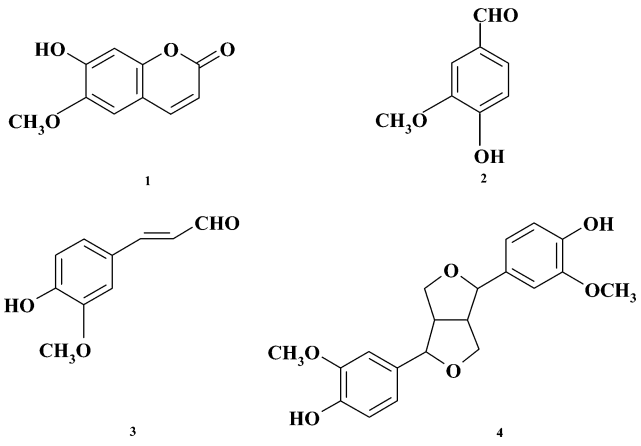

Figure 1. Chemical structures of scopoletin (**1**), vanillin (**2**), 4-hydroxy-3-methoxycinnamaldehyde (**3**), and pinoresinol (**4**).

MATERIALS AND METHODS

Plant Material. Ripe fruits from *M. azedarach* L. were collected in Córdoba, Argentina, in October 2001. A voucher specimen was deposited in the Botanical Museum of Córdoba (CORD 229, Córdoba, Argentina).

Chemicals. Mancozeb [ethylenebis(dithiocarbamic acid) manganese zinc complex] and Carboxin [5,6 dihydro-2-methyl-1,4-oxathiin-3-carboxanilide], both of technical grade, were purchased from Riedel-de Haën Co. (Seelze, Germany).

General Experimental Procedures and Apparatus. ^{1}H and ^{13}C NMR spectra were recorded in CD_3CN (Aldrich Chemical Co., Inc.) with a Bruker AC 200 spectrometer operated at 200 MHz for ^{1}H and at 50 MHz for the ^{13}C nucleus. Chemical shifts (parts per million) are relative to internal tetramethylsilane used as a reference. Gas chromatography−mass spectroscopy (GC-MS) was performed on a 17 A-QP 5000 instrument (Shimadzu, Tokyo, Japan) equipped with a NIST 107, 1998, mass spectral library. High-performance liquid chromatography (HPLC) was performed on a Waters 2690 instrument, equipped with a 250 × 4.6 mm i.d., 5 μm, Phenomenex Luna ODS reversed-phase column and photodiode array detector. The mobile phase was 1% acetic acid/MeOH in a gradient of 75:25 to 50:50, and detection was at 345 nm. Analytical and preparative thin-layer chromatography (TLC) was performed on silica gel 60 F_{254} Merck plates (Darmstadt, Germany). Silica gel was purchased from Sigma Chemical Co., Inc. (St. Louis, MO). All solvents were purchased from Merck and Fischer Scientific (Fair Lawn, NJ).

Isolation and Identification of Antifungal Principles. Air-dried kernels of ripe fruits of *M. azedarach* (2453 g) were extracted with ethanol after defatting with hexane to obtain an extract (SKE), yielding 3.17 g/100 g of seed kernels. For the isolation of the antifungal principles, a CH_2Cl_2 fraction (33.12 g), obtained by partitioning three times the SKE dissolved in 300 mL of MeOH/CH_2Cl_2/H_2O (12:3:15), was chromatographed using vacuum liquid chromatography (VLC) packed with silica gel 230−400 mesh and petroleum ether and eluted with a petroleum ether/Et_2O/Me_2CO/MeOH mixture, with increasing polarity. Ten fractions (F-1−F-10) were collected. To determine the antifungal activity of each fraction, direct bioautography was made on TLC (*21*). From F-3, an active compound obtained by sublimation was isolated and identified as vanillin (**2**), yield = 0.029 g/100 g of seed kernel, by HPLC (*21*).

From F-4 to F-9 a compound that showed an inhibition zone on direct bioautography TLC on a bright fluorescent spot (360 nm) corresponding to an R_f 0.75 ($CHCl_3$/MeCN 2:1) was isolated after successive VLC, radial preparative chromatography (eluted with $CHCl_3$/MeCN or petroleum ether/ethyl acetate mixture with increasing polarity), and finally preparative TLC developed in ethyl acetate. The fluorescent band was scraped off and eluted with CH_3CN. This compound was identified as scopoletin (**1**) (**Figure 1**), according to ^{1}H and ^{13}C NMR spectra and comparison with a GC-MS library, and the yield was 0.0018 g/100 g of seed kernel, by HPLC.

From the same fractions, two more active compounds were also isolated, after successive vacuum liquid and radial preparative chromatography carried out as above explained. The first compound was identified by spectroscopic means as 4-hydroxy-3-methoxycinnamaldehyde (**3**), yield = 0.054 g/100 g of seed kernel, by HPLC (*21*), and the second one was obtained as a white crystalline solid, yield = 0.131 g/100 g of seed kernel, by HPLC and was identified as (±)-pinoresinol (**4**) (*21*).

Scopoletin (**1**): $C_{10}O_4H_8$; t_R = 18.1 min (by HPLC); MS, *m*/*z* (rel int %) 192 (M^+, 100), 164 (31), 149 (54), 121 (29), 79 (18), 69 (69), 65 (15), 51 (41); ^{1}H NMR (200 MHz, CD_3CN) δ 3.92 (3 H, s, OMe), 6.20 (1H, d, J = 9.5 Hz, H-3), 6.84 (1 H, s, H-8), 7.11 (1 H, s, H-5), 7.77 (1H, d, J = 9.5 Hz, H-4); ^{13}C NMR (50.0 MHz, CD_3CN) δ 57.4 (ArOMe), 104.0 (C-8), 110.3 (C-5), 113.9 (C-3), 145.2 (C-4), 146.1 (C-6), 151.4 (C-8), 151.7 (C-7).

Microorganism and Growth Medium. *F. verticillioides* (Saccardo) Nirenberg strain M-7075 (a voucher specimen was deposited in the National University of Río Cuarto), isolated from maize by Dra. S. Chulze and provided by Dra. L. Giorda, was maintained as a monosporic culture in a V8 juice medium (*22*).

Antifungal Assay. A broth microdilution method (*13*) carried out in a 96-well sterilized microplate was used to determine the minimum inhibitory concentration (MIC) of compound **1**, of the other pure compounds, and of different combinations among them or combined with the synthetic fungicides Mancozeb or Carboxin. MICs were determined as the lowest concentration that produces complete growth inhibition of the tested fungus.

Four-day-old spores from *F. verticillioides* were added to sterile glucose−mineral salts medium to reach 10^5 spores/mL. Scopoletin (**1**) dissolved in filter (PVDF 0.45 μm; Millipore, Billerica, MA) sterilized dimethyl sulfoxide (DMSO) was incorporated in duplicate into each well containing the spore suspension, resulting in concentrations ranging from 0.10 to 1.70 mg/mL. The final concentration of DMSO did not exceed 2−4%. Wells containing spore suspension with the addition or otherwise of DMSO were simultaneously run as controls. The percentage of fungi growth, recorded as spore germination with respect to control (of which mycelial growth was considered as 100% of growth) was visually assessed with an inverted light microscope for each concentration of the compound. The measurements were always done by the same operator. The inhibitory concentration (IC_{50}) value was calculated by Probit analysis on the basis of the percentage of inhibition obtained at each concentration of the sample.

Synergism among the pure compounds was also measured as above by adding to each well containing the inoculum different combinations of two, three, or four of the compounds to be tested, at different concentrations ranging from 0.05 to 0.75 mg/mL (DMSO final concentration < 6%).

The synergistic effect of **1**−**4** with Mancozeb or Carboxin was also measured by adding concentrations of natural compounds ranging from 0.05 to 0.70 mg/mL and of Mancozeb and Carboxin corresponding to 0.05×10^{-3}−0.01×10^{-1} and 0.25×10^{-1}−0.50 mg/mL, respectively.

RESULTS AND DISCUSSION

The antifungal activity of seed kernel extract from *M. azedarach* has been reported in previous publications, and three compounds responsible for this activity have been isolated (*21*). In this study, another antifungal compound, the hydroxycoumarin scopoletin (**1**), was obtained from the same extract, showing a weak antifungal effect per se but, when combined with the other active compounds, a greatly unexpected enhancement of the activity.

MICs of Individual Compounds. The MIC of scopoletin (**1**) against *F. verticillioides* was 1.50 mg/mL, with its IC_{50} equivalent to 0.73 mg/mL (95% confidence interval = 0.60−0.88). The latter is similar to that of pinoresinol (**4**) (IC_{50} = 0.76 mg/mL; 95% confidence interval = 0.37−1.56) (*21*). This level of activity could be in part compared to that described by Shukla et al. (*23*), who, although obtaining an IC_{50} of compound **1** on *Fusarium fusiformis* (0.82 mg/mL) similar to that we

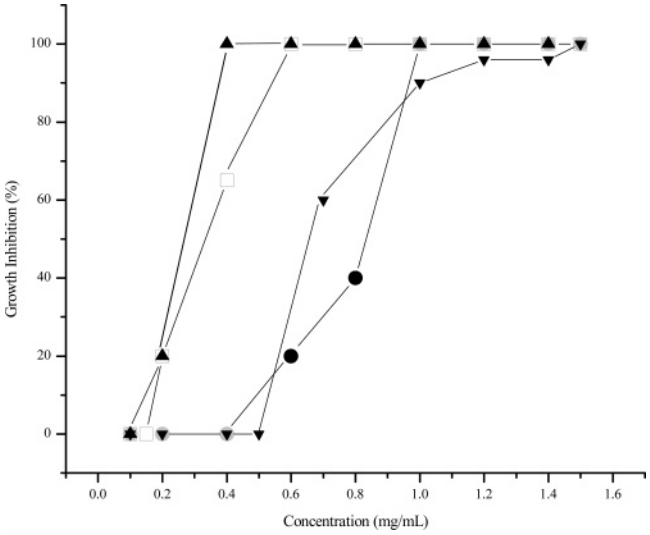

Figure 2. Inhibitory effect of different concentrations of **1** (▼), **2** (□), **3** (▲), and **4** (●) on growth of *F. verticillioides*.

Table 1. Synergistic Effect of Different Concentrations of Scopoletin (**1**), Vanillin (**2**), 4-Hydroxy-3-methoxycinnamaldehyde (**3**), and Pinoresinol (**4**) Combined in Pairs against *F. verticillioides*

compound	growth inhibition[a] (%), compound **1** (mg/mL; % of MIC)				
(mg/mL; % of MIC)	(0.75; 50)	(0.50; 33)	(0.30; 20)	(0.15; 10)	(0.08; 5)
2 (0.30; 50)	100	100	98	94	88
2 (0.20; 33)		92	94	67	
2 (0.15; 25)		98	88		
3 (0.20; 50)	100	100	100	100	100
3 (0.10; 25)			100	90	
3 (0.05; 12.5)		100	95	75	
4 (0.70; 70)	100		100	93	90
4 (0.50; 50)	100	100	100	45	45
4 (0.40; 40)	100	100	20		
4 (0.10; 10)		88	25	20	

[a] Value at 48 h from the beginning (time of complete growth of the control); average of two replicates.

obtained on *F. verticillioides*, found the inhibition produced with the highest assayed concentration, equivalent to 1.00 mg/mL, was 51.8% (*23*), whereas on *F. verticillioides* this concentration produced 90% control, as seen in **Figure 2**. On *Alternaria alternata*, scopoletin exhibited a complete inhibition at 1.00 mg/mL (*23*). On *Fusarium semitectum*, 66.2% of growth inhibition was reached at the same concentration, but the IC_{50} corresponding to 0.47 mg/mL differs from that obtained with *F. fusiformis* (*23*). The MICs of compounds **2**, **3**, and **4** corresponded to 0.60, 0.40, and 1.00 mg/mL, respectively (*21*).

Synergistic Effect among Compounds 1−4. As was reported by Carpinella et al. (*21*), the antifungal compounds **2**−**4** isolated from the SKE of *M. azedarach* exhibited a strong synergistic effect among themselves. This time we questioned if scopoletin (**1**) showed the same effect and at what level of potency it worked. To answer this question, a broth microdilution method was carried out by adding the compounds **1**−**4** in different combinations of two, three, or four, which, in most cases, were equivalent to half or less of their MIC values.

The synergistic effect between compounds **1** and **2**−**4** is shown in **Table 1**. When compound **1** was added at a concentration corresponding to 33% of its MIC value (0.50 mg/mL) and **2** at 50% of its MIC (0.30 mg/mL), a 100% inhibition in the growth of *F. verticillioides* was exhibited, and even when the concentration of compound **1** was decreased to 0.08 mg/mL, a value equivalent to 5% of its MIC value, an effect of 88% control was still observed. Inhibitions of 92 and 98% were detected when **1**, added at 33% of its MIC, was combined with compound **2** reduced to 33% (0.20 mg/mL) and 25% (0.15 mg/mL), respectively, of its MIC value. This level of inhibition was still observed when the concentration of compound **1** was decreased to 20% of its MIC (0.30 mg/mL) with compound **2** remaining at 33%. At the latter level, compound **2** alone exhibited an effectiveness in the inhibition corresponding to 20%, whereas at 25% of its MIC the inhibition was 0% (**Figure 2**). On its own, compound **1** at 33% of its MIC value or lower developed no inhibition (**Figure 2**), clearly showing the synergistic effect between the compounds.

When **1** was combined at 5% of its MIC with compound **3** at 50% of the total inhibitory concentration (0.20 mg/mL), growth inhibition was complete. When compound **1** was added at 10% of its MIC (0.15 mg/mL) and the concentration of **3** was decreased to 25% of its MIC (0.10 mg/mL), a 90% inhibition developed. The results indicate that the potentiation of the effect between **1** and **3** is stronger than that observed between **1** and **2** (**Table 1**). It is important to bear in mind that compound **3** individually, at 50 and 25% of its MIC, showed only 20 and 0%, respectively, of control of the pathogen (**Figure 2**).

When compound **1** was added at 5% of its MIC and **4** at 0.70 mg/mL, equivalent to 70% of its MIC value, 90% inhibition was detected. Only 20% inhibition was detected by combining 20% of the MIC of **1** and 40% of the MIC of **4** (0.40 mg/mL), increasing to 100% when the concentration of **1** was increased to a 33%. As can be seen in **Figure 2**, compound **4** alone at 0.40 mg/mL showed no inhibition of *F. verticillioides*.

To compare the level of efficacy of the combinations of **1** with compounds **2**−**4**, we observed that adding the former, at 10% of its MIC, to each of the other compounds, at 50% of the minimum concentration that produces total growth inhibition, showed that the most potent combination was between **1** + **3** with 100% inhibition, followed by the 94% growth inhibition exhibited by **1** + **2**, and finally **1** + **4**, which showed 45% control (**Table 1**).

When any of the compounds **2**−**4**, combined among themselves at 20 or 25% of their MICs (*21*), were replaced by compound **1** at 20% of its MIC, an increase in the inhibition of the pathogen was observed. For instance, when compound **2** was combined with compound **3**, both at 25% of their MICs, a 90% inhibition was produced (*21*). When **3** was replaced by **1**, at 20% of its MIC, the level of inhibition did not change (88%) but when **2** was the metabolite replaced, an increase to 100% control was detected (**Table 1**). This complete inhibition between **3** and **1** contrasted with a 2% control exhibited by the combination of 25% of the MIC of **3** and, in this case, a 20% of **4** (0.20 mg/mL) (*21*) in place of **1**. If compound **3** was replaced by 20% of the MIC of **1**, keeping **4** at 20%, a low increase from 2 to 20−25% would be obtained (**Table 1**). Combining **2** at 25% of its MIC with **4** at 20%, 80% inhibition was exhibited (*21*), increasing to 88% when **4** was replaced by **1** at the same quantity. These results showed that the approximate level of potency was, in decreasing order, **1** + **3**, followed by the combination of **1** + **2**, then **2** + **3** or **2** + **4**, then **1** + **4**, and finally **3** + **4**.

As was previously explained (*21*), whereas **2** + **3**, added together at 33 and 25%, respectively, of their MIC, exerted 95% inhibition on *F. verticillioides*, this inhibition increased to 100% when **1** was added to the pair at as little as 5% of its MIC value (**Table 2**). Combining **2** at 25% and **4** at 20% of their MICs,

Table 2. Synergistic Effect of Different Concentrations of Scopoletin (**1**), Vanillin (**2**), 4-Hydroxy-3-methoxycinnamaldehyde (**3**), and Pinoresinol (**4**) Combining All Three or Four against *F. verticillioides*

compound (mg/mL; % of MIC)	growth inhibition[a] (%), compound **1** (mg/mL; % of MIC)			
	(0.40; 27)	(0.30; 20)	(0.15; 10)	(0.08; 5)
2 (0.20; 33) **3** (0.10; 25)				100
2 (0.20; 33) **4** (0.40; 40)				100
2 (0.15; 25) **4** (0.20; 20)	100	94	80	80
3 (0.10; 25) **4** (0.50; 50)			88	88
3 (0.10; 25) **4** (0.20; 20)			85	85
3 (0.05; 12.5) **4** (0.70; 70)			88	90
2 (0.15; 25) **3** (0.05; 12.5) **4** (0.10; 10)		98		0
2 (0.08; 12.5) **3** (0.10; 25) **4** (0.10; 10)				95

[a] Value at 48 h from the beginning (time of complete growth of the control); average of two replicates.

80% inhibition was produced (*21*), and the same value of control remained even when **1** was added at a concentration corresponding to 10% of its MIC. However, when compound **1** was added at 27% of its MIC (0.40 mg/mL), the control was total (**Table 2**).

When 5% of the MIC of **1** was added to the combination of **3** at 25% of its MIC and **4** at 20%, inhibition increased greatly from 2 (*21*) to 85%. Adding compound **1**, at 5% of its MIC, to **3** and **4** at 12.5% (0.05 mg/mL) and 70% (0.70 mg/mL) of their respective MICs produced 90% inhibition (**Table 2**). The combination of **3** and **4** alone inhibited growth by 80% (*21*). As seen in **Table 1**, the combination of 5% of **1** and **4** at 70% of its MIC produced 90% inhibition. This means that **3** did not enhance activity in the combination of **1** and **4**. When **2**, **3**, and **4** were added at 12.5, 25, and 10%, respectively, of their MICs (0.08, 0.10, and 0.10 mg/mL, respectively), an 82% inhibition was observed (*21*), increasing to 95% when **1** was added at 5% of its MIC (**Table 2**). These results demonstrate the potentiation effect exhibited when compound **1** was present, even at very low concentrations with respect to its MIC.

As noted, even when scopoletin did not possess high levels of control per se, it showed, combined with the other antifungal compounds, an enhancement in the growth inhibitory effect, through a synergistic potentiation. Stark and Walter (*24*) reserve the term "synergism" only for when the greater activity is a result of the sum of the individual effects of two ingredients with similar modes of action, and they use "potentiation" when the two ingredients show different modes of action. We prefer to talk about "synergism of sum", as the result of the sum of the effects of each compound on the same or different targets, or "synergistic potentiation", as the enhancement of the effect resulting from the combined action of two or more compounds with different or the same modes of action, but when the final activity produced is greater than the individual sum of effects. In most of our assays, the combinations showed a synergistic potentiation, even though the metabolites act with the same or different modes of action, which must be studied in more detail. This effect, mainly displayed when the weak antifungal compound **1** was present, may be due in part to a direct effect on fungi survival or germination, by the inhibition of broad-specificity multidrug resistant pumps (MDRs), an efflux mechanism found in microorganisms which provides a barrier to antifungal metabolites (*25*, *26*), by inhibition of detoxification enzymes (*14*, *27*), or by decreasing excretion (*28*). For instance, given the antioxidant properties exhibited by compound **1** (*29*, *30*), it is possible that subinhibitory concentrations of it would inhibit the oxidation of vanillin, added at sub-MIC levels, to the inactive vanillic acid (*31*, *32*). The aldehyde function in the vanillin structure is necessary for its effectiveness against fungi (*33*). If any of the compounds **1**−**4** is a MDR inhibitor, allowing the remaining compounds to accumulate in the cell, this would make their weak to moderate activity increase and enable them to really show their previously masked strong antifungal properties, which could be as effective as those exhibited by conventional antifungals (*26*). This potentiation activity might explain why the quantity of compound **1** needed for in vitro inhibition of the growth of *Ophiostoma ulmi* is greater than the quantity found in the culture medium of inoculated cell suspensions of resistant *Ulmus pumila* (*15*), where **1** may exhibit synergism together with other five chemicals present in the resistant *Ulmus* species.

Synergistic Effect between Compounds 1−4 and Synthetic Antifungal Compounds. Compound **1** also exhibited a strong synergistic effect with the synthetic compounds Mancozeb and Carboxin. The antifungal agent Mancozeb exhibited a MIC value against *F. verticillioides* of 0.01 mg/mL. The MIC of Carboxin was difficult to determine due to its low solubility in water, but corresponded to ∼1.70 mg/mL. As seen in **Table 3**, when compound **1** was added at 20% of its corresponding MIC and Mancozeb at a concentration corresponding to 2.5% of its MIC (0.25×10^{-3} mg/mL), a complete inhibition of the reference fungus was observed. An inhibition of 90% was obtained when the concentration of Mancozeb decreased to 0.01×10^{-2} mg/mL, equivalent to only 1% of its MIC, and compound **1** was added at 27% of its MIC. In both cases the corresponding concentrations of Mancozeb exhibited, individually, 20% control of the pathogen.

When Carboxin and scopoletin (**1**) were added together, both at 0.50 mg/mL, corresponding to 29 and 33%, respectively, of their corresponding MICs, a total inhibition of the pathogen (**Table 4**) was observed; individually both compounds at this concentration showed zero effect on fungi. Even when the concentrations of **1** and Carboxin were decreased to 27 and 6% (0.10 mg/mL), respectively, of their corresponding MICs, 70% inhibition was still detected. Decreasing the concentrations of Carboxin to values equivalent to 3−1.5% of its MIC the inhibition reached values from 65 to 20% (**Table 4**). These inhibitory levels show that the synergistic effect between **1** and Mancozeb was more effective than that displayed between **1** and Carboxin. As observed, these results imply that the useful quantities of the commercial agents could be greatly reduced, at the same time decreasing their toxicity in mammals and negative environmental impact, as well as the cost of controlling diseases in corn crops.

Scopoletin can be found in many edible plants and fruits such as *Avena sativa*, *Allium ampeloprasum*, *Apium graveolens*, *Capsicum annuum*, *Capsicum frutescens*, *Daucus carota*, *Cichorium intybus*, *Citrus limon*, and *C. paradisi*, which denotes its low toxicity, guaranteeing its safe use as an antifungal or synergistic compound in combination with synthetic or other natural substances, such as vanillin. Given the strong specific

Table 3. Synergistic Effect of Different Concentrations of Scopoletin (**1**), Vanillin (**2**), 4-Hydroxy-3-methoxycinnamaldehyde (**3**), and Pinoresinol (**4**) with the Synthetic Agent Mancozeb against *F. verticillioides*

compound (mg/mL; % of MIC)	growth inhibition[a] (%), Mancozeb (mg/mL; % of MIC)				
	$(0.01 \times 10^{-1}; 10)$	$(0.05 \times 10^{-2}; 5)$	$(0.25 \times 10^{-3}; 2.5)$	$(0.01 \times 10^{-2}; 1)$	$(0.05 \times 10^{-3}; 0.5)$
1 (0.50; 33)	100		100	90	
1 (0.40; 27)			100	90	
1 (0.30; 20)	100	100	100		
2 (0.50; 83)		100	100		
2 (0.40; 67)		100			98
2 (0.30; 50)	100	100	98	97	93
2 (0.15; 25)	100	95			
3 (0.20; 50)	100	100	99	80	10
3 (0.10; 25)	100	99			
3 (0.05; 12.5)					
4 (0.70; 70)		100			
4 (0.50; 50)	100	100	94	80	
4 (0.20; 20)		90			

[a] Value at 48 h from the beginning (time of complete growth of the control); average of two replicates.

Table 4. Synergistic Effect of Different Concentrations of Scopoletin (**1**), Vanillin (**2**), 4-Hydroxy-3-methoxycinnamaldehyde (**3**), and Pinoresinol (**4**) with the Synthetic Agent Carboxin against *F. verticillioides*

compound (mg/mL; % of MIC)	growth inhibition[a] (%), Carboxin (mg/mL; % of MIC)			
	(0.50; 29)	(0.10; 6)	(0.05; 3)	$(0.25 \times 10^{-1}; 1.5)$
1 (0.50; 33)	100	80	65	30
1 (0.40; 27)	80	70	20	20
1 (0.30; 20)	20	20	20	
2 (0.50; 83)	98	95	99	97
2 (0.40; 67)	95	94	92	91
2 (0.30; 50)	93	90	88	85
3 (0.10; 25)	85			
4 (0.70; 70)	100	100	100	93
4 (0.20; 20)	88	60	0	

[a] Value at 48 h from the beginning (time of complete growth of the control); average of two replicates.

flavor that the latter negligibly toxic compound exhibits, it is not suitable for use in preventing fungus growth in manufactured food at the concentrations at which it displays inhibitory activity (*33*, *34*). Diminishing its effective doses would thus be of great advantage.

After these successful findings involving synthetic antifungal agents and compound **1**, we studied the same action with the rest of the compounds. Compounds **2**−**4** were combined with Mancozeb, and the same synergistic potentiation exhibited with compound **1** was produced. As is seen in **Table 3**, when compound **2** at 50% of its MIC value was added to Mancozeb at 5% of its MIC (0.05×10^{-2} mg/mL), a complete inhibition in pathogen growth was detected, decreasing only 3% when the Mancozeb concentration was reduced to 1% of its MIC. An almost total inhibition was observed when compound **3** and Mancozeb were combined at 25 and 5%, respectively, of their MICs (**Table 3**). When the same quantity of the synthetic compound was added to **4** at 50% of its MIC, a 100% inhibition was exhibited.

When **2**−**4** were combined with Carboxin, the effect, as in the case of **1**, was not as strong as with Mancozeb. High but not total inhibition values (**Table 4**) were observed after the addition of **2** at 83 and 67% of its MIC (0.50 and 0.40 mg/mL, respectively) to Carboxin at 29 to 1.5% of its MIC (0.50 to 0.25×10^{-1} mg/mL, respectively). The combination of **3** at 25% of its MIC value and Carboxin at 29% showed 85% growth inhibition. The most potent combination with Carboxin, even higher than that observed in combination with **1**, was produced by compound **4**. With **4** at 70% of its MIC together with Carboxin even at 3% of its corresponding MIC (0.05 mg/mL), complete inhibition was detected (**Table 4**).

These last results imply that compounds **2**−**4** could also be used to reduce the effective concentrations of toxic synthetic antifungals such as Mancozeb and Carboxin, which are widely used in conventional agriculture, which, in most agricultural countries, involves the majority of their field crops. This extensive use increases the risk of undesirable effects on the environment and on human and animal health, which could be reduced by replacing, at least in part, the synthetic substances by natural negligibly toxic, highly specific, and biodegradable antifungal or synergistic compounds.

ABBREVIATIONS

SKE, seed kernel extract; GC-MS, gas chromatography−mass spectrometry; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; VLC, vacuum liquid chromatography; MIC, minimum inhibitory concentration; DMSO, dimethyl sulfoxide; MDR, multidrug resistant pump.

ACKNOWLEDGMENT

We are grateful to Dra. S. Chulze and Dra. L. Giorda.

LITERATURE CITED

(1) Smith, A. G.; Bustamante, C. I.; Wood, C. Disseminated cutaneous and vascular invasion by *Fusarium moniliforme* in a fatal case of acute lymphocytic leukemia. *Mycopathologia* **1993**, *122*, 15−20.

(2) Smith, D. R.; White, D. G. Diseases of corn. In *Corn and Corn Improvement*, 3rd ed.; Agronomy Monograph 18; Sprague, C. F., Dudley, J. W., Eds.; ASA-CSSA-SSSA: Madison, WI, 1988; pp 687−766.

(3) Glenn, A. E.; Gold, S. E.; Bacon, C. W. Fdb1 and Fdb2, *Fusarium verticillioides* loci necessary for detoxification of preformed antimicrobials from corn. *Mol. Plant−Microbe Interact.* **2002**, *15*, 91−101.

(4) Desjardins, A. E.; Manandhar, G. D.; Plattner, R. D.; Maragos, C. M.; Shrestha, K.; Mccormick, S. P. Occurrence of *Fusarium* species and mycotoxins in Nepalese maize and wheat and the effect of traditional processing methods on mycotoxin levels. *J. Agric. Food Chem.* **2000**, *48*, 1377−1383.

(5) Desjardins, A. E.; Plattner, R. D. Fumonisin B_1-nonproducing strains of *Fusarium verticillioides* cause maize (*Zea mays*) ear infection and ear rot. *J. Agric. Food Chem.* **2000**, *48*, 5773−5780.

(6) Desai, K.; Sullards, M. C.; Allegood, J.; Wang, E.; Schmelz, E. M.; Hartl, M.; Humpf, H. U.; Liotta, D. C.; Peng, Q.; Merrill, A. H., Jr. Fumonisins and fumonisin analogs as inhibitors of ceramide synthase and inducers of apoptosis. *Biochim. Biophys. Acta* **2002**, *1585*, 188−192.

(7) Stockmann-Juvala, H.; Mikkola, J.; Naarala, J.; Loikkanen, J.; Elovaara, E.; Savolainen, K. Fumonisin B_1-induced toxicity and oxidative damage in U-118MG glioblastoma cells. *Toxicology* **2004**, *202*, 173−183.

(8) Van der Westhuizen, L.; Gelderblom, W. C. A.; Shephard, G. S.; Swanevelder, S. Disruption of sphingolipid biosynthesis in hepatocyte nodules: Selective proliferative stimulus induced by fumonisin B_1. *Toxicology* **2004**, *200*, 69−75.

(9) Tomlin, C. D. S., Ed. *The Pesticide Manual*; British Crop Protection Council: Surrey, U.K., 1997; pp 1−1606.

(10) Reigart, J. R.; Roberts, J. R. *Recognition and Management of Pesticide Poisonings*; U.S. EPA: Washington, DC, 1999; pp 137−155.

(11) Hall, C. V. Pesticide waste disposal in agriculture. In *Treatment and Disposal of Pesticide Wastes*; ACS Symposium Series 259; Krueger, R. F., Seiber, J. N., Eds.; Americal Chemical Society: Washington, DC, 1984; pp 27−36.

(12) Knight, S. C.; Anthony, V. M.; Brady, A. M.; Greenland, A. J.; Heaney, S. P.; Murray, D. C.; Powell, K. A.; Schulz, M. A.; Spinks, C. A.; Worthington, P. A.; Youle, D. Rationale and perspectives on the development of fungicides. *Annu. Rev. Phytopathol.* **1997**, *35*, 349−372.

(13) Wilson, C. L.; Solar, J. M.; El Ghaouth, A.; Wisniewski, M. E. Rapid evaluation of plant extracts and essential oils for antifungal activity against *Botrytis cinerea*. *Plant Dis.* **1997**, *81*, 204−210.

(14) Cowan, M. M. Plant products as antimicrobial agents. *Clin. Microbiol. Rev.* **1999**, *12*, 564−582.

(15) Valle, T.; López, J. L.; Hernández, J. M.; Corchete, P. Antifungal activity of scopoletin and its differential accumulation in *Ulmus pumila* and *Ulmus campestris* cell suspension cultures infected with *Ophiostoma ulmi* spores. *Plant Sci.* **1997**, *125*, 97−101.

(16) Stange, R. R., Jr.; Sims, J. J.; Midland, S. L.; McDonald R. E. Isolation of a phytoalexin, *trans-p*-coumaryl aldehyde, from *Cucurbita maxima*, Cucurbitaceae. *Phytochemistry* **1999**, *52*, 41−43.

(17) Valladares, G.; Defagó, M. T.; Palacios, S. M.; Carpinella, M. C. Laboratory evaluation of *Melia azedarach* (Meliaceae) extracts against the Elm Leaf Beetle (Coleoptera: Chrysomelidae). *J. Econ. Entomol.* **1997**, *90*, 747−750.

(18) Carpinella, M. C.; Ferrayoli, G. C.; Valladares, G.; Defago, M.; Palacios, S. M. Potent limonoid insect antifeedant from *Melia azedarach. Biosci., Biotechnol., Biochem.* **2002**, *66*, 1731−1736.

(19) Carpinella, M. C.; Defago, M. T.; Valladares, G.; Palacios, S. M. Antifeedant and insecticide properties of a limonoid from *Melia azedarach* (Meliaceae) with potential use for pest management. *J. Agric. Food Chem.* **2003**, *51*, 369−374.

(20) Carpinella, M. C.; Herrero, G. W.; Alonso, R. A.; Palacios, S. M. Antifungal activity of *Melia azedarach* fruit extracts. *Fitoterapia* **1999**, *70*, 296−298.

(21) Carpinella, M. C.; Giorda, L. M.; Palacios S. M. Antifungal effects of different organic extracts from *Melia azedarach* L. on phytopathogenic fungi and their isolated active components. *J. Agric. Food Chem.* **2003**, *51*, 2506−2511.

(22) Taylor, R. J.; Salas, B.; Secor, G. A.; Rivera, V.; Gudmestad, N. C. Sensitivity of North American isolates of *Phytophthora erythroseptica* and *Pythium ultimum* to Mefenoxam (Metalaxyl). *Plant Dis.* **2002**, *86*, 797−802.

(23) Shukla, Y. N.; Srivastava, A.; Kumar, S.; Kumar, S. Phytotoxic and antimicrobial constituents of *Argyreia speciosa* and *Oenothera biennis*. *J. Ethnopharmacol.* **1999**, *67*, 241−245.

(24) Stark, J. D.; Walter, J. F. Neem oil and neem oil components affect the efficacy of commercial neem insecticides. *J. Agric. Food Chem.* **1995**, *43*, 507−512.

(25) Morel, C.; Stermitz, F.; Tegos, G.; Lewis, K. Isoflavones as potentiators of antibacterial activity. *J. Agric. Food Chem.* **2003**, *51*, 5677−5679.

(26) Tegos, G.; Stermitz, F. R.; Lomovskaya, O.; Lewis, K. Multidrug pump inhibitors uncover remarkable activity of plant antimicrobials. *Antimicrob. Agents Chemother.* **2002**, *46*, 3133−3141.

(27) Duncan, S. H.; Flint, H. J.; Stewart, C. S. Inhibitory activity of gut bacteria against *Escherichia coli* O157 mediated by dietary plant metabolites. *FEMS Microbiol. Lett.* **1998**, *164*, 283−288.

(28) Repetto, M. Mecanismos de toxicidad. In *Toxicología Fundamental*; Repetto, M., Ed.; Ediciones Díaz de Santos: Madrid, Spain, 1997; pp 154−276.

(29) Chong, J.; Baltz, R.; Schmitt, C.; Beffa, R.; Fritig, B.; Saindrenan, P. Downregulation of pathogen-responsive tobacco UDP-Glc: Phenylpropanoid glucosyltransferase reduces scopoletin glucoside accumulation, enhances oxidative stress, and weakens virus resistance. *Plant Cell* **2002**, *14*, 1093−1107.

(30) Ng, T. B.; Liu, F.; Lu, Y.; Cheng, C. H. K.; Wang, Z. Antioxidant activity of compounds from the medicinal herb *Aster tataricus*. *Comp. Biochem. Physiol. C* **2003**, *136*, 109−115.

(31) De Wulf, O.; Thonart, P. Bioconversion of vanillin to vanillyl alcohol in a two-phase reactor. *Appl. Biochem. Biotechnol.* **1989**, *20*, 165−180.

(32) Edlin, D. A. N.; Narbad, A.; Dickinson, J. R.; Lloyd, D. The biotransformation of simple phenolic compounds by *Brettanomyces anomalus*. *FEMS Microbiol. Lett.* **1995**, *125*, 311−316.

(33) Fitzgerald, D. J.; Stratford, M.; Narbad, A. Analysis of the inhibition of food spoilage yeast by vanillin. *Int. J. Food Microbiol.* **2003**, *86*, 113−122.

(34) Kometani, T.; Tanimoto, H.; Nishimura, T.; Okada, S. Glucosylation of vanillin by cultured plant cells. *Biosci., Biotechnol., Biochem.* **1993**, *57*, 1290−1293.

Received for review October 21, 2004. Revised manuscript received February 17, 2005. Accepted February 17, 2005. This work was supported by FONCyT, PICT 7124. M.C.C. is a member of the Scientific Career of CONICET.

JF0482461