Activity of the Antifungal Protein from *Aspergillus giganteus* Against *Botrytis cinerea*

Ana Beatriz Moreno, Álvaro Martínez del Pozo, Marisé Borja, and Blanca San Segundo

First and fourth authors: Departamento de Genética Molecular, Instituto de Biología Molecular de Barcelona, CID-CSIC, Jordi Girona 18, 08034 Barcelona, Spain; second author: Departamento de Bioquímica y Biología Molecular, Facultad de Química, Universidad Complutense, 28040 Madrid, Spain; and third author: Departamento de I+D+I, Fundación Promiva, Finca La Veguilla, M501, Km 5.1, Boadilla del Monte, 28660 Madrid, Spain.

Accepted for publication 30 May 2003.

ABSTRACT

Moreno, A. B., Martínez del Pozo, Á., Borja, M., and San Segundo, B. 2003. Activity of the antifungal protein from *Aspergillus giganteus* against *Botrytis cinerea*. Phytopathology 93:1344-1353.

Botrytis blight (gray mold), caused by *Botrytis cinerea*, is one of the most widely distributed diseases of ornamental plants. In geranium plants, gray mold is responsible for important losses in production. The mold *Aspergillus giganteus* is known to produce and secrete a basic low-molecular-weight protein, the antifungal protein (AFP). Here, the antifungal properties of the *Aspergillus* AFP against various *B. cinerea* isolates obtained from naturally infected geranium plants were investigated. AFP strongly inhibited mycelial growth as well as conidial germination of *B. cinerea*. Microscopic observations of fungal cultures treated with AFP revealed reduced hyphal elongation and swollen hyphal tips. Washout experiments in which *B. cinerea* was incubated with AFP for different periods of time and then washed away revealed a fungicidal activity of AFP. Application of AFP on geranium plants protected leaves against *Botrytis* infection. Cecropin A also was active against this pathogen. An additive effect against the fungus was observed when AFP was combined with cecropin A. These results are discussed in relation to the potential of the *afp* gene to enhance crop protection against *B. cinerea* diseases.

Additional keyword: *Pelargonium*.

Fungal diseases are one of the major biotic stresses that contribute substantially to the overall loss in yield among crop plants. A search for new strategies for disease control becomes necessary with the increasing demand for crops produced with minimal application of chemical input. Some of these strategies for plant protection involve the expression of antimicrobial genes in transgenic plants. Plants produce antimicrobial proteins and peptides (antibacterial and antifungal) as part of their natural defense systems to control disease-causing microorganisms (4,14,43). There are many examples in the literature of increased resistances of plants toward pathogenic fungi by introduction of plant antifungal genes into plants (7,11,13,31,47). The outcome varies, but resistances obtained with plant antifungal genes have not supported the production of new disease-resistant cultivars suitable for commercial crops. One of the main limitations of this approach has been the relatively low level of resistance obtained with a single plant antifungal gene, which has resulted in the need to use gene combinations, i.e., simultaneous expression of chitinase and β-1,3-glucanase genes (23,54). Additionally, these transgenic plants showed resistance against a narrow spectrum of pathogens, which represents another important limitation for protecting crops. It appears that plant pathogens have already evolved tolerance to plant-derived antifungal compounds (34,40).

The production of antimicrobial proteins or peptides is not restricted to plant species but seems to be ubiquitous in nature (19, 53). Some examples of antimicrobial peptides include human defensins, insect cecropins, and magainins from amphibians. Cecropins, which were originally isolated from immune hemolymph of the Cecropia moth, are a key component of the immune response in insects (44). It is well known that cecropins possess activity against gram-positive and gram-negative bacteria. We previously reported the ability of cecropin A-derived peptides to inhibit the growth of several fungal plant pathogens (9). Antifungal genes from sources other than plants can also be considered candidate genes for plant transformation to enhance crop protection against fungal pathogens.

The genome of mycoparasitic and antagonistic fungi, which has evolved specifically to attack other fungi but not plants, represents a potential source of antifungal genes of nonplant origin. As an example, *Trichoderma* spp., which have been repeatedly used as biocontrol agents, produce fungal cell wall degrading enzymes (27). These enzymes exhibit more antifungal activity than plant chitinases and β-1,3-glucanases. Most importantly, they are effective against a much wider range of pathogens and they are not toxic to plants.

The mold *Aspergillus giganteus*, isolated from the soil of a farm in Michigan, has been reported to produce a basic low-molecular-weight protein (51 amino acids) showing antifungal properties, the so-called antifungal protein (AFP) (32,33). This protein was accidentally discovered during an anticancer screening program developed in Michigan during the 1960s (33). This AFP has been thoroughly characterized from the structural point of view. Thus, its amino acid sequence, spectroscopical features, and three-dimensional structure are known (6,24).

The fungus *Botrytis cinerea* is one of the most destructive plant pathogens. It attacks a wide variety of plants (over 200 plant species) and infects flowers, fruits, and vegetative tissues. This fungus causes gray mold, or Botrytis blight in geranium plants,

Corresponding author: B. San Segundo; E-mail address: bssgmb@cid.csic.es

*The *e*-Xtra logo stands for "electronic extra" and indicates that the online article contains supplemental material not included in the print edition. Figures 3 to 6 are in color online.

Publication no. P-2003-0908-01R

one of the important destructive diseases affecting geranium seedlings, cuttings, and stock plant production (21). The disease symptoms are characterized by gray sporulating lesions that are commonly observed under humid conditions. These lesions produce masses of conidia that become airborne and are the primary means by which the fungus is disseminated. A wet, humid greenhouse environment is ideal for the rapid growth and prolific sporulation of *B. cinerea* (22). Control of *B. cinerea* is challenging because of its abilities to survive as a saprophyte, rapidly invading host tissue and quickly producing abundant conidia that are easily distributed by air currents. Moreover, the fungus is capable of growing within a wide range of temperatures. Thus, the optimum temperature for *B. cinerea* growth is 24 to 28°C, but fungal growth occurs from 0 to 35°C. Numerous fungicides, such as benzimidazole and dicarboximide, are used to control gray mold. Many of them are preventives and must be repeatedly applied before infection symptoms appear. However, this is becoming less acceptable because it conflicts with public concern for fungicide residues and also increases the potential for the build-up of resistance in *B. cinerea* to fungicides. In this respect, resistances to these chemicals have already appeared in natural populations of *B. cinerea* (25). Biological control, on the other hand, has advantages over fungicides, but its efficacy varies depending on the timing and the environmental conditions. Finally, traditional breeding for resistant cultivars is a difficult problem, mainly because of a lack of host resistance to *B. cinerea*. Consequently, finding specific compounds exhibiting antifungal properties against *B. cinerea* is a requisite for creating geranium cultivars with improved resistance to this pathogen.

In this work, the ability of the *A. giganteus* AFP protein to inhibit *B. cinerea* growth was investigated. We show that AFP exhibits a potent antifungal activity against different *B. cinerea* isolates obtained from naturally infected geranium plants. In vitro antifungal assays revealed that AFP was capable to inhibit mycelial growth as well as conidial germination of *B. cinerea*. Application of AFP on geranium leaves completely prevented fungal growth. *B. cinerea* was also sensitive to inhibition by cecropin A, but AFP was a more potent inhibitor of this pathogen. Moreover, in assays involving combinations of AFP and cecropin A, we observed an additive activity against *B. cinerea*. This information will be useful to design strategies to enhance crop protection against *Botrytis* diseases.

MATERIALS AND METHODS

Purification of AFP. AFP was purified from the extracellular medium of *A. giganteus* MDP18894 cultures as described previously (24). Homogeneity of the protein preparation was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and amino acid composition analysis as well as by its spectroscopic features (24).

Plant and fungal material. The geranium *Pelargonium* × *hortorum* cv. Eclipse RedII was purchased from a local nursery and maintained under glasshouse conditions with a day temperature of 20 ± 4°C and a night temperature of 16 ± 4°C.

Isolates CC1, CC19, CC24, and CC27 of *B. cinerea* were obtained from tissues (flower, stem, petiole, or leaf) of geranium (*Pelargonium* × *hortorum* 'Eclipse RedII', Goldsmith Plants Inc., Gilroy, CA) plants from a commercial nursery disease outbreak in 2001 and used in further experiments. Fungi were grown on PDA (potato dextrose medium containing 15 g/liter of agar) for 10 to 15 days. Conidial suspensions were collected by adding sterile water to the surface of the mycelium. Suspensions were filtered before counting in a Bürker counting chamber and adjusted to the appropriate concentration.

Antifungal activity assays. The in vitro activity of AFP was determined by measuring the absorbance at 595 nm of fungal cultures in 96-well microtiter plates (9). The microtiter well plate assay was used to determine the AFP protein concentration required for inhibition of fungal growth. In microplate wells, 150 µl of potato dextrose broth (PDB; Difco Laboratories, Detroit) was mixed with 50 µl of each conidial suspension (at the concentration of 1×10^6 spores per ml). *Botrytis* conidia were allowed to pregerminate for 6 h at 26 to 28°C, and the absorbance was determined. Purified AFP solutions were added to the pregerminated conidia to the desired final concentrations. The microtiter plates were incubated for 24 h at 26 to 28°C and the absorbance was read. The minimal inhibitory concentration (MIC) value was defined as the lowest protein concentration that prevented *B. cinerea* growth. Bovine serum albumin (BSA) was used as control. Fungal growth was checked microscopically to confirm the spectrophotometric data. For this, fungal mycelium was stained with lactophenol cotton blue before microscopical observations.

The antifungal activity of cecropin A (Sigma Chemical, St. Louis), and combinations of AFP with cecropin A, was determined with the microtiter plate bioassay. Expected efficacy of each combination was determined by the Abbott formula (1): $I_e = X + Y - (XY/100)$, where I_e is the expected percent inhibition for a given interaction, and X and Y are the percent growth inhibited by the compounds when used alone. The nature of interaction of these antifungal compounds was determined by the interaction ratios (IRs), which were computed as $IR = I_o/I_e$ (I_o, observed percent inhibition). IRs between 0.5 and 1.5 represent additive interactions, ratios of >1.5 represent synergistic interaction, and ratios of <0.5 represent antagonist interactions (16). This criterion was followed in the experiments reported here.

The ability of AFP to inhibit conidial germination of *B. cinerea* was measured by placing conidial suspensions in PDB (3×10^3 conidia in 500 µl of PDB) at the bottom of sterile glasses of concave shape. Conidial germination was examined after 6 h of incubation in a moist chamber at 26 to 28°C. Incubations with two AFP concentrations (5 and 10 µM) were assayed. Controls without AFP were performed.

In order to examine whether AFP has fungistatic or fungicidal activity, we performed a series of wash-out experiments. Antifungal activity assays were set up as described previously. AFP was added to the 6 h pregerminated conidia to final concentrations of 10 and 20 µM and incubated for time periods of 6, 12, 18, and 24 h. After the respective incubation period had expired, fungal cultures were washed twice with 200 µl of PDB and resuspended in 50 µl of PDB. The suspension was placed on PDA plates and incubated for 4 days. Controls with BSA (10 µM), nystatin (0.1 µg/µl), or untreated were performed. The activity of AFP with *B. cinerea* conidia that had not been pregerminated was similarly assayed.

In vivo inhibition tests. Geranium plants were locally infected with conidial suspensions of *B. cinerea* by droplet inoculations. For this, 20 µl of the conidial suspension (10^6 conidia per ml in 0.25%, vol/vol, Tween 20) was applied to leaf surfaces. Then, a drop of an AFP solution (at the appropriated concentration), or sterile water containing Tween 20 (control), was deposited at the same place as the infection drop. The plants were maintained with high humidity. The progression of symptoms was followed visually.

The protective effect of AFP was also assayed by applying AFP onto geranium leaves prior to fungal inoculation. In these tests, 20 µl of a 10 µM AFP solution was applied 3 or 14 days prior to inoculating the same spot with 20 µl of the *B. cinerea* conidial suspension (10^6 conidia per ml in 0.25%, vol/vol, Tween 20). Lesion progression in regions with or without AFP treatment was followed.

RESULTS

The aim of this work was to test the potential antifungal activity of AFP against *B. cinerea*, the fungus that causes gray mold

of geranium. The ability of cecropin A to inhibit the growth of this pathogen and the effect of combinations of AFP with cecropin A have also been assayed. AFP was purified to electrophoretic homogeneity from the extracellular medium of *A. giganteus* cultures and used in our antifungal assays. Its amino acid composition and spectroscopic properties were always determined prior to use. The concentrations required for 50% inhibition (inhibitory concentration, IC_{50}) and for complete inhibition of fungal growth (MIC) in the microtiter plate assays were taken as a measure of the inhibitory potency of AFP on the *B. cinerea* isolate.

Figure 1 shows that the growth of *B. cinerea* isolate CC1 was significantly impaired in the presence of AFP. The estimated MIC value, that is, the lowest AFP concentration that prevented any detectable fungal growth, was 10 μM. At AFP concentrations below the MIC, the antifungal potency tended to decrease with time (Fig. 1A). After 24 h of incubation, a concentration of AFP ranging from 0.5 to 1.0 μM was enough to produce 50% inhibition of *B. cinerea* CC1 growth (Fig. 1B).

When the in vitro antifungal assay was extended to other *B. cinerea* isolates (CC19, CC24, and CC27) (Fig. 1B), AFP also caused inhibition of fungal growth. AFP was, however, less effective for inhibition of isolates CC19, CC24, and CC27 than for isolate CC1. Altogether, these experiments revealed that AFP concentrations in the range of 0.5 to 5 μM were needed for 50% inhibition of the various *B. cinerea* isolates assayed in this work. Fungal growth was fully inhibited by the presence of nystatin (0.1 μg/μl) in the medium, whereas fungal cultures treated with BSA displayed normal growth behavior (data not shown).

The antifungal effect of cecropin A against *B. cinerea* CC1 growth was also assayed. Cecropin A was found to possess inhibitory activity against this fungus (Fig. 2). The IC_{50} and MIC values observed, after 24 h of incubation, were 20 and 80 μM, respectively (Fig. 2B), indicating that *B. cinerea* is more sensitive to AFP than to cecropin A.

When combined, antifungal compounds might interact synergistically, additively, or antagonistically. The ability of antimicrobial compounds to act synergistically or additionally in controlling bacteria is well documented (39,52). Less attention has been paid, however, to the interactions of such compounds for controlling plant-pathogenic fungi (17). Thus, AFP and cecropin A were investigated for the effect of their combination in inhibiting the

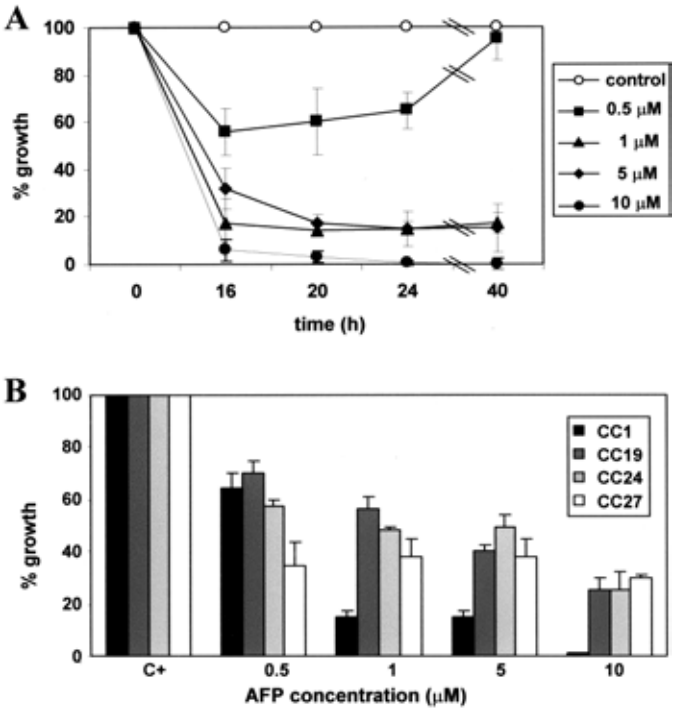

Fig. 1. In vitro antifungal activity of antifungal protein (AFP) against *Botrytis cinerea.* **A,** AFP inhibitory activity on *B. cinerea* CC1 growth with time. Absorbances (optical density at 595 nm) over time for control cultures in the absence of AFP and in the presence of 0.5, 1, 5, and 10 μM concentrations of AFP were determined. **B,** Growth of *B. cinerea* isolates CC1, CC19, CC24, and CC27 in the presence of increasing concentrations of AFP. Absorbances of fungal cultures were determined after 24 h of incubation with AFP. Fungal growth is expressed as the percent growth of control cultures (100% growth represents fungal growth in potato dextrose broth medium without AFP). Values represent the mean and the standard error for three independent experiments in triplicate.

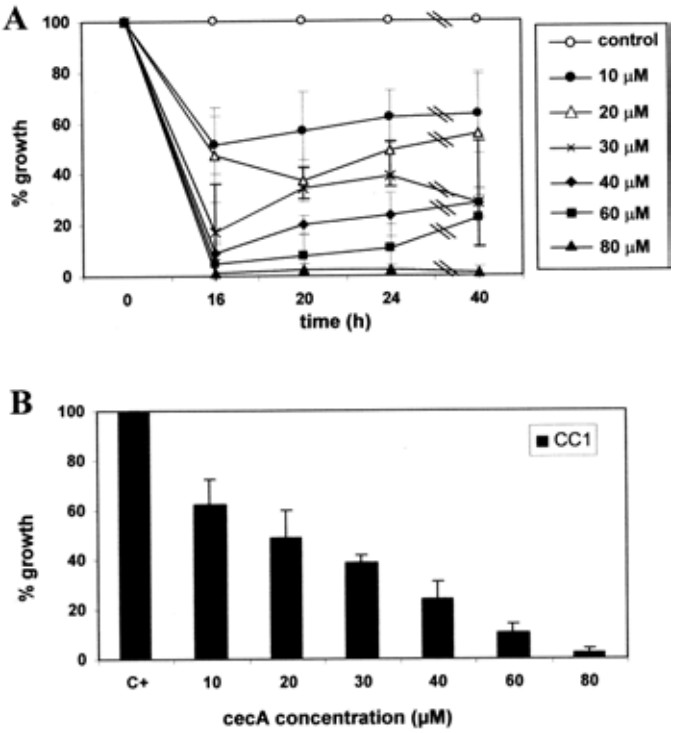

Fig. 2. Effect of cecropin A on *Botrytis cinerea* (isolate CC1) growth. **A,** Absorbances over time for control cultures and in the presence of 10, 20, 30, 40, 60, and 80 μM concentrations of cecropin A were determined. **B,** Growth of *B. cinerea* (isolate CC1) in the presence of increasing concentrations of cecropin A. Absorbances of fungal cultures were determined after 24 h with cecropin A. Fungal growth is expressed as the percent growth of control cultures (100% growth represents fungal growth in potato dextrose broth medium without cecropin A). Values represent the mean and the standard error for three independent experiments in triplicate.

TABLE 1. Additive antifungal effect of combinations of antifungal protein (AFP) and cecropin A against *Botrytis cinerea* CC1 growth

Compounds	% Growth inhibition of *B. cinerea*		
	Observed (I_o)	Expected (I_e)[a]	IR (I_o/I_e)[b]
AFP (0.75 μM)	47.8 ± 6.8	–	–
Cecropin A (5 μM)	11.8 ± 2.9	–	–
Cecropin A (10 μM)	52.7 ± 6.4	–	–
AFP (0.75 μM) + cecA (5 μM)	66.5 ± 11.5	59.9	1.11
AFP (0.75 μM) + cecA (10 μM)	93.0 ± 1.5	72.2	1.28

[a] Expected inhibition in growth was determined by the Abbott formula: $I_e = X + Y - (XY/100)$, where X and Y are the percent inhibition given by each compound when used alone. Thus, 100% growth represents fungal growth in potato dextrose broth medium. Values X and Y were obtained in three independent experiments, each consisting of three replications. The data represent average and standard error.

[b] IR = interaction ratio for the combination of AFP and cecropin A, obtained by dividing the observed percent inhibition (I_o) in growth by the expected percent inhibition (I_e). An IR of <0.5 = antagonistic, 0.5 to 1.5 = additive, and >1.5 = synergistic (16).

growth of *B. cinerea* in vitro. Table 1 shows the percent growth inhibition of *B. cinerea* CC1 isolate for each protein when tested alone or in combination. In these experiments, AFP was used at a constant concentration (0.75 μM), which is in the range of the IC_{50} value for inhibition of *B. cinerea* isolate CC1, and was mixed with two different concentrations of cecropin A that were below the MIC value (5 and 10 μM cecropin A). The IRs obtained for the mixtures of AFP and cecropin A were between 0.5 and 1.5, indicating that AFP and cecropin A acted additively against this pathogen (16).

Fungal growth inhibition mediated by AFP also was analyzed microscopically (Fig. 3). With this purpose, 24 h after addition of the AFP protein, the growing mycelium was stained with lactophenol cotton blue. *B. cinerea* grown in the presence of AFP showed reduced hyphal elongation compared with the control which displayed a much more extended mycelium. Moreover, the tips of the hyphae appeared swollen as seen in Figure 3, suggesting that polarity of the hyphal tip was affected by AFP. These alterations in the morphology of hyphae were observed in all the isolates assayed (CC1, CC19, CC24, and CC27).

The antifungal properties of AFP also were studied with respect to *B. cinerea* conidial germination. Conidia of the various *B. cinerea* isolates were mixed with AFP (5 and 10 μM AFP) and observed microscopically after 6 h of incubation (Fig. 4). The conidia that did germinate in the presence of 5 μM AFP showed a much shorter germ tube. Incubation of conidia with 10 μM AFP led to complete inhibition of conidial germination.

Moreover, wash-out experiments were conducted to determine whether AFP has a fungicidal or a fungistatic activity against *B. cinerea*. Conidia were pregerminated for 6 h at 26 to 28°C and incubated with two different concentrations of AFP (10 and 20 μM) in PDB for 6, 12, 18, or 24 h. Fungal cultures grown in the presence of AFP, and control cultures grown either in the absence of AFP or in the presence of BSA or nystatin, were washed twice with PDB and resuspended in 50 μl of PDB. Each suspension was placed in the center of PDA plates. After 4 days, fungal growth on PDA plates was checked. As shown in Figure 5A, fungal cultures incubated with AFP and washed did not grow, whereas fungal cultures treated with BSA (Fig. 5A) or untreated controls (data not shown) did grow. No fungal growth was observed in cultures treated with nystatin (Fig. 5A). These results suggest that the inhibitory effects shown by the in vitro antifungal assay were due to a fungicidal activity of AFP against this fungus. The fungicidal activity was already observed for the shortest period of treatment of fungal cultures with AFP (6 h). Each of the wash-out experiments was performed in twice.

Activity of AFP against ungerminated *B. cinerea* conidia also was studied in wash-out experiments (Fig. 5B). Conidia were maintained in the presence of 10 μM AFP for 6 h and washed with PDB twice as described previously. After 4 days of incubation, no fungal growth was found in AFP-treated conidia. Equally, fungal growth was not observed in conidia that had been incubated with nystatin. The control platings with untreated or BSA-treated conidia showed fungal growth.

From these results, it was concluded that low micromolar concentrations of AFP inhibit growth and conidial germination of *B. cinerea.* Cecropin A also was active against *B. cinerea.* The AFP protein was, however, more active against this fungus than cecro-

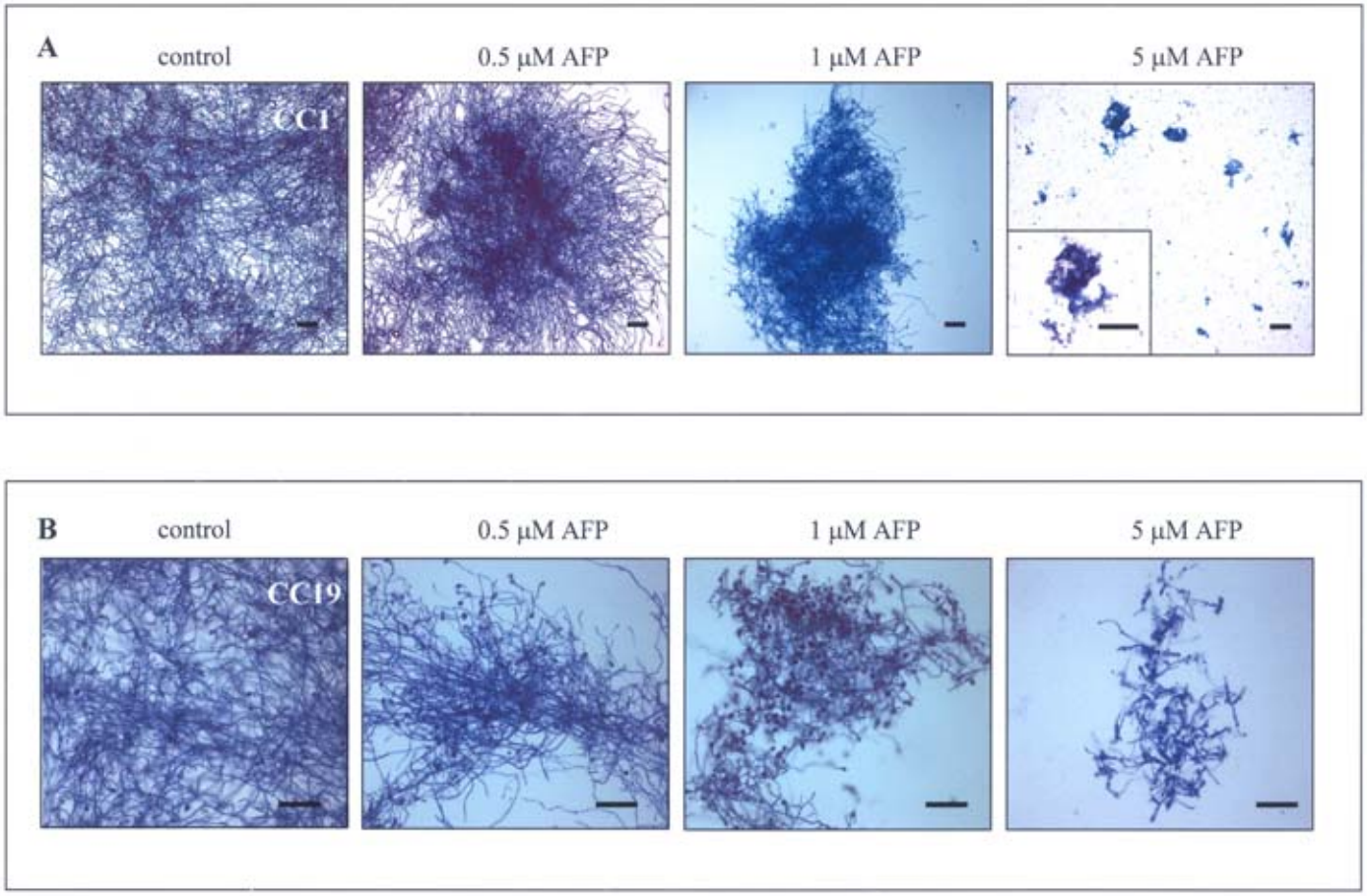

Fig. 3. Micrographs of *Botrytis cinerea* cultures grown in the presence of antifungal protein (AFP). In the presence of AFP, *B. cinerea* showed less extensive mycelia. *B. cinerea* **A,** CC1 and **B,** CC19 were grown in potato dextrose broth medium without AFP (control) or in the presence of AFP at the indicated concentrations. The morphology of mycelia of *B. cinerea* CC24 and CC27 was similarly changed (data not shown). Bars = 15 μm.

pin A. Finally, the action of AFP and cecropin A was additive against *B. cinerea*.

Activities of antifungal compounds determined in vitro provide first indication of the potency of such compounds. However, they do not always represent the effectiveness of these compounds in the in vivo situation. Thus, the effectiveness of AFP for inhibition of *Botrytis* growth in planta was assayed. Leaves of geranium plants were locally inoculated with a drop of the conidial suspension of *B. cinerea* to give rise to a macroscopically visible plant reaction. The inoculated leaf sites were immediately treated with a drop of AFP (at a concentration of 10 μM) that was deposited on the same spot as the inoculation drop. Control areas that had been infected but had not been treated with AFP also were made. Development of disease symptoms was monitored visually (Fig. 6A and B). In areas that had not been treated with AFP, necrotic lesions were clearly observed as soon as 2 days postinoculation with *B. cinerea*, whereas no apparent symptoms developed in inoculated spots that had been treated with AFP.

Postgermination protection afforded by AFP also was assayed by inoculating geranium leaves with *B. cinerea* and, after a period of 6 h (a time point in which germination had occurred), treating the infected areas with AFP. As shown in Figure 6C, symptoms of *Botrytis* infection were not detected at AFP-treated spots.

The potential for use of AFP as a protectant was further explored by applying AFP to geranium leaves prior to fungal inoculation. For this, AFP was applied 3 or 14 days before fungal inoculation. Results obtained are presented in Figure 6D. These experiments revealed that AFP remains effective for protection against *B. cinerea* infection for long periods of time (up to 14 days, the longest period of time assayed in this work).

Finally, the degree of protection of geranium plants by AFP was assayed by application of different concentrations of the antifungal protein to the inoculated leaf regions (Fig. 6E). The production and size of lesions appeared to be dose dependent. As previously found, no disease symptoms were observed at a concentration of 10 μM AFP. Treatment with 1 μM AFP was not completely effective for protection of *Botrytis* infection, although the extent of lesion expansion was significantly inhibited compared with lesions developed at the infected, non-AFP-treated areas. Decreasing the amount of AFP from 1 to 0.1 μM AFP, although causing a delay in symptom development, did not fully protect geranium leaves against *Botrytis* infection. These observations indicate that the potency determined by the in vitro assays correlates well with in planta assays.

DISCUSSION

Plants in nature are constantly challenged by pathogens, and modern agriculture is still highly dependent on chemical fungicides for their control. However, the repeated use of chemicals has several drawbacks such as their lack of specificity, increased

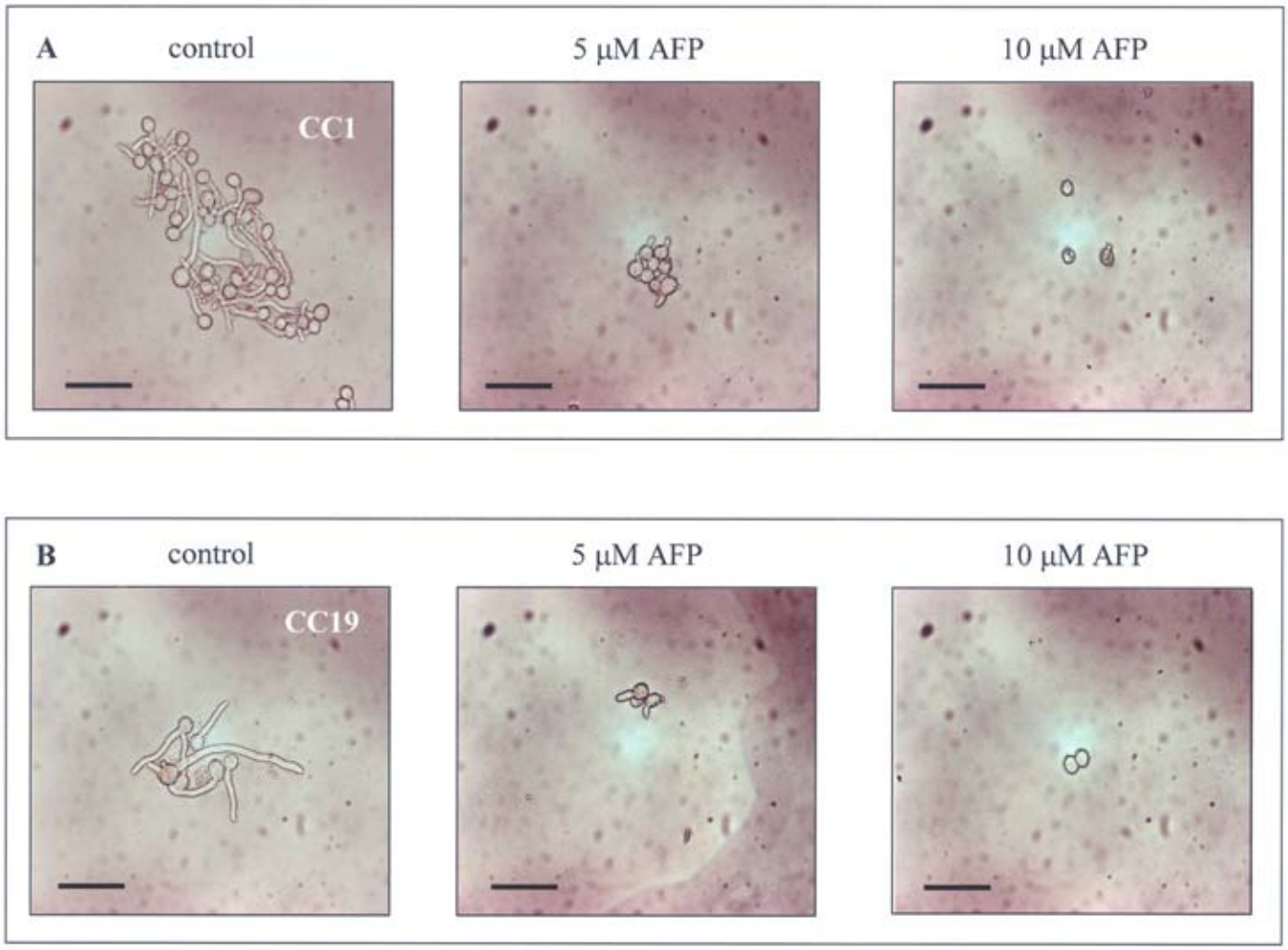

Fig. 4. Effect of antifungal protein (AFP) on germination of conidia of *Botrytis cinerea.* Conidia of *B. cinerea* **A,** CC1 and **B,** CC19 were incubated in vitro in the absence (control) or in the presence of AFP at the indicated concentrations. Micrographs were taken at 6 h and are representative observations of the many germinating conidia. A similar effect was observed in conidia of isolates CC24 and CC27 incubated with AFP (data not shown). Bars = 10 μm.

incidence of development of resistance after prolonged application, and the environmental hazards inherent to residual toxicity. Developing disease-resistant transgenic plants using genes encoding poly- and oligo-peptides showing antimicrobial properties is one of the potential alternatives to the use of chemical fungicides.

We are interested in studying the antifungal properties of compounds that are produced as part of the defense response of different organisms against phytopathogens, as well as in their application for the development of fungal resistant plants through gene transfer. Toward this end, we previously reported the ability of cecropin A-derived peptides to inhibit growth of several fungal plant pathogens (9). More recently, we reported that AFP inhibits the growth of *Magnaporthe grisea*, *Fusarium moniliforme*, and *Phytophthora infestans* (50). Taking into account that gray mold of geranium causes heavy yield losses in the greenhouse ornamental industry in Europe and North America, we have chosen *B. cinerea* as a working system to target the identification of novel antifungal proteins.

Antimicrobial peptides have been found in many different kinds of organisms such as bacteria, insects, amphibians, mammals, and plants (4,14,19,53). Certain common structural patterns are found in the antimicrobial peptides so far characterized. Some of them are cationic and amphipathic molecules (i.e., cecropin peptides) and others contain β-sheet elements stabilized by intramolecular cystine disulfide bonds as exemplified by plant and insect defensins. The AFP structure defining a small and compact β-barrel stabilized by four internal disulfide bridges together with its low molecular weight and apparent toxic character resembles other antifungal peptides found in plants, such as defensins and thionins (5,6,14). Therefore, it appears that different organisms have developed similar weapons and defense strategies to combat microbial infection.

On the other hand, Lorito et al. (29) demonstrated the utility of using fungal genes, particularly genes from mycoparasitic fungi, to increase plant resistance against fungal pathogens. Since then, other fungal and bacterial genes have been used to improve disease resistance in transgenic plants (28). The production of ani-

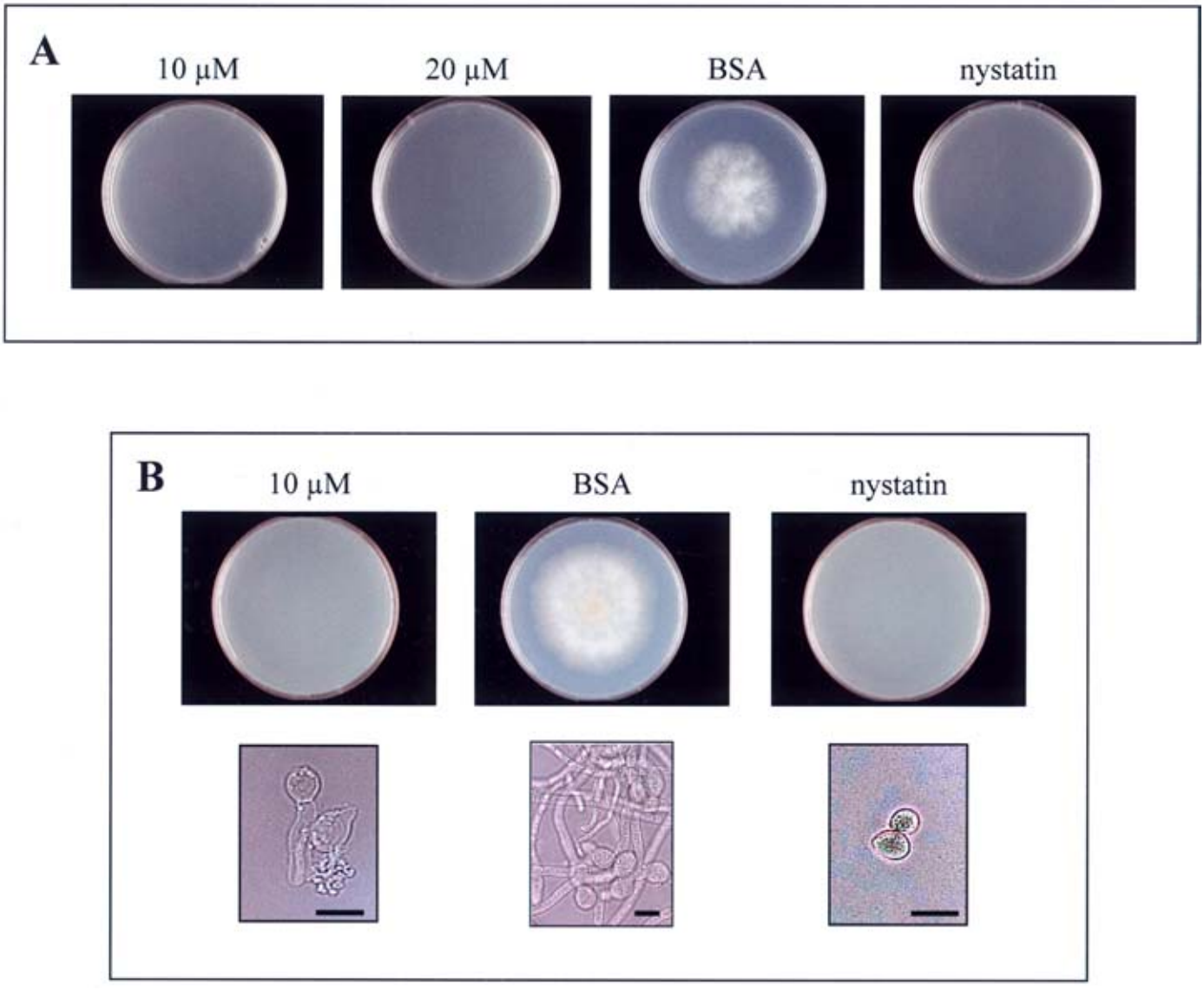

Fig. 5. Fungicidal activity of antifungal protein (AFP) on in vitro growth of *Botrytis cinerea* isolate CC1. **A,** Conidia were pregerminated for 6 h, and AFP was added at final concentrations of 10 or 20 µM. Fungal cultures were then incubated for 6 h, washed twice consecutively with potato dextrose broth (PDB), resuspended in 50 µl of PDB, and plated on potato dextrose agar (PDA) plates. Photographs were taken 4 days after plating. Controls with bovine serum albumin (BSA) or nystatin are presented. **B,** Ungerminated conidia were treated for 6 h with AFP at a final concentration of 10 µM, washed with PDB, and plated onto PDA plates as indicated in **A**. Photographs were taken 4 days after plating (top panel). Micrographs of AFP-treated conidia are presented (lower panel). Bars = 2.5 µm.

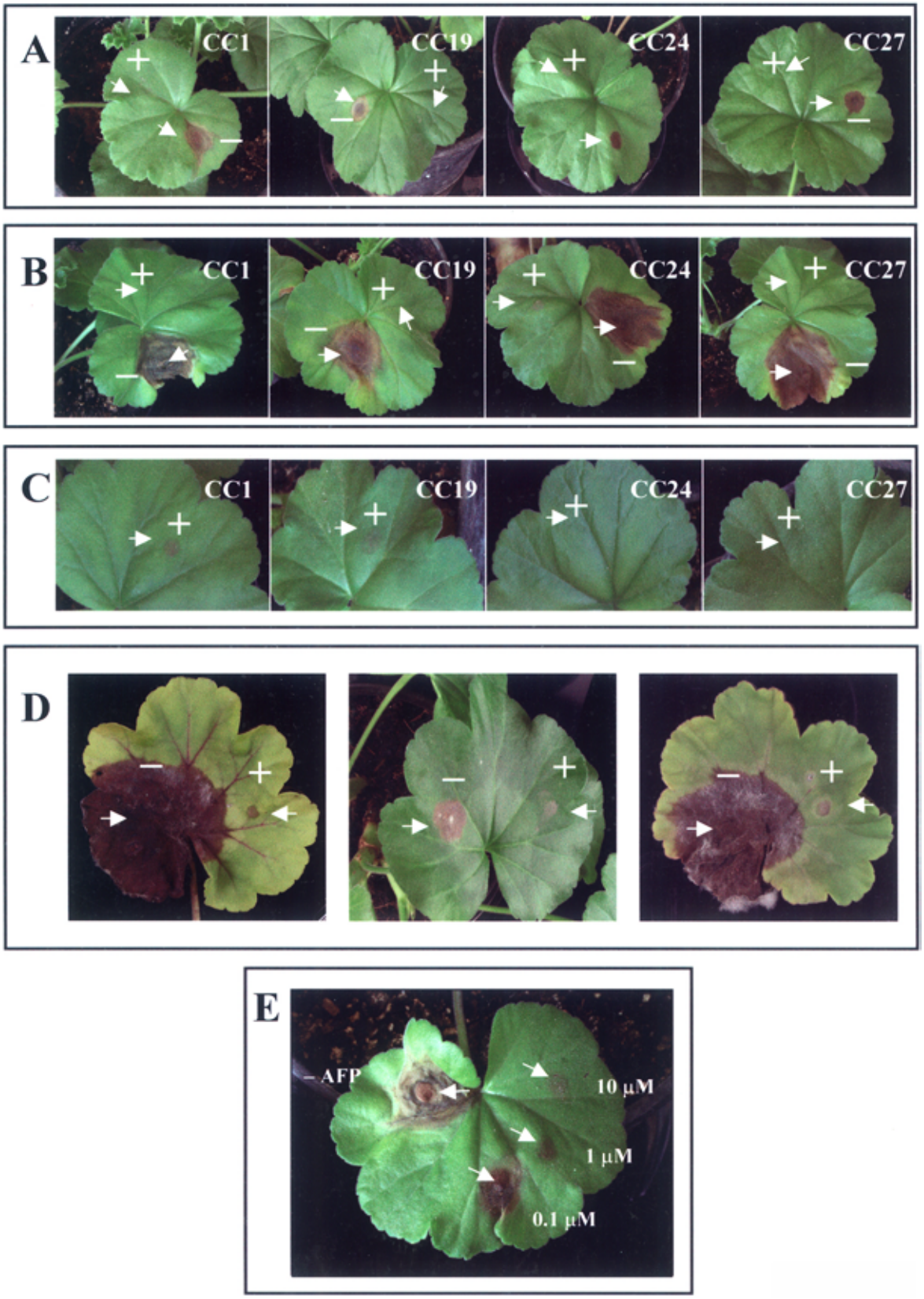

Fig. 6. Protection of *Pelargonium* plants against *Botrytis cinerea* by direct application of antifungal protein (AFP). Geranium leaves were spot inoculated with conidia of *B. cinerea* CC1, CC19, CC24, and CC27 isolates. The sites at which the conidial suspension was placed are indicated by arrows. The inoculated areas were immediately treated with a drop of 10 µM AFP solution (indicated by +) or water (indicated by –) which was deposited over the point of inoculation. Photographs were taken **A,** 3 and **B,** 6 days after inoculation. **C,** Geranium leaves were locally inoculated (as in panel **A**) and treatment with AFP was carried out 6 h postinoculation. Photographs were taken 3 days after inoculation. **D,** A drop of 10 µM AFP solution was applied onto the leaf, and 3 (left panel) and 14 (middle and right panels) days later, the same spot was inoculated with a *B. cinerea* CC1 conidial suspension. Photographs were taken 11 days (left and right panel) or 3 days (middle panel) after fungal inoculation. **E,** Leaves were inoculated with *B. cinerea* CC1 conidia and immediatelly treated with different concentrations of AFP (10, 1, and 0.1 µM). Photograph was taken 3 days after inoculation.

mal-derived or synthetic antimicrobial peptides in transgenic plants also has proved useful for obtaining resistance to phytopathogens (8,12,35). More specifically, transgenic expression of cecropin B in rice confers resistance to bacterial leaf blight (42). Enhanced disease resistance conferred by expression of an antimicrobial magainin analog in transgenic tobacco also has been reported (26).

The results presented here indicate that the AFP from the soil mold *A. giganteus* displays a potent antifungal activity against *B. cinerea*, the causal agent of Botrytis blight of geranium. AFP inhibits mycelial growth and conidial germination of *B. cinerea*. Depending on the isolate assayed, the concentrations required for 50% growth inhibition ranged from 0.5 to 5 μM. Accompanying microscopic observations of the antifungal assays provided valuable information on the effect of AFP treatments on the growth of the hyphae as well as on conidial germination. Thus, reduced hyphal elongation was commonly observed in AFP-treated fungal cultures compared with well extended mycelial growth in controls.

AFP was effective against *B. cinerea* not only in vitro but also in vivo, as demonstrated by the absence or reduction of disease symptoms on geranium leaves. Thus, our experiments with geranium plants showed that, when inoculated with *B. cinerea*, the infected leaves develop the characteristic gray mold symptoms. In contrast, treatment with AFP prevented *Botrytis* infection of geranium leaves. The protective effect of AFP is maintained for long periods of time. Presumably, the effectiveness of AFP in preventing *Botrytis* infection in geranium leaves is related to its ability to inhibit both conidial germination and hyphal growth.

The simultaneous expression of antifungal genes into a single transgenic plant and the effect of the combination of their end products is a major issue. Additive and synergistic effects are desirable, whereas antagonistic effects are not. In this respect, it is well known that antimicrobial compounds may act synergistically, additively, or rarely, antagonistically with other antimicrobial compounds (15,17,39,51,52). Our in vitro inhibition assays showed that *B. cinerea* isolate CC1 also was sensitive to the cecropin A peptide. Furthermore, we show that the combination of AFP and cecropin A has an additive effect on *B. cinerea* growth. This information will be potentially useful for designing strategies to enhance crop protection against *B. cinerea* diseases.

The mechanism of action of many antimicrobial proteins and peptides is presumed to involve the interaction of the protein and peptide with the target cell membrane followed by membrane disruption and formation of pores across microbial membranes (3,10,41,49,53). Proteins and peptides active against phytopathogenic fungi have been demonstrated to disrupt fungal membranes and cause alteration in morphology of hyphae (9,38).

AFP is a highly basic and low-molecular-weight protein structurally related to plant antifungal proteins, namely defensins and thionins. There is then the possibility that the interaction of positively charged sites of the AFP protein with negatively charged phospholipids of susceptible fungal membranes results in an inhibitory effect of AFP on phytopathogens. The observed interaction of AFP with phospholipid membranes supports this possibility (24). Using an assay based on the uptake of the fluorescent dye SYTOX Green, Theis et al. (48) recently reported that AFP causes membrane permeabilization in *A. niger*. At present, the exact mechanism by which AFP exerts its antifungal activity on *B. cinerea* growth is however unknown.

In addition, a nucleic acid interacting ability of AFP has been recently reported (30). Binding of AFP to DNA promotes charge neutralization and condensation of DNA. The similarities observed at the level of three-dimensional structure between AFP and the oligonucleotide/oligosaccharide binding (OB fold)-containing proteins support this activity of AFP. The activity of AFP on promoting DNA condensation and interacting with lipid membranes may well be related to its antifungal activity. In this respect, other nucleic acid binding proteins also interact with acidic phospholipids. This would be the case, for example, of the DnaA protein, the initiator of DNA replication in *Escherichia coli* (20).

Even though *B. cinerea* is one of the most common diseases of greenhouse-grown ornamental plants (*Poinsettia*, *Cyclamen*, and *Pelargonium*, etc.), very little effort has been devoted to obtain *B. cinerea* resistance through genetic engineering in ornamental plants. Only expression of the antimicrobial protein, *Ace*-AMP1, in scented geranium has been reported to enhance resistance to *B. cinerea* leaf infection (2). In other studies, resistance to *Botrytis* infection in transgenic tobacco, carrot, cucumber, or tomato plants has been described (18,29,36,37,45,46).

However, from a practical standpoint, the level of an antifungal protein in transgenic plants would depend on rates of synthesis and secretion to the appropriate subcellular compartment. The success of a transgenic approach by expressing an antifungal protein in conferring resistance to the transgenic plant also depends on its stability and resistance to proteolytic activities in the transgenic plant. In this regard, the stability of AFP and its resistance to proteolytic degradation (24) makes it plausible to design protective strategies for expression of the AFP gene in transgenic plants. Alternatively, considering that AFP is a protein that can be easily purified in large amounts from the extracellular medium of *A. giganteus* cultures (24,33), it offers an attractive and economical process for its rapid and convenient production. Direct application of AFP, either by surface application or by spraying, for the protection of geranium plants against Botrytis blight also may be considered. To conclude, the high antifungal potency of AFP, together with the protection observed after application of this protein on geranium leaves, suggests that the *afp* gene may be a promising candidate for crop protection, particularly for protection of geranium plants against *B. cinerea*.

ACKNOWLEDGMENTS

This work was supported by grants BIO2000-1682-02-01, BMC2000-0551, FIT-010000-2000-13, and FIT-010000-2001-13 from the Ministerio de Ciencia y Tecnología and within the framework of the Centre de Referència de Biotecnología (CeRBA) from the Generalitat de Catalunya.

LITERATURE CITED

1. Abbott, W. S. 1925. A method of computing effectiveness of an insecticide. J. Econ. Entomol. 18:265-267.
2. Bi, Y.-M., Cammue, B. P. A., Goodwin, P. H., KrishnaRaj, S., and Saxena, P. K. 1999. Resistance to *Botrytis cinerea* in scented geranium transformed with a gene encoding the antimicrobial protein *Ace*-AMP1. Plant Cell Rep. 18:835-840.
3. Blondelle, S. E., and Houghten, R. A. 1992. Design of model amphipathic peptides having potent antimicrobial activities. Biochemistry 31:12688-12694.
4. Broekaert, W. F., Cammue, B. P., De Bolle, M. F., Thevissen, K., De Samblanx, G. W., and Osborn, R. W. 1997. Antimicrobial peptides from plants. Crit. Rev. Plant Sci. 16:297-323.
5. Bruix, M., Jiménez, M. A., Santoro, J., González, C., Colilla, F. J., Méndez, E., and Rico, M. 1993. Solution structure of gamma 1-H and gamma 1-P thionins from barley and wheat endosperm determined by 1H-NMR: A structural motif common to toxic arthropod proteins. Biochemistry 32:715-724.
6. Campos-Olivas, R., Bruix, M., Santoro, J., Lacadena, J., Martínez del Pozo, A., Gavilanes, J. G., and Rico, M. 1995. NMR solution structure of the antifungal protein from *Aspergillus giganteus*: Evidence for cysteine pairing isomerism. Biochemistry 34:3009-3021.
7. Carmona, M. J., Molina, A., Fernández, J. A., López-Fando, J. J., and García-Olmedo, F. 1993. Expression of the α-thionin gene from barley in tobacco confers enhanced resistance to bacterial pathogens. Plant J. 3:457-462.

8. Cary, J. W., Rajasekaran, K., Jaynes, J. M., and Cleveland, T. E. 2000. Transgenic expression of a gene encoding a synthetic antimicrobial peptide results in inhibition of fungal growth in vitro and in planta. Plant Sci. 154:171-181.
9. Cavallarin, L., Andreu, D., and San Segundo, B. 1998. Cecropin A-derived peptides are potent inhibitors of fungal plant pathogens. Mol. Plant-Microbe Interact. 11:218-227.
10. Christensen, B., Fink, J., Merrifield, R. B., and Mauzerall, D. 1988. Channel-forming properties of cecropins and related model compounds incorporated into planar lipid membranes. Proc. Natl. Acad. Sci. USA 85:5072-5076.
11. Datta, K., Muthukrishnan, S., and Datta, S. 1999. Expression and function of PR-protein genes in transgenic plants. Pages 261-277 in: Pathogenesis-Related Proteins in Plants. S. K. Datta and S. Muthukrishnan, eds. CRC Press, Boca Raton, FL.
12. DeGray, G., Rajasekaran, K., Smith, F., Sanford, J., and Daniell, H. 2001. Expression of an antimicrobial peptide via the chloroplast genome to control phytopathogenic bacteria and fungi. Plant Physiol. 127:852-862.
13. Epple, P., Apple, K., and Bohlmann, H. 1997. Overexpression of an endogenous thionin gives enhanced resistance in *Arabidopsis thaliana* against *Fusarium oxysporum.* Plant Cell. 9:509-520.
14. García-Olmedo, F., Molina, A., Alamillo, J. M., and Rodríguez-Palenzuela, P. 1998. Plant defense peptides. Biopolymers 47:479-491.
15. Giacometti, A., Cirioni, O., Barchiesi, F., Fortuna, M., and Scalise, G. 1999. In vitro activity of cationic peptides alone and in combination with clinically used antimicrobial agents against *Pseudomonas aeruginosa*. J. Antimicrob. Chemother. 44:641-645.
16. Gisi, U. 1996. Synergistic interaction of fungicides in mixtures. Phytopathology 86:1273-1279.
17. Gonzalez, C. F., Provin, E. M., Zhu, L., and Ebbole, D. J. 2002. Independent and synergistic activity of synthetic peptides against thiabendazole-resistant *Fusarium sambucinum*. Phytopathology 92:917-924.
18. Hain, R., Reif, H., Krause, E., Langebartels, R., Kindl, J., Vornam, B., Wiese, W., Schmelzer, E., Schreier, P., Stocker, R., and Stenzel, K. 1993. Disease resistance results from foreing phytoalexin expression in a novel plant. Nature 361:153-156.
19. Hancock, E. W., and Lehrer, R. 1998. Cationic peptides: A new source of antibiotics. Trends Biotechnol. 16:82-88.
20. Hase, M., Yoshimi, T., Ishikawa, Y., Ohba, A., Guo, L., Mima, S., Makise, M., Yamaguchi, Y., Tsuchiya, T., and Mizushima, T. 1998. Site-directed mutational analysis for the membrane binding of DNA A protein. J. Biol. Chem. 273:28651-28656.
21. Hausbeck, M. K. 1993. Botrytis blight. Pages 223-228 in: Geranium IV. J. White, ed. Ball Publishing, Geneva.
22. Hausbeck, M. K., and Moorman, G. W. 1996. Managing *Botrytis* in greenhouse-grown flower crops. Plant Dis. 80:1212-1219.
23. Jach, G., Gornhardt, B., Mundy, J., Logemann, J., Pinsdorf, E., Leah, R., Schell, J., and Maas, C. 1995. Enhanced quantitative resistance against fungal disease by combinatorial expression of different barley antifungal proteins in transgenic tobacco. Plant J. 8:97-103.
24. Lacadena, J., Martínez del Pozo, A., Gasset, M., Patiño, B., Campos-Olivas, R., Vázquez, C., Martínez-Ruiz, A., Mancheño, J. M., Oñaderra, M., and Gavilanes, J. G. 1995. Characterization of the antifungal protein secreted by the mold *Aspergillus giganteus*. Arch. Biochem. Biophys. 324:237-281.
25. Leroux, P., Chapeland, F., Giraud, T., Brygoo, T., and Gredt, M. 1999. Resistance to sterol biosynthesis inhibitors and various other fungicides in *Botrytis cinerea.* Pages 297-303 in: Modern Fungicides and Antifungal Compounds II. H. Lyr, P. E. Russell, H. W. Dehne, and H. D. Sisler, eds. Intercept, Andover, UK.
26. Li, Q., Lawrence, C. B., Xing, H.-Y., Babbitt, R. A., Troy Bass, W., Maiti, I. B., and Everett, N. P. 2001. Enhanced disease resistance conferred by expression of an antimicrobial magainin analog in transgenic tobacco. Planta 212:635-639.
27. Lorito, M., Harman, G. E., Hayes, C. K., Broadway, R. M., Tronsmo, A., Woo, S. L., and Di Pietro, A. 1993. Chitinolytic enzymes produced by *Trichoderma harzianum*: Antifungal activity of purified endochitinase and chitobiosidase. Phytopathology 83:302-307.
28. Lorito, M., and Scala, F. 1999. Microbial genes expressed in transgenic plants to improve disease resistance. J. Plant Pathol. 81:73-88.
29. Lorito, M., Woo, S. L., García Fernandez, I., Colucci, G., Harman, G. E., Pintor-Toro, J. A., Filippone, E., Muccifora, S., Lawrence, C. B., Zoina, A., Tuzun, S., and Scala, F. 1998. Genes from mycoparasitic fungi as a source for improving plant resistance to fungal pathogens. Proc. Natl. Acad. Sci. USA 95:7860-7865.
30. Martínez del Pozo, A., Lacadena, V., Mancheño, J. M., Olmo, N., Oñaderra, M., and Gavilanes, J. G. 2002. The antifungal protein AFP of *Aspergillus giganteus* is an oligonucleotide/oligosaccharide binding (OB) fold-containing protein that produces condensation of DNA. J. Biol. Chem. 277:46179-46183.
31. Molina, A., and García-Olmedo, F. 1997. Enhanced tolerance to bacterial pathogens caused by transgenic expression of barley lipid transfer protein LTP2. Plant J. 12:669-675.
32. Nakaya, K., Omata, K., Okahashi, I., Nakamura, Y., Kolkenbrock, H., and Ulbrich, N. 1990. Amino acid sequence and disulfide bridges of an antifungal protein isolated from *Aspergillus giganteus*. Eur. J. Biochem. 193:31-38.
33. Olson, B. H., and Goerner, G. L. 1965. α-Sarcin, a new antitumour agent: Isolation, purification, chemical composition, and identity of a new amino acid. Appl. Microbiol. 13:314-321.
34. Osborn, R. W., De Samblanx, G. W., Thevissen, K., Goderis, I., Torrekens, S., Van Leuven, F., Atteborough, S., Rees, S. B., Broekaert, W. F. 1995. Isolation and characterization of plant defensins from seeds of Asteraceae, Fabaceae, Hippocastanaceae and Saxifragaceae. FEBS Lett. 368:257-262.
35. Osusky, M., Zhou, G., Osuska, L., Hancock, R. E., Kay, W. W., and Misra, A. 2000. Transgenic plants expressing cationic peptide chimeras exhibit broad-spectrum resistance to phytopathogens. Nat. Biotechnol. 18:1162-1166.
36. Plunja, Z. K., and Raharjo, S. H. T. 1996. Response of transgenic cucumber and carrot plants expressing different chitinase enzymes to inoculation with fungal pathogens. Plant Dis. 80:999-1005.
37. Powell, A. L., van Kan, J., ten Have, A., Visser, J., Greeve, L. C., Bennett, A. B., and Labavitch, J. M. 2000. Transgenic expression of pear PGIP in tomato limits fungal colonization. Mol. Plant-Microbe Interact. 13:942-950.
38. Reed, J. D., Edwards, D. L., and González, C. F. 1997. Synthetic peptide combinatorial libraries: A method for the identification of bioactive peptides against phytopathogenic fungi. Mol. Plant-Microbe Interact. 10:537-549.
39. Segura, A., Moreno, M., Madueño, F., Molina, A., and García-Olmedo, F. 1999. Snakin-1, a peptide from potato that is active against plant pathogens. Mol. Plant-Microbe Interact. 12:16-23.
40. Sela-Buurlague, M. B., Ponstein, A. S., Bres-Vloemans, S. A., Melchers, L. S., van den Elzen, P. J. M., and Cornelissen, B. J. C. 1993. Only specific tobacco (*Nicotiana tabacum*) chitinases and β-1,3-glucanases exhibit antifungal activity. Plant Physiol. 101:857-863.
41. Shai, Y. 1995. Molecular recognition between membrane spanning polypeptides. Trends Biochem. Sci. 20:460-464.
42. Sharma, A., Sharma, R., Imamura, M., Yamakawa, M., and Machii, H. 2000. Transgenic expression of cecropin B, an antibacterial peptide from *Bombix mori*, confers enhanced resistance to bacterial leaf blight in rice. FEBS Lett. 484:7-11.
43. Somssich, I. E., and Halbrock, K. 1998. Pathogen defence in plants: A paradigm of biological complexity. Trends Plant Sci. 3:86-90.
44. Steiner, H., Hultmatk, D., Engstrom, A., Bennich, H., and Boman, H. G. 1981. Sequence and specificity of two antibacterial proteins involved in insect immunity. Nature 292:246-268.
45. Tabei, Y., Kitade, S., Nishizawa, Y., Kikuchi, N., Kayano, T., Hibi, T., and Akutsu, K. 1998. Transgenic cucumber plants harboring a rice chitinase gene exhibit enhanced resistance to gray mold (*Botrytis cinerea*). Plant Cell Rep. 17:59-164.
46. Terakawa, T., Takaya, N., Horiuchi, H., Koike, M., and Takagi, M. 1997. A fungal chitinase gene from *Rhizopus oligosporus* confers antifungal activity to transgenic tobacco. Plant Cell Rep. 16:439-443.
47. Terras, F. R. G., Eggermont, K., Kovaleva, V., Raikhel, N. V., Osborn, R. W., Kester, A., Rees, S. B., Torrekens, S., van Leuven, F., Vanderleyden, J., Cammue, B. P. A., and Broekaert, W. F. 1995. Small cysteine-rich antifungal proteins from radish: Their role in host defense. Plant Cell 7:573-588.
48. Theis, T., Wedde, M., Meyer, V., and Stahl, U. 2003. The antifungal protein from *Aspergillus giganteus* causes membrane permeabilization. Antimicrob. Agents Chemother. 47:588-593.
49. Thevissen, K., Osborn, R. W., Acland, D. P., and Broekaert, W. 2000. Specific binding sites for an antifungal plant defensin from dahlia (*Dahlia merckii*) on fungal cells are required for antifungal activity. Mol. Plant-Microbe Interact. 13:54-61.
50. Vila, L., Lacadena, V., Fontanet, P., Martínez del Pozo, A., and San Segundo, B. 2001. A protein from the mold *Aspergillus giganteus* is a potent inhibitor of fungal plant pathogens. Mol. Plant-Microbe Interact. 14:1327-1331.
51. Vorland, L. H., Osbakk, S. A., Perstolen, T., Ulvatne, H., Rekdal, O., Svendsen, J. S., and Gutteberg, T. J. 1999. Interference of the antimicrobial peptide lactoferricin B with the action of various antibiotics

against *Escherichia coli* and *Staphylococcus aureus*. Scand. J. Infect. Dis. 31:173-177.
52. Westerhoff, H. V., Zasloff, M., Rosner, J. L., Hendler, R. W., De Waal, A., Vaz Gomes, A., Jongsma, P. M., Riethorst, A., and Juretic, D. 1995. Functional synergism of the magainins PGLa and magainin-2 in *Escherichia coli*, tumor cells and liposomes. Eur. J. Biochem. 228:257-264.
53. Zasloff, M. 2002. Antimicrobial peptides of multicellular organisms. Nature 415:389-395.
54. Zhu, Q., Maher, E. A., Masoud, S., Dixon, R. A., and Lamb, C. J. 1994. Enhanced protection against fungal attack by constitutive co-expression of chitinase and glucanase genes in transgenic tobacco. Bio/Technology 12:807-812.