

INDUCTION OF PHENOLIC COMPOUNDS IN TWO CULTIVARS OF CUCUMBER BY TREATMENT OF HEALTHY AND POWDERY MILDEW-INFECTED PLANTS WITH EXTRACTS OF *Reynoutria sachalinensis*

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Abstract—Accumulation of phenolic compounds (*p*-coumaric, caffeic, and ferulic acids and *p*-coumaric acid methyl ester) was followed in susceptible (Mustang) and tolerant (Flamingo) cucumber (*Cucumis sativus*) cultivars. The objective was to determine whether these compounds played a role in resistance against powdery mildew following a prophylactic treatment with Milsana (leaf extracts from the giant knot weed *Reynoutria sachalinensis*, polygonaceae). This treatment significantly reduced the incidence of powdery mildew in both cultivars. Phenolic compounds were extracted from leaves. In the hydrolyzed fraction containing phenolic aglycones, levels of *p*-coumaric, caffeic, and ferulic acids and of *p*-coumaric acid methyl ester increased in all treatments (with leaf extracts of *R. Sachalinensis*, powdery mildew, or both) except the control, one or two days after treatment. In the fraction containing free phenolics, from the tested compounds, only ferulic acid showed an increase in cv. Flamingo (tolerant), and was particularly evident following treatments. On the other hand, the amounts of hydroxycinnamic acids increased rapidly in the two cultivars following Milsana treatment, suggesting their role in disease reduction. All compounds showed antifungal activity when tested against common pathogens of cucumber (*Botrytis cinerea*, *Pythium ultimum*, and *P. aphanidermatum*), but in general methyl esters were more fungitoxic than their corresponding free acids. This study suggests that

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cucumber is able to release antifungal compounds that are instrumental in repressing powdery mildew infection. This response is seemingly independent from the level of genetic resistance associated with each cultivar.

Key Words—*Cucumis sativus*, phenolic, phytoalexin, Milsana, induced resistance, aglycone.

INTRODUCTION

Plants challenged by pathogens may exhibit several biochemical defense responses (Goodman et al., 1986) such as enzyme synthesis (Stahmann and Demorest, 1973), wall deposition of lignin and suberin (Hammerschmidt and Kuc, 1982, Hammerschmidt et al., 1985, Borg-Olivier and Monties, 1993, Stein et al., 1993), and accumulation of secondary metabolites (Benner, 1993; Bennett and Wallsgrave, 1994). In cucumber, enzyme synthesis and lignification occur as components of systemic acquired resistance (Siegrist et al., 1994). Until recently, secondary metabolites acting as phytoalexins were generally considered to be nonexistent in cucurbits. In 1995, we demonstrated that Milsana (Compo, Germany), a commercial formulation of leaf extracts from *Reynoutria sachalinensis* F. Schmidt stimulated the production of phenolic compounds in glycosylated form in cucumber (Daayf et al., 1995, 1996). Their accumulation was associated with resistance to powdery mildew, caused by *Sphaerotheca fuliginea*, which indicated that they acted as phytoalexins when released in their active deglycosylated form (Daayf et al., 1997b). Of the several compounds that were observed, we characterized one as *p*-coumaric acid methyl ester (*p*-CAME) (Daayf et al., 1997a), while most of the others appeared to be hydroxycinnamic derivatives (Daayf et al., 1997b).

In an attempt to understand better the biological role of these antifungal compounds in cucurbits, the objectives of this work were threefold: (1) to compare the accumulation of *p*-CAME and of specific hydroxycinnamic acids in susceptible (cv. Mustang) and tolerant (cv. Flamingo) cultivars of cucumber infected or not with *S. fuliginea*, (2) to assess the effect of Milsana on the variation of the accumulation of these compounds, and (3) to evaluate the fungitoxicity of these hydroxycinnamic acids and of some close derivatives on growth of three fungal pathogens of cucumber. We also report the identities of some previously unknown compounds.

METHODS AND MATERIALS

Plant Material and Mode of Infection. Seeds of cucumber (*Cucumis sativus* L.) cultivars Mustang and Flamingo were sown in LC-1 Horticultures, fertilized daily with a nutrient solution of N-P-K (7-11-27), and 3-week-old plants transferred to individual pots containing peat and vermiculite. Thirty-two plants were

placed in a growth chamber and divided equally into four groups separated by plastic curtains. Plants from two groups were inoculated artificially by shaking off conidia from powdery mildew-infected leaves. Upon appearance of the first signs of powdery mildew, Milsana (M^+) and water (M^-) were applied each to eight infected (B^+) and eight healthy (B^-) plants, which resulted in four different treatments: B^-M^- , B^-M^+ , B^+M^- , and B^+M^+ . The Milsana treatment comprised an application of leaf extracts from *Reynoutria sachalinensis* with the recommended dosage of concentrated extracts diluted in water (2%).

Estimation of Disease Severity. The severity of powdery mildew was estimated one, two, and seven days after treatment by calculating the percent infected leaf area for all leaves on each plant and the mean for each treatment $\pm$ standard deviation ($P < 0.05$). In addition, means were compared by analysis of variance (ANOVA), at $P = 0.05$, with the software Genstat 5 (IACR, Rothamsted, UK).

Extraction and Fractionation of Phenolic Compounds. Leaves from four plant replicates were used for extraction from each of the four tested treatments (B^-M^- , B^-M^+ , B^+M^- , and B^+M^+) one, two, and seven days after treatment. The method of extraction was adopted as described by Daayf et al. (1997b), allowing for the determination of free and glycosidic-linked phenolics. Chlorophylls, carotenoids, waxes, and lipids were contained in fraction I. Free phenolics were contained in fraction II, and aglycones obtained from glycosidically bound phenolics were in fraction III.

HPLC Apparatus and Determination of Compounds. Compounds were separated by HPLC. The chromatograph was equipped with a photodiode array detector, an autosampler (Waters model 717), and fitted with a C18 reverse-phase column (Waters model RCM (8×100 mm)). Results were analyzed with the program Millenium Software 2.1. The column was eluted with a gradient of 0–100% acetonitrile as follows: [time (minutes)/acetonitrile (percent)/flow (milliliters per minute)] = [0/0/1, 10/0/0.9, 20/15/0.9, 40/15/0.9, 50/25/0.9, 60/25/0.65, 70/40/0.5, 80/50/0.5, 85/70/0.5, 90/80/0.9, 95/80/1, 100/0/1]. Two column cartridges were used to insure optimum separation of molecules. *p*-Coumaric (*p*-CA), *m*-coumaric (*m*-CA), *o*-coumaric (*o*-CA), ferulic (FA), sinapic (SA), and caffeic (CA) acids were identified based on their retention times, their absorbance spectra (220–400 nm), and cochromatography with commercial standards (Sigma Chemicals, St-Louis, Missouri). Standard and extracted compounds had the following retention times (minutes): *p*-CA: 51, *m*-CA: 63, *o*-CA: 73, CA: 38, FA: 59, SA: 58, *p*-CAME: 92, *m*-CAME: 89, and *o*-CAME: 94. Concentration of each compound was estimated on the basis of five-point calibration curves established with corresponding standards and averaged from three separate HPLC runs. Concentration means were compared by analysis of variance with the software Genstat 5 (IACR, Rothamsted, UK).

Esterification. Methyl esters of *p*-CA (*p*-CAME), *m*-CA (*m*-CAME), and

TABLE 1. POWDERY MILDEW INFECTION OF CUCUMBER CULTIVARS MUSTANG (SUSCEPTIBLE) AND FLAMINGO (TOLERANT) IN RESPONSE TO TREATMENT WITH MILSANA^a

Days after treatment	Percent infection			
	M ⁻ B ⁻	M ⁻ B ⁺	M ⁺ B ⁻	M ⁺ B ⁺
Flamingo				
1	0 ± 0 (a)	5 ± 1 (b)	0 ± 0 (a)	5 ± 1 (b)
2	0 ± 0 (a)	5 ± 2 (b)	0 ± 0 (a)	5 ± 1 (b)
7	2 ± 1 (ab)	30 ± 3 (c)	2 ± 1 (a)	9 ± 3 (b)
Mustang				
1	0 ± 0 (a)	5 ± 3 (b)	0 ± 0 (a)	5 ± 2 (b)
2	1 ± 2 (a)	7 ± 2 (b)	0.5 ± 2 (a)	6 ± 2 (ab)
7	2 ± 2 (a)	43 ± 2 (d)	2.5 ± 3 (ab)	11 ± 2 (b)

^aControl consisted of water application (B⁻M⁻), B⁺ inoculation with powdery mildew pathogen, M⁺, treatment with Milsana extract. Each value represents the mean infection rate of eight plants (%) ± SD. Data were submitted to analysis of variance. Values followed by the same letter do not differ significantly at *P* ≤ 0.05 (LSD: 4.4).

o-CA (*o*-CAME) were synthesized as follows: in a condenser-equipped flask containing 20 ml of methanol, we added 1 g of *p*-CA, *m*-CA, or *o*-CA (Sigma) and 10 ml (0.12 mol) of BF₃–MeOH complex (Aldrich). The mixture was heated 60 min at 60°C. After cooling to room temperature, the methanol was removed under vacuum. The residue was diluted with water and extracted with ethyl acetate (3 × 20 ml). The organic fractions were combined, washed with water, dried over anhydrous magnesium sulfate, filtered, and evaporated under vacuum. The residue was purified by recrystallization or flash chromatography. Analysis of the synthesized molecules gave the following spectral data: EI-MS (70 eV), *m/z* (relative intensity): *p*-CAME 178 M⁺(65), 179 M⁺1(8), 147 M⁺-CH₃O(100), 119 M⁺-C₂H₃O₂(28), 91 M⁺-C₄H₇O₂); *m*-CAME 178 M⁺(19), 179 M⁺+1(2), 146 M⁺-CH₃OH(100), 118 M⁺-C₂H₄O₂(99), 91 M⁺-C₄H₇O₂(42); *o*-CAME 178 M⁺(78), 179 M⁺+1(10), 146 M⁺-CH₃OH(100), 119 M⁺-C₂H₃O₂(35), 91 M⁺-C₄H₇O₂(40).

The identity of these molecules was confirmed on the basis of data stored in the GC-MS compounds bank.

Effects of Compounds on Fungal Growth. Authentic samples of *p*-CA, *m*-CA, *o*-CA, CA, FA, SA, *p*-CAME, *m*-CAME, and *o*-CAME were tested for their fungitoxicity against *Pythium ultimum*, *P. aphanidermatum*, and *Botrytis cinerea* as root and foliar pathogens. Briefly, for each replicate, a mycelial disk (3 mm) was placed in the center of a Petri dish and three aliquots of each compound were placed 4 cm away from the fungal disk. Five concentrations (0, 1, 3, 5, or 10 µg/ml) were tested. Growth of mycelium was then observed daily or hourly, depending on the speed of fungal growth. Reported results were obtained for

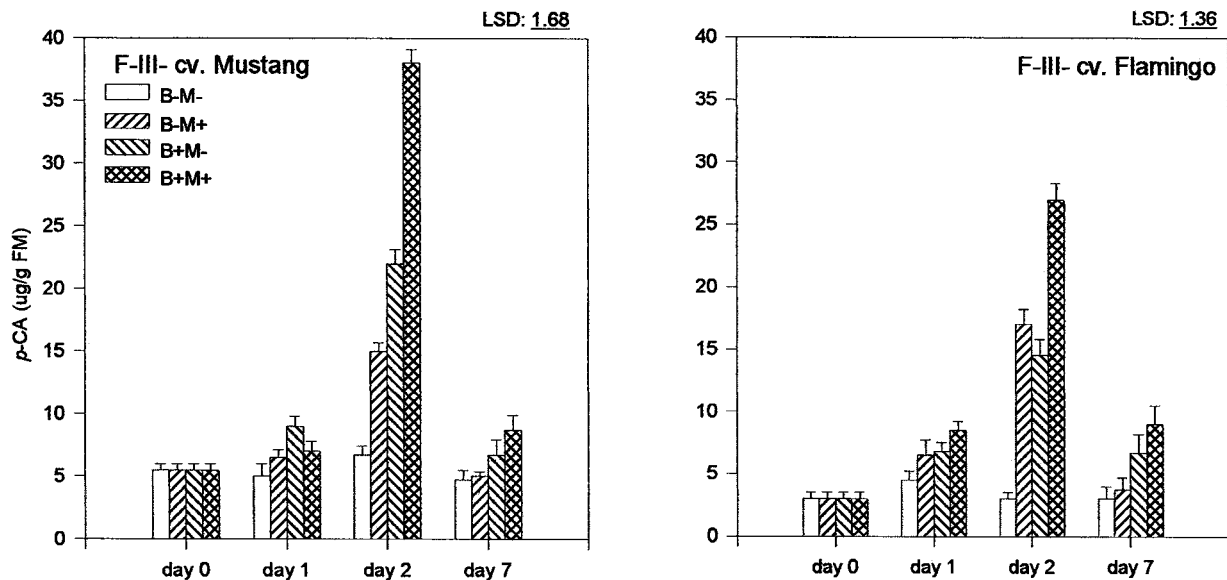
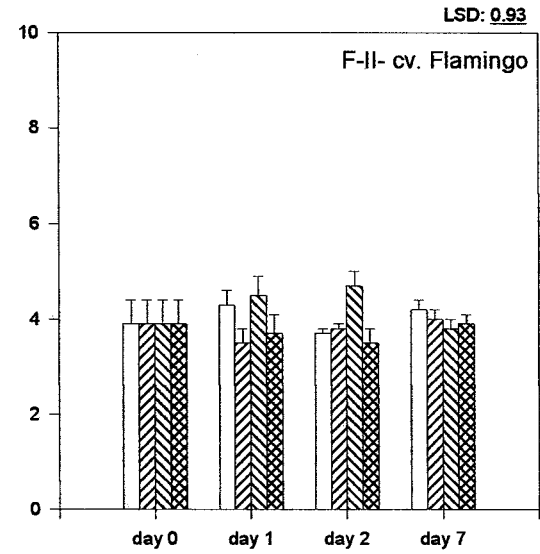
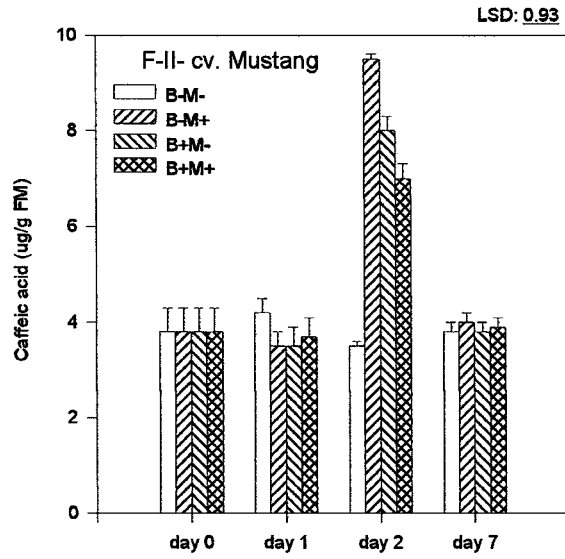


FIG. 1. Concentrations of *p*-coumaric acid over time in its aglycone form (FIII) from cucumber leaves obtained from healthy control (B^-M^-), healthy Milsana-treated (B^-M^+), powdery mildew-infected (B^+M^-), and Milsana-treated (B^+M^+) plants of two cultivars: Mustang (susceptible) and Flamingo (tolerant). Each value is the mean $\pm$ SD of three separate HPLC runs. FM: fresh weight. LSD: least significant difference for days $\times$ treatments based on ANOVA at $P = 0.05$. F-II- and F-III- represent results for free phenolics and for aglycone phenolics obtained after hydrolysis, respectively.



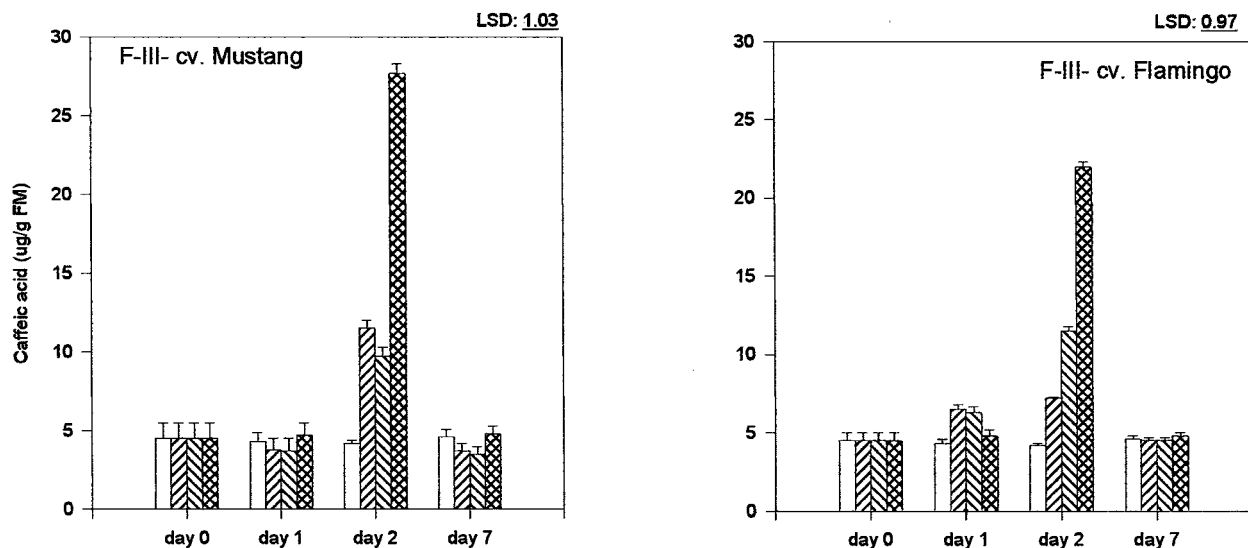


FIG. 2. Concentrations of caffeic acid over time in its free (FII) and aglycone (FIII) forms, from cucumber leaves obtained from healthy control ($B^{-}M^{-}$), healthy Milsana-treated ($B^{-}M^{+}$), powdery mildew-infected ($B^{+}M^{-}$), and Milsana-treated ($B^{+}M^{+}$) plants of two cultivars: Mustang (susceptible) and Flamingo (tolerant). Each value is the mean $\pm$ SD of three separate HPLC runs. FM: fresh weight. LSD: least significant difference for days $\times$ treatments based on ANOVA at $P = 0.05$. F-II- and F-III- represent results for free phenolics and for aglycone phenolics obtained after hydrolysis, respectively.

each fungus when the control culture (0 μg) reached confluence on the Petri dish. This experiment was replicated three times and means were compared by analysis of variance with the software Genstat 5 (IACR, Rothamsted, UK).

RESULTS AND DISCUSSION

Pathogenicity Test. Application of Milsana resulted in a reduction of powdery mildew incidence in the tested cultivars, relative to control, seven days after the treatment (Table 1). Untreated plants of cv. Flamingo (tolerant) showed a lower rate of infection than those of cv. Mustang (susceptible), but these differences subsided in treated plants (B^+M^+). These results confirm the prophylactic properties of Milsana as previously reported (Daayf et al., 1995, Dik and VanDerStraay, 1995). When cucumber leaves were analyzed for their phenolic content, high variations in the concentrations of *p*-CAME and of some hydroxycinnamic acids were shown in cucumber leaves. These concentrations were affected by infection with *S. fuliginea*, by the Milsana treatment, and by the cultivar tested.

Changes in Phenolic Compounds. In the fraction containing free phenolics (fraction II), *p*-CA was present in traces only. In the hydrolyzed fraction (fraction III), *p*-CA aglycone concentration increased the first day and mostly the second day after treatment in both cultivars (Figure 1). These increases were higher in B^+M^+ , with higher accumulation in the susceptible cv. Mustang. On day 7 after treatment, these concentrations dropped significantly.

Concentration of caffeic acid (CA) in fraction II showed no difference among treatments in cv. Flamingo, while an increase was recorded the second day in cv. Mustang in all treatments but the control (Figure 2). In fraction III, the increase in CA levels induced by powdery mildew infection and/or Milsana application occurred on day 2 after treatment in the two cultivars (Figure 2) with a higher level in cv. Mustang. As with *p*-CA, the highest level was found in the treatment B^+M^+ .

FA and SA showed very similar retention times and absorbance spectra; as a result they were eluted as two distinct peaks only when present in very low quantities. However, they could be distinguished on the basis of their absorbance spectra. They each have maximum absorbance at 325.1 nm, but only FA has a shoulder at 295 nm. The concentration of FA estimated in fraction III was shown to increase on day 2 in all treatments except the control (Figure 3). This increase was particularly important in the tolerant cv. Flamingo (Figure 3), which may explain in part the difference in powdery mildew resistance between the two cultivars. Hydroxycinnamic acids are known to have antimicrobial activity (Baranowski and Nagel, 1982), and some of them, including FA and SA, are components of the lignification process, which has been shown to occur as a

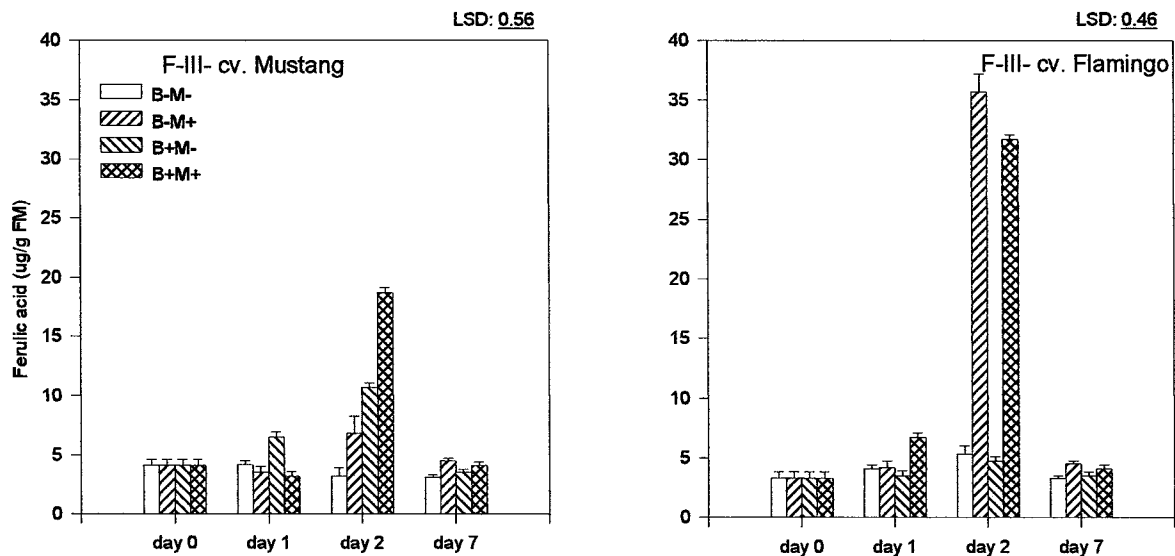
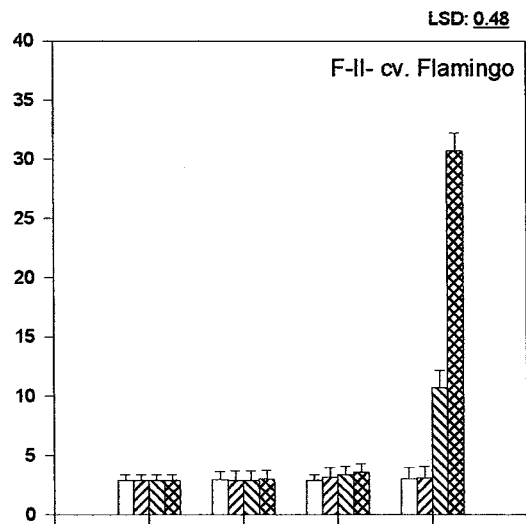
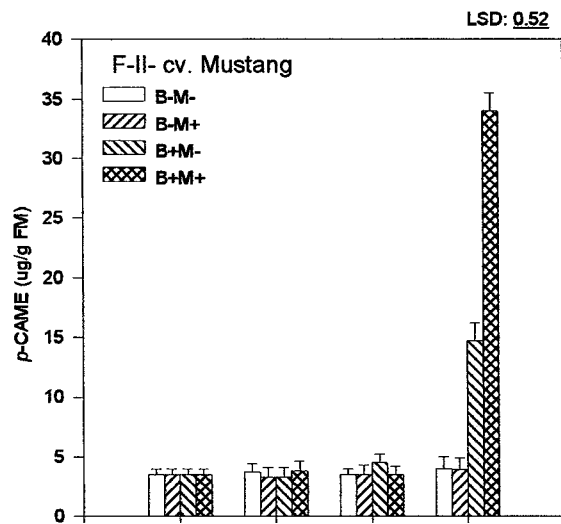


FIG. 3. Concentrations of ferulic acid over time in its aglycone form (FIII) from cucumber leaves obtained from healthy control (B^-M^-), healthy Milsana-treated (B^-M^+), powdery mildew-infected (B^+M^-), and Milsana-treated (B^+M^+) plants of two cultivars: Mustang (susceptible) and Flamingo (tolerant). Each value is the mean $\pm$ SD of three separate HPLC runs. FM: fresh weight. LSD: least significant difference for days $\times$ treatments based on ANOVA at $P = 0.05$. F-II- and F-III- represent results for free phenolics and for aglycone phenolics obtained after hydrolysis, respectively.



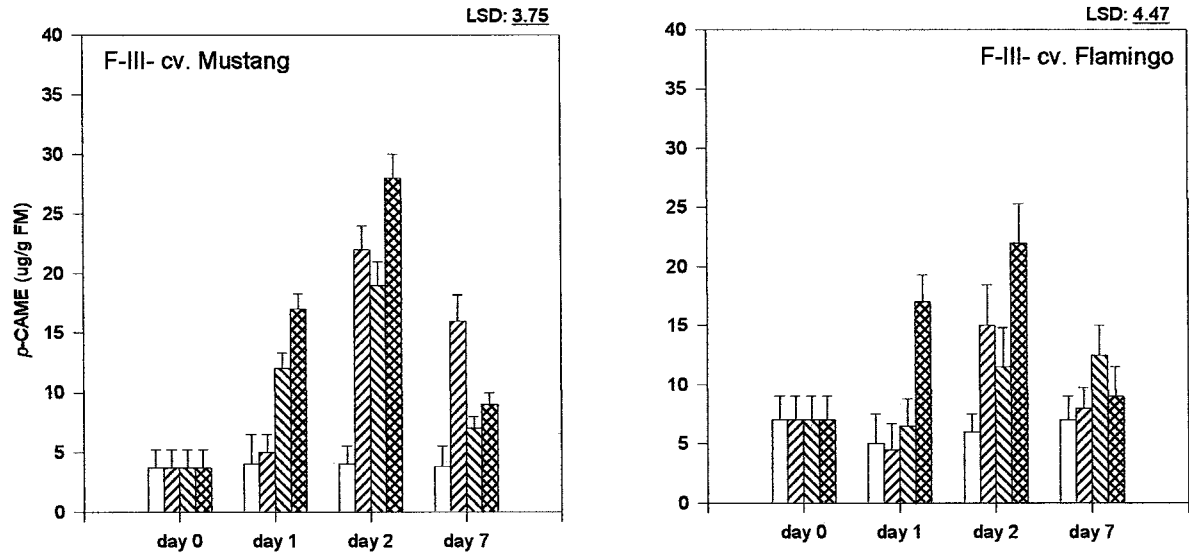


FIG. 4. Concentrations of *p*-coumaric acid methyl ester (*p*-CAME) over time in its free (FII) and aglycone (FIII) forms, obtained from healthy control ($B^{-}M^{-}$), healthy Milsana-treated ($B^{-}M^{+}$), powdery mildew-infected ($B^{+}M^{-}$), and Milsana-treated ($B^{+}M^{+}$) cucumber leaves of two cultivars: Mustang (susceptible) and Flamingo (tolerant). Each value is the mean $\pm$ SD of three separate HPLC runs. FM: fresh weight. LSD: least significant difference for days $\times$ treatments based on ANOVA at $P = 0.05$. F-II- and F-III- represent results for free phenolics and for aglycone phenolics obtained after hydrolysis, respectively.

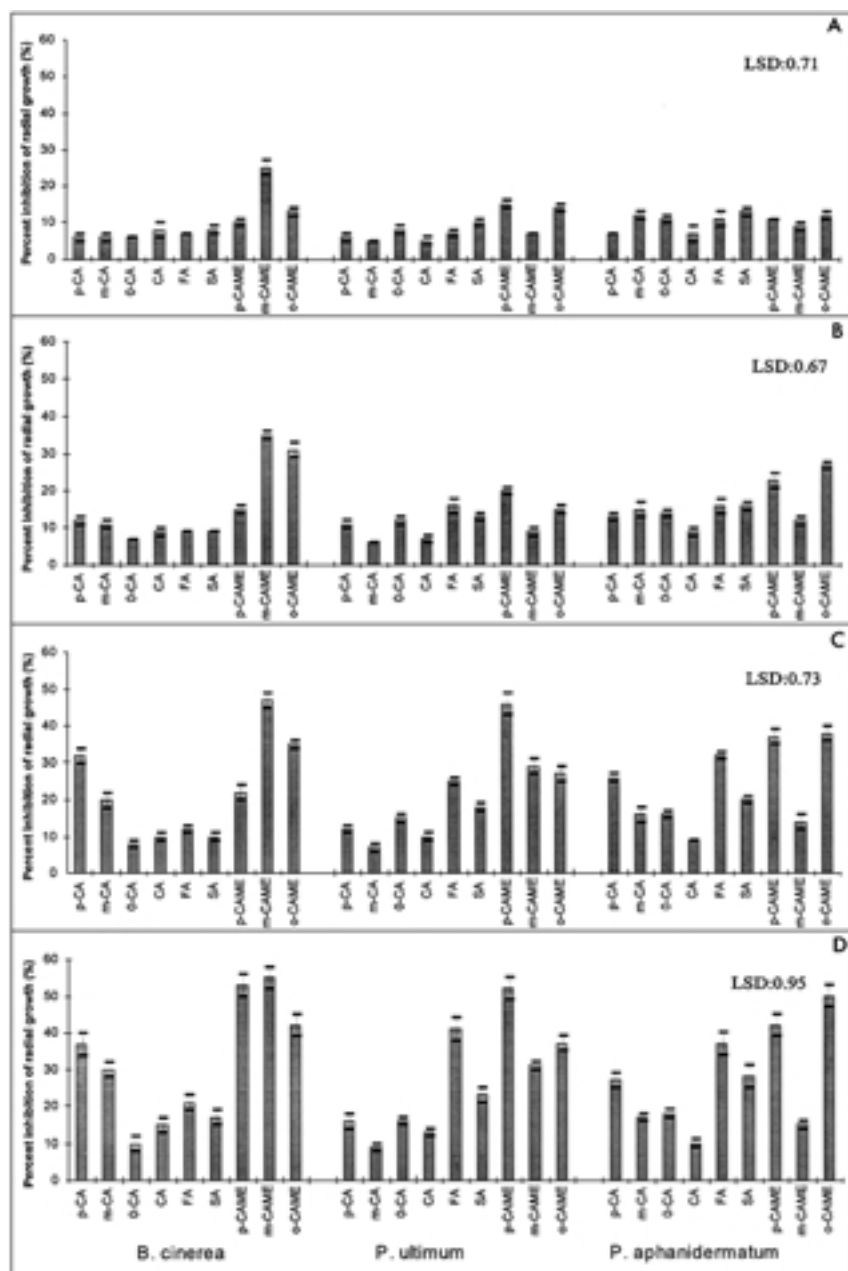
cucumber defense reaction in response to microbial attack (Hammerschmidt and Kuc, 1985; Stein, 1993). However, in our study, the increase of FA in the tolerant cultivar was associated only with treatment with Milsana (Figure 3).

Concentrations of *p*-CAME, previously described as a phytoalexin-like compound in cv. Mustang (Daayf et al., 1997a, 1997b), also were evaluated along with those of the isomers *m*-CAME and *o*-CAME. The latter two were not detected in the tested cultivars in either fraction. One and two days after treatment, no significant variation could be recorded in concentration of *p*-CAME in fraction II (Figure 4). On the other hand, analysis of these fractions seven days after treatment showed a marked increase in this compound within infected leaves of the two cultivars (Figure 4). The highest levels were reached when plants had both treatments (inoculated and treated with Milsana) (B⁺M⁺). In fraction III, an increase in *p*-CAME level occurred one day after treatment in cv. Mustang B⁺M⁻ and B⁺M⁺ and in cv. Flamingo B⁺M⁺. Accumulation of *p*-CAME in response to infection and treatment with Milsana was more marked the second day after treatment and was higher in cv. Mustang (Figure 4).

Antifungal Activity of Phenolic Compounds. When tested for their antifungal activity, *p*-CA, *m*-CA, and *o*-CA were more fungitoxic on *B. cinerea*, a foliar and stem pathogen, than on *P. ultimum* and *P. aphanidermatum*, which are root rot agents. FA and SA had the opposite effect (Figure 5), and CA was found to be the least fungitoxic of the tested compounds. Methyl esters generally showed higher inhibitory activities than their corresponding acids (Figure 5), which confirm data previously reported for other microorganisms (Baranowski and Nagel, 1982, 1984). In addition, alkyl esters inhibit fungal (Macko et al., 1972) as well as bacterial growth (Baranowski and Nagel, 1982, 1983) and also have antioxidant activities in food products (Baranowski and Nagel, 1984). *p*-CAME was previously reported in the rhizome of *Costus speciosus* as a constitutive principle with antifungal activity against *Cladosporium cucumerinum*, *Colletotricum*, *Curvularia* sp., and *Penicillium* sp. (Bandara et al., 1988). However, methyl esters are rarely cited as components of plant tissues (Bandara et al., 1988, Daayf et al., 1997b). This pattern of accumulation is in line with the results obtained previously with cv. Mustang (Daayf et al., 1997b).

The accumulation of compounds with antifungal activity suggests the non-

FIG. 5. (Opposite) Percent in vitro inhibition of radial growth of three fungal pathogens of cucumber by different phenolic compounds. A, B, C, and D represent results at four concentrations (1, 3, 5, and 10 µg/ml). Percent corresponds to reduction of fungal radial growth as compared to the control (medium with no phenolics added). *p*-CA, *m*-CA, and *o*-CA: *p*-, *m*-, and *o*-coumaric acids; CA, FA, and SA: caffeic, ferulic, and sinapic acids; and *p*-CAME, *m*-CAME, and *o*-CAME: *p*-, *m*-, and *o*-coumaric acid methyl esters, respectively. LSD: least significant difference based on ANOVA at *P* = 0.05.



specific role that cucumber antifungal compounds may play in the defense process against pathogens. Indeed, in addition to their possible protective role against powdery mildew (Daayf et al., 1995, 1996, 1997a,b), it appears that these compounds can have activity against both foliar (*B. cinerea*) and root (*P. ultimum* and *P. aphanidermatum*) pathogens. Considering that only foliar extracts were tested here, it is nonetheless relevant to compare these results with those of Chérif et al. (1994), who demonstrated accumulation of antifungal compounds in crude extracts of cucumber roots in response to a *Pythium* attack.

The cultivars we tested could not be distinguished in this study on the basis of differential accumulation of phenolics that may be related to their differences in susceptibility to powdery mildew. Both cultivars reacted to the infection and to the Milsana application by increasing concentrations of their antifungal compounds. This suggests that tolerance of cv. Flamingo is predetermined also by other factors, such as cuticle thickness and lignification, which would be in line with FA production.

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