The Effects of Plant Extracts of *Reynoutria sachalinensis* on Powdery Mildew Development and Leaf Physiology of Long English Cucumber

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ABSTRACT

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An aqueous formulation of concentrated extracts (Milsana flüssig) from leaves of the giant knotweed, *Reynoutria sachalinensis*, applied weekly at a concentration of 2%, provided control of powdery mildew (*Sphaerotheca fuliginea*) on long English cucumber that was as effective as benomyl. In two separate experiments, this treatment significantly reduced the severity of powdery mildew compared to control plants. Fruit yield was not affected by the treatment, even though repeated applications of Milsana induced a greener and glossier coloration of the leaves, which became brittle to the touch. A rapid and distinct accumulation of fungitoxic phenolic compounds occurred in leaves treated with Milsana, especially in infected leaves. A slight inhibition of conidial germination was the only direct effect of Milsana on *S. fuliginea*. These results support the hypothesis that Milsana may act indirectly by inducing plant defense reactions and that it may be useful in the integrated management of cucumber powdery mildew.

Cucumber powdery mildew, caused by *Sphaerotheca fuliginea* (Schlechtend.:Fr.) Pollacci (20) generally reduces yield of cucumber (*Cucumis sativus* L.) produced in the greenhouse by interfering with photosynthesis and respiration of the leaves (12). Control of this disease depends mostly on fungicides, although considerable effort has been invested in developing biocontrol methods. In fact, powdery mildew is currently the sole pest that prevents long English cucumber from being produced without synthetic chemicals. In this context, a biological alternative would fulfill growers' efforts to achieve pesticide-free production.

Prophylactic treatment with extracts from leaves of *Reynoutria sachalinensis* F. Schmidt (Nakai) has been proposed to prevent infection of cucumber (*C. sativus*) by *S. fuliginea* (11). In addition, these extracts protect tomato, apple, and begonia against powdery mildew (10). The hyphal density of powdery mildew colonies on leaves treated with extracts 24 h prior to inoculation is lower, and fewer conidiophores are produced (10).

In 1990, a wettable powder formulation of these extracts was commercialized for control of powdery mildew and other pathogens (Milsana, Compo, Münste, Germany). The extraction procedure was time-consuming, however, so an aqueous formulation was developed (Milsana flüssig). In spite of the commercial potential of this product, there is little scientific information concerning its properties and benefits. For instance, there are no or very limited data on the use and efficacy of the new aqueous formulation, its efficacy over time under conditions of natural infection, its efficacy under large-scale experiments, its relative efficacy versus fungicides, its effect on yield, or even its mode of action.

Biochemical studies of Milsana-treated plants showed increased chlorophyll values and activities of enzymes, such as peroxidase and β-1,3-glucanase, as well as an increase in ethylene production (10). Recent results showed that chitinase production and activity against powdery mildew of cucumber did not necessarily correlate, indicating that chitinase may not play a role in the induction of resistance (15). Kowalewski (13) could not detect phytoalexins or hypersensitive reactions in treated plants. The activity of several enzymes, such as peroxidases, PPO, and PAL, which are involved in the production or the metabolism of phenolics increased (10,18), but no antifungal compounds of a phenolic nature were isolated or related to the phenomenon. To date, the mechanism by which these extracts protect cucumber against powdery mildew is unknown.

In light of the limited information supporting the putative role and mode of action of Milsana, the objectives of the present study were to: i) determine the efficacy of an aqueous formulation of Milsana in protecting long English cucumber against powdery mildew under semicommercial conditions; ii) compare the efficacy of the treatment to that of the fungicide benomyl; iii) evaluate the impact of a Milsana treatment on cucumber yield; and iv) investigate the production of antifungal compounds by cucumber in response to the treatment.

MATERIALS AND METHODS

Plant material and mode of infection. Seeds of cucumber cv. Mustang were sown in LC-1 Horticubes and fertilized daily with a nutrient solution of N-P-K (7-11-27) for 3 weeks in a greenhouse. Plants were transferred to gullies fed by a base nutrient solution as described by Chérif and Bélanger (5).

The experiment consisted of a control treatment in which plants were sprayed with water, a fungicide treatment with benomyl (Benlate 50W, 0.5 g a.i./liter), and a Milsana treatment with the manufacturer's recommended dosage (2%) of concentrated extracts diluted in water. Plants were sprayed with 10 liters per row per treatment. Four rows, each composed of eight plants, were used for each treatment, and each section of 32 plants was separated by a guard row. Plants were infected naturally, and after the first signs of the disease were observed, each treatment was applied weekly. Two separate experiments were conducted: one extending from 29 June through 24 August 1993 and the other from 1 March through 11 July 1994.

Evaluation of disease incidence and yield. In both experiments, the severity of powdery mildew was estimated weekly by calculating the percentage of infected leaf area for all leaves on each plant and the mean for each treatment. In addition, in the second experiment, cucumber fruit was harvested three times a week and graded according to the standard system applied in Canada (2), based on grades of 1, 2, or 3 (grade 1 = superior quality). The accumulated production was calculated every 2 weeks and used for comparison among treatments over the experimental period.

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The effects of the treatments on powdery mildew severity were analyzed by analysis of variance (ANOVA), and means were separated by Duncan's multiple range test ($P < 0.05$) using the software Super-ANOVA (Abacus Concepts, Berkeley, Ca).

Analysis of foliar bioactive compounds. Cucumber leaves were tested for the presence of antifungal phenolic compounds 24 h after the first treatment. Three to four leaves were collected from plants that represented the following situations: (C) control, healthy leaves; (I) infected leaves showing an infected area of ~20%; (CM) healthy leaves treated with Milsana; and (IM) infected leaves, showing an infected area of ~20%, treated with Milsana.

A modified extraction method of Dercks and Buchenauer (7) was adopted to allow for the determination of free and glycosidic-linked phenolics. Fresh foliar material (FM) was mixed with 80% acidified methanol (pH 2.0) 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% acidified 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 roto-evaporated at 38°C, and the aqueous residue was partitioned three times with 30 ml of ethyl ether anhydrous.

Free phenolic constituents were contained in the ether fraction (fraction II). The aqueous fraction still contained phenolic esters and glycosides. Acid hydrolysis was performed in a water bath at 100°C. The aqueous fraction was diluted with an equal volume of 4 N HCl and hydrolyzed for 1.5 h. After cooling, the hydrolysate was partitioned against anhydrous ethyl ether as described earlier (fraction III, containing aglycones). The concentration of fractions II and III was 20 g of FM per ml.

Chromatogram inhibition assay. The fractions were evaluated for fungitoxicity directly on chromatograms. Aliquots (10 µl) from fractions II and III (C, I, CM, and IM) were spotted on silica gel thin-layer chromatography (TLC) plates (silica gel 60 F-254, Sigma Chemical Co., St. Louis) and developed with dichloromethane:hexane:methanol (6:4:1) (BDH Inc. Toronto, Canada). After drying for 1 to 2 h, plates were covered with a concentrated conidial suspension of *Cladosporium cucumerinum*, mixed (1:1, vol/vol) in a 20 g/liter solution of potato-dextrose agar (PDA), using a modified method of Bailey and Burden (3). The plates were incubated in a humid chamber for 48 to 72 h, and zones of inhibition appeared as white spots against a dark background formed by conidia and mycelium of *C. cucumerinum*. To verify whether Milsana had any direct fungitoxic properties, chromatogram inhibition assays were performed with Milsana or with Milsana hydrolyzed as described above.

Effects of extracts on germination of *S. fuliginea* conidia. To corroborate the fungitoxicity of phenolic compounds against *S. fuliginea*, conidia were collected first from leaf powdery mildew colonies with a sterile 2% glucose solution, and 150 µl of this suspension (10^7 conidia per ml) was incubated, for each treatment, in Eppendorf tubes containing 20 µl of leaf extracts from fraction III. Twenty microliters of methanol was added to control Eppendorf tubes.

Percent germination of *S. fuliginea* conidia after 48-h incubation was determined by observing 30 conidia for each replicate of each treatment. Four replicates were used for each concentration of leaf extracts. A conidium was considered germinated when the length of the germ tube equaled or exceeded that of the conidium itself.

RESULTS

Evolution of disease severity and yield. The severity of powdery mildew on long English cucumber plants differed between the two experiments. In the first trial (Fig. 1A), powdery mildew developed rapidly on control plants and reached 100% of the leaf area within 8 weeks. Both benomyl and Milsana were effective in controlling the disease and kept disease sev-

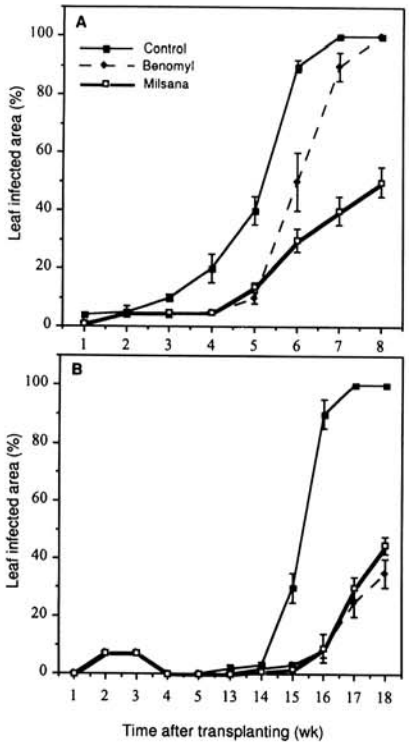

Fig. 1. Powdery mildew severity over time on long English cucumber plants treated with extracts of *Reynoutria sachalinensis* (Milsana flüssig), with benomyl, or with water (control). **(A)** Trial period: June through August 1993; **(B)** trial period: March through July 1994. Vertical bars represent standard error of the mean. Each treatment was applied weekly.

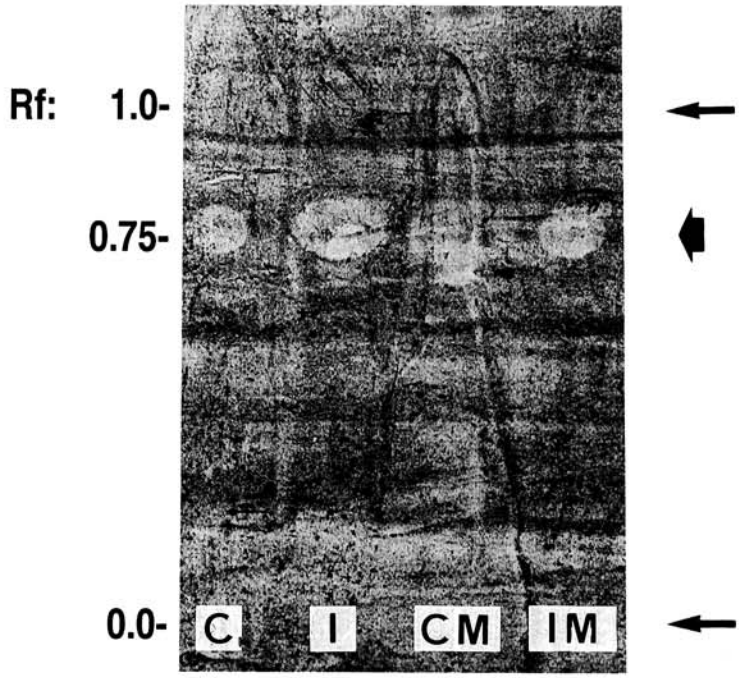

Fig. 2. Thin layer chromatography of 20 µl of free phenolics (fraction II) extracted from healthy control (C), powdery mildew-infected (I), Milsana-treated (CM), and infected Milsana-treated (IM) long English cucumber leaves. The plate was developed with dichloromethane:hexane:methanol (6:4:1, vol/vol/vol) and, after drying, was sprayed with a conidial suspension of *Cladosporium cucumerinum* and incubated for 72 h in a humid chamber. White spots indicate zones of fungitoxicity. Arrow at Rf 0.75 represents fungitoxic zone observed in all treatments. Arrows at Rfs 0.0 and 1.0 represent baseline and solvent front, respectively.

erity at <20% 5 weeks after transplanting. Subsequently, the efficacy of benomyl appeared to break down. This resulted in rapid development of disease severity that reached 100% after 8 weeks. In spite of the high inoculum level within the greenhouse, disease severity in Milsana-treated plants was only 50% at the end of the experiment (Fig. 1A).

In the second experiment (Fig. 1B), the first appearance of powdery mildew colonies occurred 2 weeks after transplanting. However, these signs subsided rapidly to reappear only during week 13. Disease severity did not exceed 5% until week 14 but climbed rapidly to 30% at the end of the following week in control plants. Disease severity remained below 5% with benomyl and Milsana treatments until 16 weeks after transplanting. Subsequently, control plants became covered with powdery mildew colonies, while benomyl and Milsana treatments maintained a disease level of ~25 and 40% at 17 and 18 weeks, respectively (Fig. 1B).

Cucumber yield was evaluated in the second trial. At the end of the experimental period, cumulative yield of grade 1 fruit with benomyl and Milsana exceeded that of control plants by 21 and 18%, respectively. These differences were obtained mostly toward the end (weeks 14 through 18) of the experiment, when control plants were more severely affected by powdery mildew. Although the yield of Milsana-treated plants was similar to that of benomyl-treated plants, their leaves developed a distinct morphology and appearance, characterized by a greener and glossier color, and were brittle to the touch.

Chromatogram inhibition assay. Free phenolics extracted from cucumber leaves of healthy control leaves, powdery mildew-infected leaves, healthy leaves treated with Milsana, and infected leaves treated with Milsana displayed a faint zone of fungitoxicity based on the TLC bioassay with *C. cucumerinum* (Fig. 2). This zone was revealed at an approximate Rf = 0.75 and was apparently uniform in intensity among the different samples, indicating no differential activity among the plant material tested.

In contrast, fungitoxicity revealed in fraction III, containing aglycones from the hydrolyzed fraction, varied greatly among the treatments (Fig. 3). Extracts from control plants developed only two small fungitoxic spots on the TLC bioassay (Rf = 0.84 and 0.93) that were present also in roughly the same concentration in extracts from all other treatments (Fig. 3). Another fungitoxic zone (Rf = 0.37), not revealed in control leaves, was observed in extracts of all other treatments, but the zone of inhibition was much larger in infected leaves treated with Milsana. Finally, extracts from infected leaves treated with Milsana developed a unique zone of fungitoxicity (Rf = 0) not observed in the other treatments (Fig. 3). No zones of fungitoxicity were detected in crude or hydrolyzed extracts obtained from Milsana concentrates (results not shown), indicating that the product itself had no antifungal activity.

Effects of extracts on germination of *S. fuliginea* conidia. Percent germination of conidia of *S. fuliginea*, when mixed with aglycones from cucumber leaf extracts, varied among treatments. Aglycone extracts from all treatments significantly inhibited germination compared to the water control, in which a germination rate of 63% was observed. Germination was 43% in extracts from healthy control leaves compared to 37 and 31%, respectively, in extracts from infected and Milsana-treated plants (both significantly [$P = 0.05$] lower than the control). Germination of conidia in extracts from infected leaves from Milsana-treated plants, which displayed the strongest antifungal activity on the chromatograms, was only 20%, significantly lower than all other values.

DISCUSSION

The results presented in this study are the first to describe comprehensively the effects of a prophylactic treatment with plant extracts of *R. sachalinensis* (Milsana flüssig) on cucumber yield and the severity of powdery mildew under semicommercial conditions. Previous reports demonstrated that a preventive treatment followed by artificial inoculation delayed the development of powdery mildew colonies on a number of hosts for ~7 days (10,11). In our experiments, critical information was obtained on long-term effects on disease incidence and yield in comparison with a fungicide treatment. In our first trial, the Milsana treatment reduced disease severity from 100% in the control and benomyl treatments to 50% 8 weeks after transplanting.

The same level of protection was provided by Milsana in the second trial, in which its efficacy was comparable to benomyl. Cucumber yield was unaffected by the Milsana treatment in comparison with conventional disease management methods. These results are important because Milsana-treated plants developed morphological modifications (e.g., greener and glossier leaves, brittle to the touch) that could have had a negative impact on growth and yield. These modifications could be attributed, in part, to an increase in chlorophyll production, as previously reported by Herger and Klingauf (10), but it is unlikely that the protection provided by Milsana is related to this particular phenomenon. In any event,

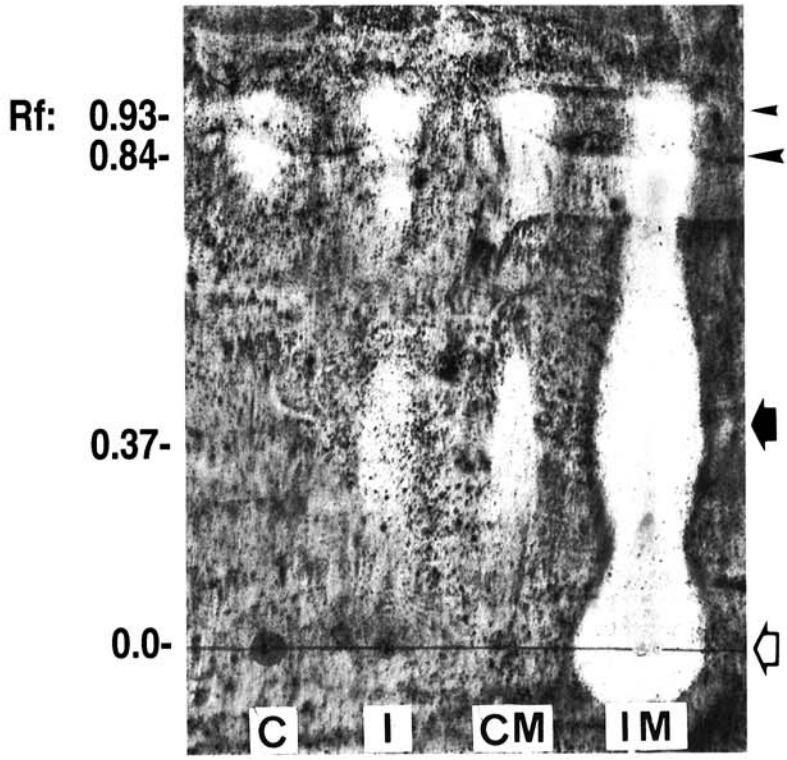

Fig. 3. Thin layer chromatography of 20 µl of aglycones from glycosidically bound phenolics after hydrolysis with HCl. Extracts were from healthy control (C), powdery mildew-infected (I), Milsana-treated (CM), and infected Milsana-treated (IM) long English cucumber leaves. The plate was developed with dichloromethane:hexane:methanol (6:4:1, vol/vol/vol) and, after drying, was sprayed with a conidial suspension of *Cladosporium cucumerinum* and incubated for 72 h in a humid chamber. White spots indicate zones of fungitoxicity. Arrowheads at Rfs 0.84 and 0.93 represent fungitoxic zones observed in all treatments. Arrow at Rf 0.37 indicates fungitoxic zone present in all treatments, except C; the IM treatment reacted most intensely. Arrow at Rf 0.0 represents fungitoxic zone observed only in IM treatment.

this phenomenon is not expected to be a deterrent, because the greener color conferred a healthier appearance to the plants. On the other hand, while it is impossible at this time to quote a specific price for Milsana, its use is expected to be more expensive than currently used fungicides. A grower would have to weigh the benefits of eliminating chemical fungicides for powdery mildew control versus a slightly higher cost of production.

Based on our chromatogram bioassay, production of free phenolics (fraction II) in cucumber leaves could not explain the differential protective action between control and Milsana treatments. Whereas a small zone of antifungal activity was detected in that fraction, it appeared to be constitutively present in all samples observed. Glycosidically bound phenolics, hydrolyzed into their free form (fraction III) and displayed antifungal activity in different forms and intensity among the treatments. Milsana appeared to induce a rapid and more abundant accumulation of these compounds, especially in cases in which leaves were infected. This fungitoxicity of aglycone fractions was corroborated against conidia of *S. fuliginea*.

The role of aglycones in specific plant defense reactions has been reported in other plant-pathogen interactions (8,9). Involvement of these aglycone phenolics supposes a preliminary hydrolysis of their glycosylic form, a phenomenon that was recently reported by Chérif et al. (4,6), who described a similar production of antifungal compounds in cucumber, coupled with an increase in β-glycosidase activity.

The chromatogram inhibition assay with Milsana concentrates, either crude or hydrolyzed, revealed no fungitoxic activity. This corroborates previous results by Herger and Klingauf (10) who showed that *R. sachalinensis* extracts caused only a slight inhibition of conidia germination of *S. fuliginea* and had no fungitoxic properties when tested on *Botrytis cinerea* and adds evidence for an indirect mode of action by the extracts. Investigations by Kowalewski and Herger (14) concerning the chemical nature of the active constituents excluded proteins, terpenoids, phenolics, and regular sugars as active ingredients. They concluded that the resistance-inducing factor was most likely a carbohydrate with a hydrophobic part, but complete characterization of inducing and induced molecules was still being investigated.

The exploitation of natural defense mechanisms expressed by plants has become the focus of intensive research because of the current pressure to reduce synthetic chemicals for plant disease control (19). As a result, several prophylactic treatments of plants with different substances have been reported to induce resistance against bacterial, viral, and fungal diseases. Indeed, natural resistance has been induced by treating host plants with culture filtrates from nonpathogenic fungi and bacteria (14), with nonpathogenic (1) or pathogenic strains (21), with compost extracts (17), or with plant extracts (16). Plant extracts can play an important role in integrated or ecological farming systems, but they also offer a good supplement for conventional agriculture. As such, exploiting intrinsic plant defense systems with natural products such as Milsana may offer an alternative to synthetic chemical fungicides for integrated control of diseases.

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