Evidence of Phytoalexins in Cucumber Leaves Infected with Powdery Mildew following Treatment with Leaf Extracts of *Reynoutria sachalinensis*[1]

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Phenolic compounds extracted from cucumber (*Cucumis sativus* L.) leaves were separated and analyzed for their differential presence and fungitoxicity in relation to a prophylactic treatment with Milsana (Compo, Münster, Germany) against powdery mildew (*Sphaerotheca fuliginea*). Based on our extraction and purification procedures, at least eight separate phenolic compounds with antifungal activity were identified as intrinsic components of cucumber plants. Of these compounds, six displayed a significant increase in concentration as a result of elicitation with Milsana, this being particularly evident when the plant was stressed by the pathogen. The combined amounts of these antifungal compounds in treated plants was nearly five times the level found in control plants. One week after Milsana application, some of the antifungal compounds obtained through hydrolysis of their glycosidic links were also detected in their free form, indicating that they are likely liberated from conjugated phenolics by enzymatic hydrolysis in planta. To our knowledge, these results provide the first direct evidence that cucumber plants produce elevated levels of phytoalexins in response to an eliciting treatment after infection.

Several defense mechanisms are known to be induced in plants challenged by pathogens (Lyon et al., 1995). Such mechanisms culminate in a number of physical and biochemical changes, including lignification and suberization of the plant cell wall (Hammerschmidt and Kuc, 1982; Hammerschmidt et al., 1985; Stein et al., 1993), deposition of callose (Benhamou, 1992), de novo synthesis of pathogenesis-related proteins (Carr and Klessig, 1989), and biosynthesis and accumulation of secondary metabolites (Bennett and Wallsgrove, 1994), namely phytoalexins (Bailey and Mansfield, 1982; Darvill and Albersheim, 1984; Ebel, 1986). With the exception of the latter, such reactions have been reported to occur as components of systemic acquired resistance in cucumber (Siegrist et al., 1994). Surprisingly, although they are commonly found in many plants, phytoalexins have never been reported to play a role in the resistance expressed by cucumber upon chal-

lenge with a microorganism (Dixon and Paiva, 1995). This situation contrasts with the concept that plants have developed a generalized response system to stress.

In recent studies, evidence was provided that Milsana (Compo, Münster, Germany), a commercial formulation of extracts from leaves of the giant knotweed *Reynoutria sachalinensis* F. Schmidt (Nakai), significantly reduced the incidence of powdery mildew (*Sphaerotheca fuliginea* Schlecht. ex. Fr Poll.) on cucumber (*Cucumis sativus* L.) plants under both small- and large-scale conditions (Herger et al., 1988; Daayf et al., 1995; Dik and Van der Straay, 1995). The active ingredients are believed to be natural elicitors that induce the plant's natural defense mechanisms.

Although a number of morphological and biochemical modifications have been reported to take place in cucumber leaves in response to Milsana, no direct association has been made with the reported prophylactic properties. Some biochemical modifications such as increased chlorophyll values (Herger and Klingauf, 1990) may explain changes in leaf morphology following Milsana application (Daayf et al., 1995), but these are unlikely to be related to the protective properties of the extracts. In contrast, other biochemical changes, including increases in the activity of peroxidases, polyphenoloxidases, and Phe ammonia-lyase (Herger and Klingauf, 1990; Schneider and Ullrich, 1994), imply the capacity of the plant to stimulate its phenylpropanoid pathway. However, no phytoalexins could be detected in cucumber plants treated with Milsana (Kowalewski, 1993).

In a recent study, we were able to show that Milsana stimulated the production of fungitoxic phenolic compounds in cucumber (Daayf et al., 1995). The bulk of the fungitoxic activity was found in phenolics in their aglycone form. The presence of these aglycones appeared to correlate with the prophylactic properties of the product. In an attempt to determine whether these compounds were phytoalexins and could play a role in the resistance induced by Milsana in cucumber, the objectives of the present study

Abbreviations: FII, fraction II; FIII, fraction III; LW, long-wavelength UV light; *p*-CAME, *para*-coumaric acid methyl ester; Rt, retention time (in min) as observed by HPLC in a water-acetonitrile gradient; SW, short-wavelength UV light.

were (a) to isolate and purify phenolic compounds produced by powdery mildew-infected cucumber leaves in response to Milsana treatment; (b) to assess their fungitoxicity; and (c) to evaluate their concentrations in infected plants treated with Milsana in comparison with control plants.

MATERIALS AND METHODS

Extraction and Fractionation of Compounds

Seeds of cucumber (*Cucumis sativus* L. cv Mustang) were sown in LC-1 Horticubes (Smith-Oasis, Kent, OH) and fertilized daily with a nutrient solution of N-P-K (7:11:27, v/v) for 3 weeks under greenhouse conditions. Plants were then transferred to gullies fed by a base nutrient solution, as described by Chérif and Bélanger (1992).

Plant material used for extraction and fractionation of compounds came from cucumber plants that were part of an experiment (experiment 1) aimed at evaluating the protective role of Milsana against powdery mildew. This experiment comprised a control treatment, in which plants were sprayed with water, or a treatment with Milsana, in which plants were given the recommended dosage of concentrated extracts diluted in water (2%). For each treatment, four rows of eight plants each were used, and each section of 32 plants was separated by a guard row. Plants were placed in a greenhouse and observed daily for the presence of *Sphaerotheca fuliginea* colonies. In this experiment, the natural presence of powdery mildew spores in the greenhouse ensured leaf infection without artificial inoculation. Upon appearance of the first signs of infection, the treatments were applied weekly, and disease incidence was assessed as a percentage of leaf infected area by calculating the percentage for all leaves on each plant and the mean for each treatment, as described by Daayf et al. (1995).

Four to five lower cucumber leaves (approximately 100 g) were sampled 1 d after treatment from leaves that showed no signs of infection prior to the water (M−S−) or Milsana application (M+S−) and leaves that were infected (4%) prior to the treatments (M−S+ and M+S+). A modified extraction method of Dercks and Buchenauer (1986) was adopted, as described by Daayf et al. (1995), allowing for the determination of free and glycoside-linked phenolics. The foliar material was homogenized with 80% methanol at 10 mL/g, protected from oxidation by replacing oxygen with nitrogen and eliminating light, and extracted for 48 h on a rotary shaker. After extraction, the methanolic homogenate was filtered, and the residue was washed with 20 mL of 80% methanol. Chlorophyll, carotenoids, lipids, and waxes were removed by partitioning against light petroleum ether three times (fraction I). The methanolic fraction containing the phenolic constituents was evaporated at 38°C, and the aqueous residue was partitioned three times with 30 mL of anhydrous ethyl ether.

Free phenolic compounds were found in the ether fraction (FII). The aqueous fraction was diluted with an equal volume of 4 N HCl and acid hydrolysis was performed for 90 min in a water bath at 100°C. After cooling, the hydro-lysate was partitioned against anhydrous ethyl ether (FIII). Concentrations of FII and FIII were adjusted to 20 g of fresh material per milliliter of methanol.

Detection, Separation, and Quantification of Compounds

The scheme of separation of compounds of interest is shown in Figure 1. FIII (aglycones) from infected plants treated with Milsana (M+S+) was separated first because it had exhibited the highest level of fungitoxicity on TLC bioassay (Daayf et al., 1995). To this end, a concentrate of this fraction was eluted through a silica-gel flash column (40 × 3 cm), with 200 mL of each of the following solvent systems: dichloromethane:ethyl acetate (95:5, v/v), (90:10, v/v), (85:15, v/v), (80:20, v/v), (70:30, v/v), (60:40, v/v), and (40:60, v/v); 100 mL of dichloromethane:ethyl acetate (20:80, v/v); and 100 mL of each of the following systems: dichloromethane:methanol (80:20, v/v), (50:50, v/v), and (0:100, v/v). This separation led to 185 subfractions, and those showing bands with equivalent R_F values in TLC assays were pooled together to yield 21 subfractions (A–U). Detection of these bands was carried out using the universal detection reagent (75 g of ammonium molybdate, 4 g of ceric sulfate, and 500 mL of 10% sulfuric acid:H_2O, v/v), which showed dark spots after heating treated thin-layer chromatograms.

Each fraction was tested for fungitoxic activity directly on thin-layer chromatograms against *Cladosporium cucumerinum* Ellis and Arth., as described previously (Daayf et al., 1995). The fractions (20- to 50-μL aliquots) were spotted on silica-gel TLC plates (Silica Gel 60 F-254, Merck) and developed with dichloromethane:hexane:methanol (BDH, Inc., Toronto, Canada). After drying for 1 to 2 h, the plates were covered with a concentrated conidial suspension of *C. cucumerinum* mixed (1:1, v/v) in a 20 g/L solution of potato dextrose agar. The plates were then incubated in a humid chamber for 48 to 72 h, and zones of inhibition appeared as white spots against a dark background formed by spores and mycelium of *C. cucumerinum*. To assess whether fungitoxicity would result from Milsana itself, simultaneous TLC and bioassays were performed with Milsana alone and with Milsana hydrolyzed under the same conditions as the plant extracts at corresponding concentrations.

The bioactive fractions were then chromatographed by preparative TLC for further separation. Bands were reisolated and eluted, each resulting in corresponding subfractions (e.g. Ca, Cb, . . . from fraction C). These were again tested for their bioactivity against *C. cucumerinum*. Subfractions showing no fungitoxicity were eliminated, and those showing fungitoxicity were kept for two-dimensional TLC with dichloromethane:hexane:methanol (6:4:1, v/v) for the first chromatography dimension, and with different solvents for the second dimension, both of which depend on the polarity of compounds to be separated. For each active subfraction (Ca, Cb, . . . from bioactive fractions), four simultaneous two-dimensional TLC steps were carried out for the following purposes: (a) observation of their behavior under SW and LW, R_F estimation, and fungitoxicity; (b) application of ammonium hydroxide vapors, which reinforce or modify fluorescence of
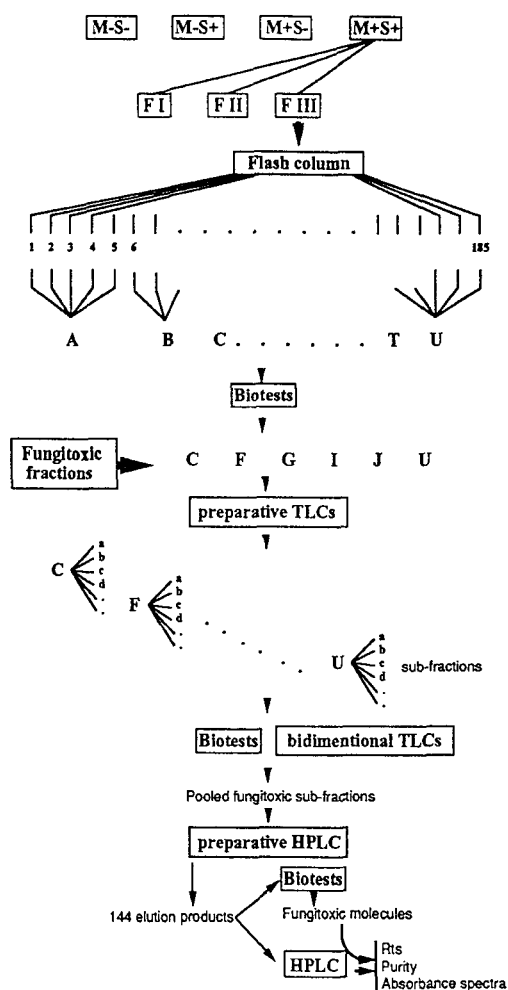
Figure 1. Flowchart describing techniques used in separation and isolation of antifungal compounds from cucumber plants. FIII (agly-cones) from powdery mildew-infected cucumber leaves treated with Milsana (M+S+) exhibited the highest level of fungitoxicity, and was used for further separation of fungitoxic compounds. A first separation was carried out by eluting this fraction through a silica-gel flash column with 200 mL of each of the following solvent systems: dichloromethane:ethyl acetate (95:05, v/v), (90:10, v/v), (85:15, v/v), (80:20, v/v), (70:30, v/v), (60:40, v/v), and (40:60, v/v); 100 mL of dichloromethane:ethyl acetate (20:80, v/v); and 100 mL of each of the following systems: dichloromethane:methanol (80:20, v/v), (50:50, v/v), and (0:100, v/v). One-hundred-eighty-five subfractions were obtained and were pooled into 21 fractions based on equivalent TLC profiles. After bioactivity tests against *C. cucumerinum*, fractions C, F, G, I, J, and U were retained based on their bioactivity. Each fraction was then fractionated on bidimensional TLC for chemical detections and calculation of R_F values. Final purification of fungitoxic compounds was carried out using preparative HPLC. All elution products were tested again for their fungitoxicity, and fungitoxic molecules were then HPLC-eluted to confirm their Rts, absorbance spectra, and purity.

certain phenolics; (c) spray of Neu's reagent (sodium 2-aminoethyl-diphenyl-borate, 1% in methanol), which detects flavonoids and some caffeic acid derivatives (Andary et al., 1984); and (d) spray of Benedikt's reagent (mixture 1, 1.6 g of $CuSO_4 \cdot 5\ H_2O$, fill to 15 mL of H_2O; mixture 2, 15 g of sodium citrate, 13 g of anhydrous Na_2CO_3, 1 g of $Na[CO_3]_2$, fill to 65 mL of hot H_2O; mixtures 1 and 2 are combined and completed to 100 mL), which quenches fluorescence of orthodiphenolics and increases that of monophenolics (Reznik and Egger, 1961).

Compounds of each subfraction (e.g. Cb, Cc, Cd, . . . etc. from fraction C) showing fungitoxicity were pooled together to prevent losses before subsequent separation. Final purification was then carried out with a reverse-phase HPLC column (C_{18}) as follows. Each pooled fungitoxic fraction was eluted using the solvent gradient described previously. All HPLC elution products of each fungitoxic fraction were numbered and concentrated using a speed vacuum to be tested separately for their fungitoxicity against *C. cucumerinum*. Molecules exhibiting biological activity were then re-chromatographed with a C_{18} reverse-phase column to confirm their Rt and absorbance spectrum, which was simultaneously calculated by the photodiode array detector system.

Concentrations of compounds were calculated as micrograms (or microgram standard equivalents) per gram of fresh material after having established a calibration curve using standards. In the case of unknown compounds, quantification was based on equivalents of standards with close chemical characteristics, as described by Higgins et al. (1995). Concentrations were averaged from amounts detected in three separate HPLC runs.

Kinetics of *p*-CAME Accumulation

One of the antifungal compounds, which was chemically characterized as *p*-CAME (Daayf, et al. 1997), was used for kinetics studies. For this purpose, a second experiment was run under controlled conditions. Cucumber seeds were sown as described above, and 3-week-old plants were 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 to ensure simultaneous and uniform infection of plants 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 to eight infected (S+) and eight healthy (S−) plants. Disease incidence was calculated 1, 2, 7, and 21 d after treatments, as described previously.

Four to five leaves were sampled 1, 2, and 7 d after application of Milsana from each of the four treatments (M−S−, M−S+, M+S−, and M+S+) (see Table II). *p*-CAME was extracted as described earlier and localized by HPLC of total extracts by comparison of Rt and absorbance spectra of separated compounds with those of total extracts. Its concentration was followed in both FII and FIII 1, 2, and 7 d after treatment. Values and sɒs were averaged from three separate HPLC runs.

TLC and HPLC Apparatus

TLC was conducted on 0.5 mm of silica gel (Sigma) on glass plates for preparative TLC, and on 0.2 mm of silica gel (Sigma) on aluminum sheets for analytical TLC. Observation of plates was carried out with UV lamps (model UVS-11 for SW and model UVL-21 for LW, UV Products, Inc., San Gabriel, CA). Purification of compounds was made by HPLC on a delivery system using a controller (model 600E, Waters) equipped with a photodiode array detector, allowing for direct determination of peak absorbance spectra, and an autosampler (model 717, Waters), and fitted with an 8×100 mm C_{18} reverse-phase column (model RCM, Waters) for analytical chromatography or with a 25×200 mm C_{18} reverse-phase column (model RCM, Waters) for preparative HPLC. In the latter case, the solvent flow used for analytical chromatography was multiplied by 6. Results were analyzed using Millenium Software, version 2.1 (Millipore). The column was eluted with a gradient from 0 to 100% acetonitrile as follows: (time [min]/acetonitrile [%]/flow [mL/min] = 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, and 100/0/1). For the analytical HPLC, two-column cartridges were used in tandem to ensure optimum separation of molecules.

RESULTS

Extraction and Fractionation of Phenolic Compounds

In the first experiment, the presence of powdery mildew spores in the greenhouse ensured leaf infection without artificial inoculation by the experimenter. In this experiment, cucumber leaves showed an average level of infection of 4% prior to the treatments. One week later, this level had reached 15 and 10% in the water and Milsana treatments, respectively. After 7 weeks, control plants were infected at 100% compared with 40% for Milsana-treated plants. Of all of the fractions tested 1 d after treatment (M−S−, M−S+, M+S−, and M+S+), FIII (aglycones) from M+S+ exhibited by far the highest fungitoxicity level against *C. cucumerinum*. Elution of a concentrate (from 100 g fresh weight) of FIII from M+S+ through a flash column yielded 185 subfractions, which were pooled into 21 subfractions (A–U) on the basis of their TLC profiles following detection with ceric acid-ammonium molybdate reagent (Fig. 1). When these 21 fractions were tested individually for fungitoxicity, only six displayed activity: C, F, G, I, J, and U (Fig. 1).

Detection, Separation, and Quantification of Antifungal Compounds

Separation of the six bioactive fractions on TLC indicated the presence of several compounds within each fraction with different properties under UV light at SW and LW (Fig. 2, A and B). When the same thin-layer chromatograms were tested for fungitoxicity, it was clear that only certain compounds within each lane were bioactive (Fig. 2C). Consequently, the use of preparative TLC allowed for the subfractioning of the six individual fractions, which led to
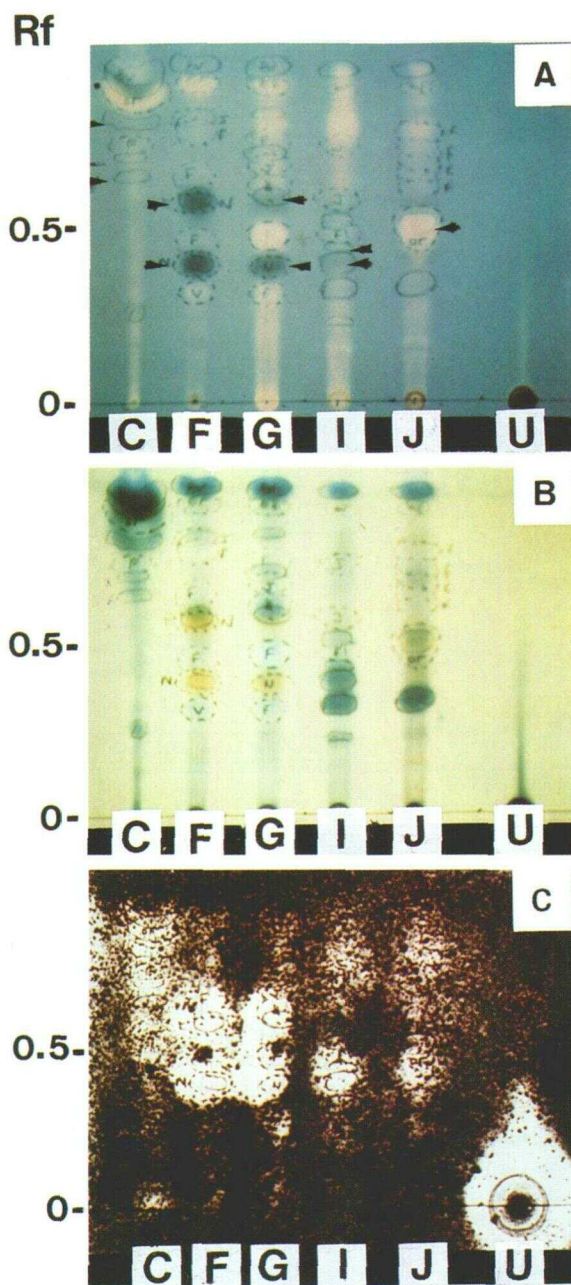

Figure 2. TLC of six fungitoxic fractions (C, F, G, I, J, and U) (30-μL aliquots from 20 g fresh weight/mL) extracted from powdery mildew-infected cucumber leaves 1 d after treatment with Milsana. A, Observed under LW. B, The same chromatogram observed under SW. C, The same chromatogram sprayed with a conidial suspension of *C. cucumerinum*; white spots indicate zones of fungitoxicity. To obtain fractions eluted here, a concentrated sample of powdery mildew-infected leaves treated with Milsana (M+S+) was fractionated by flash chromatography on silica gel, and bioactive fractions were then chromatographed on TLC to get information on bioactive compounds and to remove most of the nonbioactive compounds.

a further screening of the fungitoxic compounds (Fig. 1). For example, bioactive fraction C was divided into 11 entities (Ca–Ck), and among these only 5 (Cb, Cc, Cd, Ce, and Ch) displayed fungitoxicity as measured by the bioas-

say (Fig. 3). These subfractions from fractions C, F, G, I, J, and U presented different chemical characteristics following two-dimensional TLC (Fig. 1), which are summarized in Table I.

When all of the active subfractions were pooled into corresponding fractions and concentrated, the latter were eluted by preparative HPLC, leading to a total of 144 elution products (Fig. 1), which were each assigned a number (1–144). They were further tested for bioactivity and 8 were found to yield Rts of these bioactive molecules (Table I). Comparison of their Rts and absorbance spectra with those observed for total FIII from all four sets of sampled leaves (S−M−, S−M+, S+M−, and S+M+) led to the precise localization of their peaks on the total high-performance liquid chromatograms (Fig. 4).

For quantitative and comparative purposes, these chromatograms were analyzed based on equivalent amounts of fresh leaf material, as shown for total FIII from S−M− and S+M+ (Fig. 4). From these chromatograms, at least 21 peaks were identified as being common to both, but only 6 of them (1, 4, 10, 12, 16, and 20) contained active molecules when tested for fungitoxicity (Fig. 1; Table I). Many of these peaks displayed a marked increase in concentration in M+S+ compared with M−S− (Fig. 4), which indicates that they behaved like phytoalexins. For instance, bioactive peaks 10, 12, 16, and 20 showed an increase in concentration ranging from 2- to 9-fold compared with their counterpart in M−S−.

One day after Milsana treatment, bioactive compound 72 (peak 10) displayed a 7-fold increase in concentration in M+S+ (65 ± 5 μg m-coumaric acid equivalents/g fresh weight) compared with M−S− (9 ± 4 μg m-coumaric acid equivalents/g fresh weight) (Fig. 4). Compound 57 (peak 12) was roughly twice as high in M+S+ (15 ± 2.5 μg p-coumaric acid equivalents/g fresh weight) compared with M−S− (7 ± 1.5 μg p-coumaric acid equivalents/g fresh weight). Peak 16, containing three active compounds, exhibited a 9-fold increase in M+S+ (45 ± 8 μg p-coumaric

Rf

Figure 3. Chromatogram inhibition assay with *C. cucumerinum* of subfractions from fraction C extracted from infected cucumber leaves treated with Milsana after separation with dichloromethane:hexane:methanol (6:7:1, v/v). White spots indicate zones of fungitoxicity. From the 11 subfractions tested, only 5 exhibited bioactivity (Cb, Cc, Cd, Ce, and Ch).

acid equivalents/g fresh weight) compared with M−S− (5 ± 4 μg p-coumaric acid equivalents/g fresh weight). Compound 17 (peak 20), recently identified as p-CAME (Daayf, et al., 1997) showed a 2.5-fold increase in M+S+ (30 ± 5 μg/g fresh weight) compared with the control (13 ± 2 μg/g fresh weight). A sum calculation of all of the compounds with phytoalexin properties in this experiment (given as equivalents of phenolic compounds) gave approximately 155 μg equivalents/g fresh weight in M+S+ versus 34 μg equivalents/g fresh weight in M−S−, which translated into a nearly 5-fold increase.

Analysis of active molecules in the other two treatments (M−S+ and M+S−) indicated a similar pattern of presence. The difference was that peaks that increased in M+S− and M−S+ were the same ones that increased in M+S+, but did so to a smaller extent (results not shown).

In general, higher amounts of antifungal compounds were consistently observed 1 d after Milsana application in FIII from M+S+. Analysis of compounds in FII at this time showed no such differential accumulation among treatments. On the other hand, some of these active molecules separated from FIII were present in their free form in FII extracted 1 week after Milsana treatment.

Kinetics of p-CAME Accumulation

In a second experiment, plants were kept in a growth chamber and artificially inoculated, and the kinetics of p-CAME accumulation were followed in both its free (FII) and conjugated form (FIII) over 7 d (Fig. 5). From these results, it is clear that the compound reached its highest concentration in M+S+ in both FII and FIII. Another point of interest was the negative correlation between the increase of p-CAME in FII and FIII over time. In particular, in FIII of all tested materials, p-CAME reached its highest concentration within the first 2 d (Fig. 5). Concentrations then dropped to initial values 7 d after treatment. By contrast, p-CAME in FII remained low on d 1 and 2 after treatment and reached its highest level on d 7. The concentration in FII after 7 d was comparable to that found early in FIII, suggesting a conversion into the active form in planta. The rapid accumulation of p-CAME in FIII of M+S+ and subsequent conversion into its free form correlated with a mere 4% increase in visible infection over the next 6 d (Table II). In contrast, infection increased 34% in M−S+ plants, which displayed a much lower accumulation of p-CAME in both FII and FIII over the same time. This trend was maintained over a period of 3 weeks. Whereas 90% of the leaf surface was visibly infected in M−S+ plants, only 45% was infected in M+S+ plants.

DISCUSSION

Results presented in this study provide the first direct evidence, to our knowledge, that cucumber plants produce elevated levels of phytoalexins in response to an eliciting treatment after infection. Considering that Milsana (a) is an efficient prophylactic treatment against cucumber powdery mildew (Herger et al., 1988; Daayf et al., 1995; Dik and Van der Straay, 1995); (b) contains no fungitoxic com-
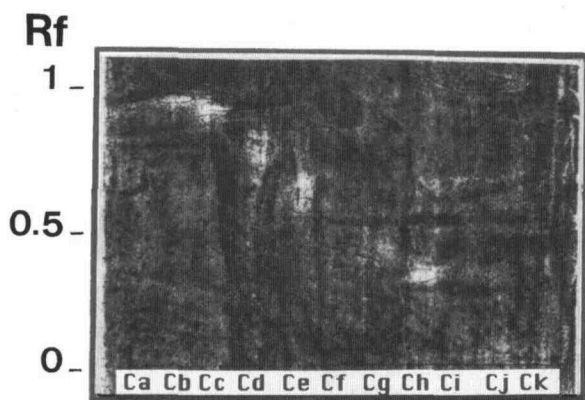
Table I. *Chromatographic and other chemical characteristics of fungitoxic compounds isolated from Milsana-treated and infected cucumber leaves and of some phenolic standards*

Peak	Cpd	LW	SW	R_F	Rt	NH$_4$OH	Neu	Benedikt	λ_{max}	λ_{min}
1	121	·	+	0.05	6	·	·	·	·	·
4	100	fl	−	0.37	30	−	+	−	285 (330″)	240
10	72	·	+	0.32	42	·	−	−	285	240
12	57	·	+	0.35	52	·	−	−	310.7	250
16	81	−	+	0.4	75	−	−	−	310	250
16	37	dk	+	0.34	75	−	−	−	360	270
16	38	dk	+	0.50	76	−	−	−	325	265
20	17	bl*	+	0.70	92	gb	−	−	310.7	250
p-Coumaric		bl*	+	0.35	51	gb	−	−	310.7	250
m-Coumaric		fl	+	0.25	63	o	−	−	281	250
o-Coumaric		y	+	0.32	73	+	−	−	275/325	240/310
p-CAME		bl*	+	0.7	92	+	−	−	310.7	250
Caffeic		bl	+	0.22	38	+	−	+	325 (295″)	265
Ferulic		bl	+	0.43	59	+	−	−	325 (295″)	260
Sinapic		bl	+	0.36	58	+	−	−	325	265
Naringenin		y	+	0.80	93	+	+	−	290 (330″)	250

Cpd, Compound; −, not detectable; +, detectable; NH$_4$OH, ammonium hydroxide vapors; Neu, flavonoids' reagent (−, no reaction, +, modification of fluorescence), Benedikt, orthopiphenolic's reagent (−, no quenching, +, quenching); λ_{max}, maximum absorbance wavelength (nm); λ_{min}, minimum absorbance wavelength (nm); bl, blue fluorescent; dk, dark; fl, fluorescent; gb, green-blue fluorescent; o, orange; y, yellow. ·, Not determined; ″, shoulder; *, pale blue. R_F values correspond to migration of compounds on silica gel-aluminum plates with solvent system (dichloromethane:hexane:methanol, 6:4:1, v/v).

pounds, as indicated here and previously (Herger and Klingauf, 1990); and (c) induces accumulation of fungitoxic phenolic compounds, it appears that these compounds contribute to cucumber's increased resistance to powdery mildew, at least in the context of a prophylactic treatment with Milsana. This was shown in the reduction of powdery

Figure 4. Reverse-phase HPLC analysis on C_{18} column of aglycone fractions from control (M−S−, top) and infected Milsana-treated (M+S+, bottom) long English cucumber leaves 1 d after Milsana treatment. Elution was performed with a gradient of 0 to 100% acetonitrile. Volumes of M−S− and M+S+ used for these injections (25 μL) correspond to the same quantity of fresh leaf material (20 g fresh weight/mL).

mildew infection in both experiments 1 and 2, in the rapid accumulation of antifungal phenolics in cucumber leaves in response to infection and a treatment with Milsana, and in the kinetics of *p*-CAME accumulation. These results are of particular significance, since they provide support for the production of antifungal compounds with induction of cucumber defense mechanisms. Previously, lignification and production of chitinases were the main defense reactions reported to occur in cucumber (Hammerschmidt and Kuc, 1982; Hammerschmidt et al., 1985; Siegrist et al., 1994).

Based on our extraction and purification procedures, at least eight separate phenolic compounds with antifungal activity were identified as intrinsic components of cucumber plants. In addition, several of them increased in concentration as a result of an elicitation with Milsana, this being particularly true when the plant was stressed by the pathogen. This phenomenon is a classical manifestation of induced resistance, in which an elicitor will magnify, in time and amount, the defense response of the plant to fend off a pathogen (Benhamou, 1996). As such it is somewhat surprising that cucumber, albeit a thoroughly studied model of induced resistance (Siegrist et al., 1994), was never before reported to produce phytoalexins or antifungal compounds.

One explanation may reside in the diversity of fungitoxic phenolics observed in the present study. Although our work has led to the chemical characterization of one of these phenolics (Daayf et al., 1997) it also indicates that a minimum of five or six additional compounds increased in response to infection, which is characteristic of phytoalexins. In earlier studies in which phytoalexins were found to be important components of resistance, the activity was usually ascribed to one molecule or a few closely related
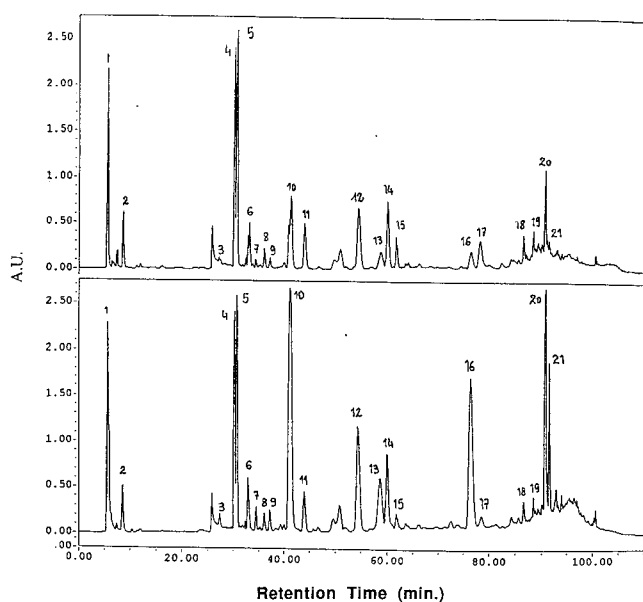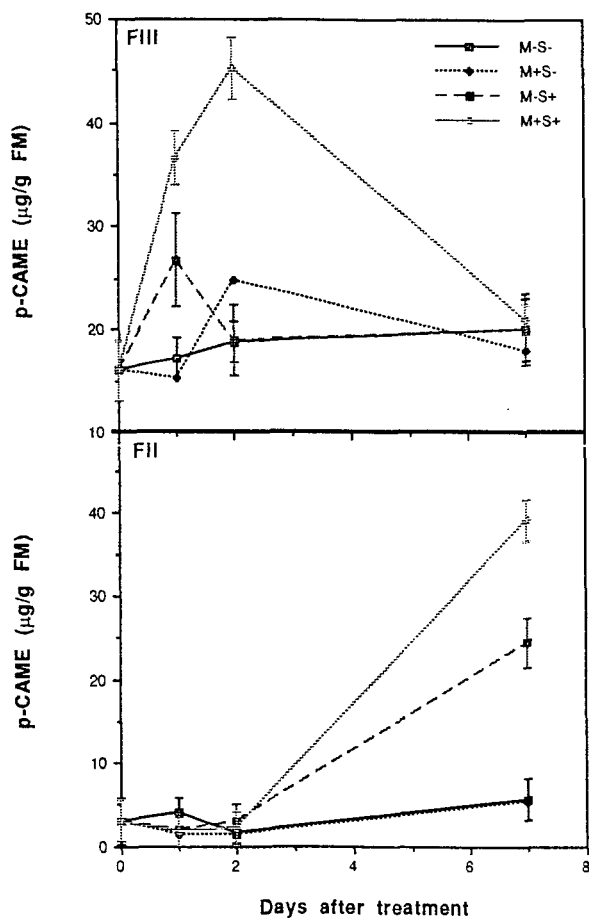
Figure 5. Concentrations of *p*-CAME over time in its conjugated (FIII) and free (FII) form in cucumber leaves obtained from healthy control (M−S−), powdery mildew-infected (M−S+), healthy Milsana-treated (M+S−), and powdery mildew-infected and Milsana-treated (M+S+) plants. Each value is the mean ± SD of three separate HPLC runs. FM, Fresh weight.

molecules. For example, the *Pisum sativum*-pisatin model is probably the best known and the most studied because the phytoalexin is unique to pea, it has very high specific activity, and its presence correlates very well with resistance (Cruickshank, 1963; Kuc, 1995). In contrast, the concept that several phytoalexins can be simultaneously involved in resistance has been well documented recently, notably in groundnut (Strange and Subba Rao, 1994). In this regard, we believe that the cumulative presence of all of the phytoalexins observed here is probably necessary for them to play an important part in resistance in cucumber. For instance, in the specific case of *p*-CAME, our results have shown that it has antifungal properties and that its kinetics of accumulation correlate well with cucumber resistance. However, in situ accumulation probably does not reach sufficient levels to account for all of the observed resistance. On the other hand, the combined amount of antifungal compounds detected (155 µg/g fresh weight) is in agreement with previous reports associating the concentration of a single phytoalexin or a group of phytoalexins with plant resistance (Yoshikawa et al., 1978; Dahiya and

Rimmer, 1988; Beimen et al., 1992; Edwards et al., 1995). In addition, the nearly 5-fold increase in concentration (155 versus 34 µg/g fresh weight) falls well within the range of reported increases for a number of phytoalexins (Graham et al., 1990; Rouxel et al., 1991; Strange and Subba Rao, 1994; Edwards et al., 1995). In this context, we have shown previously that the combined aglycones from the M+S+ treatment induced a significant reduction in germination of *S. fuliginea* conidia compared with all other treatments (Daayf et al., 1996). Previous efforts to isolate phytoalexins from cucumber may have been hampered by attempts to focus on one individual molecule (e.g. coniferyl alcohol) that would confer all of the activity (Hammerschmidt and Kuc, 1982).

From a biological point of view, this multiplicity of active compounds may confer a distinct advantage to the plant. Recent studies have shown that fungi can develop the ability to detoxify a phytoalexin (VanEtten et al., 1989, 1995), and that phytoalexin degradation could be important in determining compatibility (Kuc, 1995). Conceivably, pathogens are less likely to develop simultaneous resistance against a variety of antifungal molecules.

In this study, hydrolysis of the phenolics prior to testing their fungitoxicity was instrumental in identifying the molecules of interest, and thus may also explain prior difficulties in identifying phytoalexins in cucumber. Hydrolyzed fractions (FIII) were considerably more active than free phenolics (FII), especially shortly after treatment. In the case of *p*-CAME, the subsequent presence in its free form (FII) (Fig. 5) in leaf extracts indicates that conversion into an active form takes place in planta, presumably through the activity of β-glucosidases. Previous work by our research group has established that postinfection synthesis of β-glucosidases was stimulated in cucumber following elicitation (Chérif et al., 1994). Our findings are consistent with reports linking conjugated defense-related phenolics with resistance in an increasing number of plants (Higgins et al., 1995). In fact, other studies have suggested that conjugated phytoalexins or conjugated phytoalexin precursors that are present constitutively in the cell prior to infection are more important for resistance than phytoalexins that are produced de novo in response to infection (Graham et al., 1990; Graham and Graham, 1991; Weidemann et al., 1991; Geibel and Feucht, 1993).

Very little is known about the constituents of the Milsana formulation that confer its prophylactic properties. Al-

Table II. *Percent S. fuliginea infection of long English cucumber plants in response to a treatment with Milsana (extracts from R. sachalinensis)*

Days after Treatment	Control[a]		Milsana	
	M−S−	M−S+	M+S−	M+S+
1	0 ± 0	5 ± 3	0 ± 0	5 ± 3
2	1 ± 2	8 ± 2	1 ± 2	5 ± 3
7	2 ± 2	39 ± 3	1.5 ± 3	9 ± 2

[a] Control (M−S− and M−S+) consisted of water application. M, Milsana; S, powdery mildew. S+ plants were artificially inoculated with *S. fuliginea* 3 d prior to treatments. Each value represents the mean percent infection of eight plants ± SD.

though a direct fungicidal activity has been ruled out, it would be interesting to identify the active molecule(s) that acts as elicitor. In this sense, one may question if the production of phytoalexins in cucumber is specific to a treatment with Milsana, since previous work with soluble silicon had already suggested the presence of antifungal, soluble phenolics in the general defense responses of cucumber (Chérif et al., 1992, 1994; Bélanger et al., 1995). One may also ask whether Milsana-mediated, induced resistance occurs locally and systemically. Should it provide systemic protection, it could represent an interesting model in which to study signal perception and transduction in plant disease response (Lamb, 1994) and the role of signal molecules such as salicylic acid and jasmonic acid (Farmer and Ryan, 1990; Klessig and Malamy, 1994).

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