Inhibition of Sporulation and Ultrastructural Alterations of Grapevine Downy Mildew by the Endophytic Fungus *Alternaria alternata*

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ABSTRACT

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One hundred twenty-six endophytic microorganisms isolated from grapevine leaves showing anomalous symptoms of downy mildew were tested on grapevine leaf disks as biocontrol agents against *Plasmopara viticola*. Among the 126 microorganisms, only five fungal isolates completely inhibited the sporulation of *P. viticola*; all of them were identified as *Alternaria alternata*. Ultrastructural analyses were carried out by transmission electron microscopy to observe cellular interactions between *P. viticola* and *A. alternata* in the grapevine leaf tissue. Cytological observations indicated that, even without close contact with *A. alternata*, the *P. viticola* mycelium showed severe ultrastructural alterations,

such as the presence of enlarged vacuoles or vacuoles containing electron-dense precipitates. Haustoria appeared necrotic and irregularly shaped or were enclosed in callose-like substances. Therefore, a toxic action of *A. alternata* against *P. viticola* was hypothesized. To examine the production of toxic low-molecular-weight metabolites by *A. alternata*, we analyzed the fungal liquid culture by thin layer chromatography and proton magnetic resonance spectroscopy. The main low-molecular-weight metabolites produced by the endophyte were three diketopiperazines: *cyclo*(L-phenylalanine-*trans*-4-hydroxy-L-proline), *cyclo*(L-leucine-*trans*-4-hydroxy-L-proline), and *cyclo*(L-alanine-*trans*-4-hydroxy-L-proline). When applied at different concentrations to both grapevine leaf disks and greenhouse plants, a mixture of the three diketopiperazines was very efficacious in limiting *P. viticola* sporulation.

Additional keyword: antagonists.

Plasmopara viticola (B. et C.) Berl. et De Toni causes grapevine downy mildew, an economically important disease that is particularly destructive in parts of the world with warm, wet weather during the growing season. In temperate regions, up to 15 chemical treatments may be necessary to control the disease (49).

The pathogen attacks all green tissues of the grapevine, with the most severe damage to leaves, inflorescences, and young branches. Leaf lesions appear as yellow spots on the upper leaf epidermis and white fungal sporulation on the lower one. Young lesions normally have an oily appearance (they are also called "oil spots"), although they may also be angular and located between the veins, especially on less susceptible varieties, on old leaves or when environmental conditions are less favorable to disease development. Lesions become brown and dry with age.

Biocontrol agents could be an alternative to chemical pesticides in viticulture, with benefits to consumers, growers, and the environment. Due to their high costs and difficulties in application and effectiveness, only a few biocontrol agents are used successfully against diseases (5,6,19). In particular, several organisms

have shown promising results as biocontrol agents against grapevine downy mildew, i.e., *Epicoccum nigrum* (29), *Acremonium byssoides* (9), and *Fusarium proliferatum* (3,21).

Higher plants are commonly colonized by endophytic fungi, which sometimes form communities specific to a certain host-environment interaction (37). Endophytic fungi include weakly parasitic strains living in the host tissues which may become pathogenic as the plant's physiological state deteriorates (38). Interestingly, endophytic and pathogenic fungi can coexist in plant tissues, although the nature of their interactions is unknown in most cases (40). Endophytes that colonize host tissues before the pathogens may produce pathogen-inhibiting metabolites and other defense-related compounds that hinder expression of the disease (41).

Anomalous *P. viticola* symptoms in sensitive *Vitis vinifera* L. (grapevine) leaves have recently been reported (33), suggesting the possible presence of beneficial endophytes in grapevine tissues (17).

The aims of the present study were to (i) isolate endophytic microorganisms from grapevine leaves collected in the field, and test them as biocontrol agents against *P. viticola* on leaf disks; (ii) identify and study the cytological relationships in grapevine leaf tissues between *P. viticola* and sporulation-inhibiting endophytic species; and (iii) extract and identify low-molecular-weight metabolites (7) produced by these active endophytes, and test their effects on *P. viticola* on leaf disks and greenhouse grapevine leaves.

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MATERIALS AND METHODS

Isolation of fungal endophytes from grapevine leaves.

Grapevine leaf samples were collected in an abandoned vineyard in the Province of Grosseto, Tuscany (central Italy), in September 2002. Fungal endophytes were isolated from atypical grapevine downy mildew lesions present on *V. vinifera* leaves as previously reported, i.e., leaves with slow development or late appearance of leaf spots; atypical coloration of leaves or mosaic; or absence of symptoms in plants apparently identical to those showing typical *P. viticola* oil spots (33).

Leaf material was sampled from seven grapevine plants. Five leaves were collected per plant, stored at 4°C in the dark, and processed within 24 h. The leaves were washed with water, surface-sterilized by immersion in 3% sodium hypochlorite for 4 min, and then rinsed twice in distilled H₂O for 1 min (16).

Four fragments (about 5 × 5 mm) per leaf were placed in 90 mm petri dishes containing potato dextrose agar (PDA) and incubated for 48 h at 25°C under cold UV light with a photoperiod of 12 h. The isolated fungal colonies were subcultured five times on fresh PDA plates to obtain pure cultures. Petri dishes were checked periodically for about 1 month; the isolated fungal colonies were then maintained on PDA until tested as biocontrol agents against *P. viticola*. Subsequently, all colonies were transferred onto slants (16 × 160 mm) containing PDA and maintained at 4°C in the dark.

Test of endophyte activity against *P. viticola* on leaf disks.

Wild *P. viticola* populations were isolated from naturally infected leaves of 2-year-old *V. vinifera* plants, cv. Pinot gris, grown in an untreated vineyard (Rovereto, Trento, Italy). The pathogen was maintained by weekly spraying of a suspension of sporangia washing from sporulating lesions onto leaves from uninfected grapevines cv. Pinot gris and grown in a greenhouse at 21°C with a 12-h photoperiod. The plants were maintained in the greenhouse until oil spot symptoms appeared on the leaves. Then, after 12 h of incubation at 20°C and 100% relative humidity (RH) in the dark, new sporangia were collected from the leaves and used as inoculum. The aqueous suspension of sporangia was adjusted to 4.25×10^5 sporangia per ml with a hemacytometer.

A liquid culture of each endophyte was prepared by incubating inoculum of actively growing mycelia (5 mm diameter) in 20 ml of nutrient broth (8 g/liter of commercial nutrient broth [Difco Laboratories, Detroit, MI] plus 50 g/liter of sucrose [Sigma Chemical Co., St. Louis, MO]) on an orbital shaker (speed 120 orbits/min) for 48 h at 20°C. The test of biocontrol activity was performed according to Pertot et al. (36). Briefly, for each endophyte, five leaf disks (2 cm diameter), cut from different grapevine seedling leaves grown in a greenhouse, were floated on 900 µl of endophyte liquid culture for 5 min and then on the suspension containing *P. viticola* sporangia (4.25×10^5 sporangia per ml) overnight at 20°C. Control leaf disks were incubated on the following suspensions, used as positive (1 and 3) and negative controls (2 and 4): 1, nutrient broth plus 4.25×10^5 sporangia of *P. viticola* per ml; 2, nutrient broth; 3, distilled water plus 4.25×10^5 sporangia of *P. viticola* per ml; and 4, distilled water.

All leaf disks were kept in moist chambers at 20°C for 7 days. We evaluated the inhibition of *P. viticola* sporulation by observing the presence or absence of sporulation on leaf disks under a stereomicroscope. The biocontrol test was replicated six times.

Interaction between *P. viticola* and active endophytes in grapevine leaf disks. Only the endophytes that completely suppressed *P. viticola* (total inhibition of sporulation) were identified and used in the subsequent trials. Using the leaf disk assay described above, the interactions between each pathogen-suppressive endophyte and *P. viticola* on leaf disk tissues was observed with a transmission electron microscope (TEM).

Small samples (1 × 3 mm) from the inoculated leaf disks were fixed in 3% glutaraldehyde, rinsed in buffer, postfixed in 1%

osmium tetroxide in 0.1 M potassium phosphate for 2 h at 4°C, dehydrated in ethanol, and embedded in Epon-Araldite resin according to the method described by Musetti et al. (33). Several serial ultrathin sections of at least 80 samples (from corresponding pathogen-suppressed leaf disks tissues) were stained with uranyl acetate and lead citrate and observed under a PHILIPS CM 10 transmission electron microscope (Philips Scientifics, the Netherlands), operated at 80 kV.

Production and identification of low-molecular-weight metabolites by the active endophyte *Alternaria alternata*. The production of low-molecular-weight metabolites by the active endophytes, identified as *A. alternata* (Fr.) Keissl., was evaluated using a liquid culture of the fungus. Flasks containing 250 ml of nutrient broth (prepared as indicated above) were inoculated with a plug from the periphery of a 7-day-old petri dish culture of *A. alternata*. The broth was incubated on an orbital shaker at 20°C for 48 h, and then frozen (−40°C) and lyophilized until extraction. The lyophilized broth (20 g) was dissolved in water and filtered, and then the aqueous solution was extracted with *n*-butanol. This organic phase was then fractionated by preparative layer chromatography on a silica plate (Merck-Kieselgel, Germany; 60 PF₂₅₄) and eluted with a mixture of *n*-butanol/acetic acid/water, 60:15:25, to give three bands named A, B, and C in increasing order of polarity. Fractions A (54 mg) and B (48 mg) were purified further by thin layer chromatography (TLC) on a silica plate and eluted with a mixture of dichloromethane and methanol (CH₂Cl₂/MeOH, 9:1) to provide the pure compounds 1 (1.5 mg, R_f 0.48) and 2 (5 mg, R_f 0.43). Fraction C (83 mg) was similarly subjected to TLC (CH₂Cl₂/MeOH, 85:15) to provide another pure compound, 3 (3.5 mg, R_f 0.40). The isolated metabolites were characterized by proton nuclear magnetic resonance (NMR) spectroscopy using a Bruker AV400 (Germany) (¹H and 2D-NMR spectra at 400 MHz) instrument and the residual solvent signals as internal standard (δ in ppm, CHD₂OD = 3.31, CHCl₃ = 7.26, D₂O = 4.90). Mass spectrometry data were obtained on a Kratos MS80 (UK) with a home-built acquisition system. Optical rotations were measured on a JASCO-DIP-181 polarimeter using a 10 cm cell. The molecular formulae of the main low-molecular-weight metabolites produced by *A. alternata* in liquid culture were established by electron impact mass spectrometry (EIMS) peaks and high-resolution EIMS measurements.

Pre- and postinfection applications of *A. alternata* low-molecular-weight metabolites to *P. viticola*-infected leaf disks. Aqueous solutions of the three metabolites extracted from *A. alternata* liquid culture were mixed 1:1:1 to obtain two final concentrations, 0.33 and 2 mM. In preinfection trials, leaf disks (1 cm diameter) cut from greenhouse grapevine plant leaves were floated for 5 min on 600 µl of solution containing the mixed metabolites at the different concentrations. The leaf disks were then incubated on a suspension containing *P. viticola* sporangia (4.25×10^5 sporangia per ml) overnight at 20°C.

Control leaf disks were incubated first on a suspension of distilled water and then on a suspension containing 4.25×10^5 sporangia of *P. viticola* per ml; as chemical control, leaf disks were treated with an aqueous solution of Cu(OH)₂, at 2 g/liter, and then incubated on the solution containing 4.25×10^5 sporangia of *P. viticola* per ml.

For the postinfection experiment, grapevine leaf disks were incubated on a suspension containing *P. viticola* sporangia (4.25×10^5 sporangia per ml) for 2 h at 20°C; they were then floated for 5 min on 600 µl of the mixture of the three metabolites at the same concentrations used in the preinfection trial.

All the leaf disks were kept in moist chambers at 20°C for 7 days. To assess the effects of the metabolites on *P. viticola* development, we quantified the production of sporangia on leaf disks. Sporangia were washed from each leaf disk with 2 ml of distilled water applied with a plastic wash-bottle. The total number of sporangia per leaf disk was calculated by examining the

suspension with a hemacytometer. For each inhibition treatment, five leaf disks cut from five different greenhouse grapevines were used, and each experiment was replicated three times.

Disease severity (percent area) was expressed as percentages of downy mildew-infected leaf area and was arcsine-transformed (for homogeneity of variance) before analysis of variance using SPSS (SPSS, Inc., Chicago, IL). The Duncan test was used to determine significance of differences among treatments (21).

Pre- and postinfection effectiveness of *A. alternata* low-molecular-weight metabolites against *P. viticola* on greenhouse grapevine leaves. Healthy 2-year-old grapevine plants of cv. Pinot gris, grown in a greenhouse at 21°C with a 12-h photoperiod, were used. In the preinfection trials, 6 drops (each 10 µl) of the same metabolite mixture and concentrations used in the previously described leaf disk tests were placed on three replicates of healthy leaves of three grapevine plants. Drops of distilled water were used as an untreated control. The drops were allowed to dry and the grapevine leaves were inoculated with an aqueous suspension of *P. viticola* sporangia (same concentration as in the previous tests) applied by a vaporizer.

For the postinfection experiment, grapevine leaves were inoculated with an aqueous suspension of *P. viticola* sporangia at the same concentration as in the previous tests. Inoculum was applied with a sprinkler. At 2 or 24 h after inoculation, 6 drops (each 10 µl) of the same metabolite mixtures and concentrations used in the preinfection tests were placed on three replicates of inoculated leaves of three grapevine plants. Drops of distilled water on each leaf were used as untreated controls. All the plants were maintained in a greenhouse at 20°C, 100% RH, for 7 days. We evaluated the inhibition of *P. viticola* sporulation by observing the presence or absence of sporulation on the treated leaf areas under a stereomicroscope.

RESULTS

Isolation of fungal endophytes from grapevine leaves and test of activity against *P. viticola* on leaf disks. One hundred twenty-six fungal endophytes were isolated from a total of 140 grapevine leaf segments. They belonged to the genera *Alternaria*, *Aspergillus*, *Fusarium*, *Phoma*, and *Stemphylium*. All the isolates were tested as possible antagonists of *P. viticola*, and they showed varying effectiveness in inhibiting its sporulation (Fig. 1).

In the first trial, 49 of 126 organisms were totally effective in inhibiting *P. viticola* sporulation. These were tested in a second

trial and 12 of 49 showed total effectiveness. The 12 effective endophytes were tested a third and a fourth time and the number of totally effective fungi was 6 of 12 and 5 of 6, respectively. In the last two trials, the five tested organisms were completely effective.

The five endophytes that totally inhibited *P. viticola* sporulation on leaf disks in all six trials (Fig. 2A) were identified as *A. alternata*.

Consistent *P. viticola* sporulation was observed on leaf disks incubated with control solutions 1 and 3 (Fig. 2B). No sporulation appeared on leaf disks incubated with control solutions 2 and 4.

Interaction between *P. viticola* and active endophytes in grapevine leaf disks. TEM analysis of control leaf disks inoculated only with *P. viticola* showed hyphae of the pathogen in the substomatal zone and in the intercellular space of spongy parenchyma. Hyphae appeared vacuolated (Fig. 3A). Well-structured haustoria were also observed (Fig. 3B, ha). TEM observations of leaf tissues inoculated only with the endophytic fungus *A. alternata* (Fig. 3C and D, arrows) showed no damage to the leaves and the tissues appeared well preserved (Fig. 3E and F).

Ultrastructural observations of leaf disks incubated with *A. alternata* and then inoculated with *P. viticola* showed that both fungi penetrated into the host tissues. However, physical contact between the two fungi was never observed. Even without close contact between the two organisms, the *P. viticola* mycelium (Fig. 4A to F, P) showed severe ultrastructural alterations and marked cytoplasmic modifications, such as enlarged vacuoles (Fig. 4A and B, v), vacuoles containing electron-dense precipitates (Fig. 4C and D, arrows), detachment of the plasmalemma from the cell wall (Fig. 4C, black arrow), and lysis of the mitochondrial cristae (data not shown). Haustoria appeared necrotic and irregular in shape (Fig. 4E, ha), or they were enclosed in plant material consisting of a callose-like substance (Fig. 4F, c) and electron-opaque extrahaustorial matrix (Fig. 4E and F, arrows).

Production of low-molecular-weight metabolites by the grapevine endophyte *A. alternata*. The molecular formulae of the low-molecular-weight metabolites produced by *A. alternata*, the NMR data, proton chemical shifts, and coupling constants were identical to those reported in the literature for three compounds belonging to the diketopiperazines family (DKPs). Comparison of optical rotation values allowed us to define the stereochemistry of their component amino acids. The metabolites were identified as 1 = *cyclo*(L-phenylalanine-*trans*-4-hydroxy-L-proline) C₈H₁₂N₂O₃, MW 184; 2 = *cyclo*(L-leucine-

Fig. 1. Effectiveness of the 126 endophytes in inhibiting *Plasmopara viticola* sporulation on grapevine leaf disks. Only the endophytes showing 100% effectiveness were tested in the following trial.

trans-4-hydroxy-L-proline) C₁₁H₁₈N₂O₃, MW 226; and 3 = *cyclo*(L-alanine-*trans*-4-hydroxy-L-proline) C₁₄H₁₆N₂O₃, MW 260.

Effects of pre- and postinfection applications of *A. alternata* low-molecular-weight metabolites on *P. viticola*-infected leaf disks. In the preinfection trials, applications of the mixture of the three DKPs to grapevine leaf disks totally inhibited the production of *P. viticola* sporangia at both concentrations (Table 1; Fig. 5A). Likewise, postinfection application of the two concentrations of the DKP mixture also inhibited sporangia production in comparison to control-inoculated leaf disks (Table 1).

Consistent sporulation was observed on the untreated control leaf disks (Fig. 5B, arrows), while in Cu(OH)₂-treated samples, scarce (0.33% leaf disk surface area) *P. viticola* sporangia were observed on the leaf disk surfaces compared with the control (Fig. 5C, arrows).

Pre- and postinfection effectiveness of *A. alternata* diketopiperazines against *P. viticola* in greenhouse grapevine plants. After incubation for 7 days, *P. viticola* sporulation was not observed on the six areas of all grapevine leaves previously treated

with the drops of the DKP mixtures at concentrations of 0.33 and 2 mM (Fig. 6, arrows); in contrast, sporulation was present on the water-treated areas. Similarly, both concentrations of the DKP mixture were effective in inhibiting *P. viticola* sporulation on grapevine leaves in the postinfection experiments.

DISCUSSION

In recent years, interest in nonpathogenic microorganisms that can induce disease resistance or protect plants against pathogens has increased among researchers. Endophytes could be highly promising as biocontrol agents, since they are extremely widespread colonizers of various plant species, often without any apparent negative effects (45). The importance of endophytes is emphasized by the ability of some of them to induce defense mechanisms in host plants or to provide active plant defense (17), even occupying the microhabitat of some pathogens (37). Moreover, endophytes may produce toxins against insects and growth hormones useful to the plant's metabolism (13).

Here, we tested 126 grapevine endophytes as possible antagonists of *P. viticola*. They showed varying effectiveness at inhibiting the pathogen. Only *A. alternata*, a known grapevine endophyte (12,18,25), was completely effective in inhibiting *P. viticola* sporulation on grapevine leaf disks in these trials. For this reason, we conducted further studies on the interaction between the pathogen and *A. alternata*.

Ultrastructural analysis of grapevine leaf disk tissues revealed that *A. alternata* did not cause leaf tissue damage. By contrast, *P. viticola*, inoculated on leaf disks previously treated with *A. alternata*, exhibited marked structural changes in the presence of the endophyte even without close contact between the two fungi, such as abnormal vacuolization, accumulation of electron-dense material in the vacuoles, and appearance of necrotic haustoria. Similar ultrastructural modifications have been reported in other fungal pathogens treated with antagonists (2,23), indicating a possible involvement of toxic metabolites in the antagonistic activity. Since contact between the two organisms was never observed, a mechanism of direct hyperparasitism of *P. viticola* by *A. alternata* can be excluded (4,20,21,29,34). However, a mode of action based on the production of toxic compounds can be hypothesized. Indeed, *A. alternata* is known to produce several plant-host-specific toxic compounds (39,48). We chose to isolate and study low-molecular-weight compounds for a variety of reasons. Firstly, the chemistry of peptide synthesis is very consolidated, allowing the chemist to append a variety of chiral functional groups in highly optimized and reproducible manners. Secondly, it is relatively straightforward to sample the conformational elements of diversity and force side chains into different topographical relationships. Finally, a variety of active low-molecular-weight cyclic peptides have been observed to bind to a wide range of receptors (7).

The three low-molecular-weight toxic metabolites extracted from *A. alternata* liquid culture belong to the DKP family. The same compounds have been extracted from an undescribed Jaspidae sponge of the Fiji Islands (1), from a skin tissue extract of rabbits (27) and from a *Ruegeria* strain of bacteria associated with the sponge *Suberites domuncula* (32). Other molecules belonging to the DKP family, i.e., maculosin and its analogues, have been extracted from *A. alternata* affecting spotted knapweed (*Centaurea maculosa*) (7,44).

DKPs have also been extracted from fermentation broth of other fungi, such as *Penicillium italicum* (42), *Penicillium aurantiogriseum* (31), *Aspergillus fumigatus* (14,15), *Aspergillus niger* (35), and from lichens (24). Thus, DKPs appear to be ubiquitous compounds conserved among different kingdoms.

Moreover, DKPs are stable to proteolysis and have a wide variety of biological activities, i.e., cell cycle inhibitors, used in medicine as antibiotics, synthetic vaccines and in cancer

Fig. 2. A, Grapevine leaf disks treated with liquid culture of *Alternaria alternata* and then inoculated with *Plasmopara viticola*: sporulation of the pathogen is not present. B, Sporangia of *P. viticola* were observed on control leaf disks inoculated only with *P. viticola*.

Fig. 3. **A**, Transmission electron micrographs of control leaf tissue inoculated only with *Plasmopara viticola*: the hyphae are localized in the spongy parenchyma and appear vacuolated. **B**, Haustoria (ha) are well structured (w = wall; m = mitochondria). **C and D**, Transmission electron micrographs of control leaf tissue inoculated only with *Alternaria alternata*: the fungus (arrows) is localized in the intercellular space. **E and F**, Transmission electron micrographs of control leaf tissue treated only with *A. alternata*: the tissue does not present alterations. Cells and organelles appear well preserved (Ch = chloroplast; N = nucleus; m = mitochondria). The scale bars represent **A**, 5.5 μ m, **B**, 0.5 μ m, **C**, 1.5 μ m, **D**, 2.5 μ m, **E**, 2.5 μ m, and **F**, 1.9 μ m.

Fig. 4. Transmission electron micrographs of grapevine leaf tissue inoculated with *Alternaria alternata* and *Plasmopara viticola*. The *P. viticola* mycelium (P; arrow = haustorium) has severe ultrastructural alterations. **A and B**, Vacuoles (v) are enlarged. **C and D**, Plasmalemma is detached from the wall (black arrow); vacuoles contain electron-dense precipitates (arrows). **E and F**, Haustoria (ha) are necrotic, with an irregular shape or surrounded by callose (c) and electron-opaque extrahaustorial matrix (arrows). The scale bars represent **A**, 4.5 μm , **B**, 1.12 μm , **C**, 4.5 μm , **D**, 0.5 μm , **E**, 1.12 μm , and **F**, 0.35 μm .

chemotherapy (14). DKPs also have agricultural applications, acting as herbicides (7) and germination promoters for rice seeds under low-temperature stress, as well as resistance inducers in rice seedlings against water stress (26). Antifungal activity has been demonstrated for most of them: *Gliocladium* sp. produces a DKP that kills *Pythium* by coagulation of proteins in the cyto-

TABLE 1. Effect of three compounds belonging to the diketopiperazines family (DKPs) on sporulation of *Plasmopara viticola* on grapevine leaf disks

Treatment ^a	Severity (percent area) ^b
DKPs 0.33 mM preinfection	0.00 ± 0 a ^c
DKPs 2 mM preinfection	0.00 ± 0 a
DKPs 0.33 mM postinfection	0.00 ± 0 a
DKPs 2 mM postinfection	0.00 ± 0 a
Cu(OH) ₂	0.33 ± 0.33 a
Untreated	100.00 ± 0 b

^a DKPs were applied 5 min before (preinfection) or 2 h after infection (post-infection) by *Plasmopara viticola*.

^b Severity: percentage of downy mildew-infected surface in grapevine leaf disks.

^c Numbers are means of *n* samples ±SE, where *n* = 15. Mean values followed by the same letter are not significantly different for *P* < 0.05 (Duncan test).

plasm (10), while DKPs extracted from marine fungi show potent activity against *Pyricularia oryzae* (11).

In our experiments, the three DKPs extracted from *A. alternata* reduced *P. viticola* sporulation in grapevine leaf disks, and these results were confirmed using plants maintained in the greenhouse. The antifungal activity was not concentration-dependent, at least with the concentrations used in our experiments (0.33 and 2 mM). In fact, both concentrations had the same inhibitory effects. Several DKPs and other antimicrobial peptides have been found to be active over a range that includes the above concentrations (7,28,44).

The DKPs were active against the pathogen only in the treated areas of grapevine leaves (where the droplets were placed), which could be explained by the poor translocation capacity of DKPs (7).

It has been reported that some *A. alternata*-produced DKPs, i.e., maculosin and its analogues, cannot penetrate inside the leaves through the cuticle but only in aqueous solution through opened stomata and hydathodes (7). They were also unable to move far from the place of penetration (7). Similarly, *P. viticola* penetrates in the host leaf tissues through stomata and, during the first 12 to 15 h after penetration, the mycelium is localized in the substomatal air spaces and develops slowly, until the first haustorium is formed (30). Therefore, this period is suitable for

Fig. 5. A, Grapevine leaf disks treated with the mixture of the three compounds belonging to the diketopiperazines family at a concentration of 0.33 mM and then inoculated with *Plasmopara viticola*: sporulation of the pathogen is not present. B, Consistent sporulation was observed on untreated control leaf disks (arrows). C, Cu(OH)₂-treated samples presented scarce *P. viticola* sporangia (arrows).

the application of the DKP treatment. In our experiments, stimulation of *P. viticola* sporulation was never observed after DKP treatments, either in leaf disks or in greenhouse grapevine leaves, and no necrotic lesions or other phytotoxicity symptoms were observed on DKP-treated grapevine leaf tissues.

In addition to inhibition by DKPs, space and/or nutrient competition may be involved in the *A. alternata*–*P. viticola* relationship. Indeed, several other authors have reported this type of competition between microorganisms (8,19,43,46,47).

A. alternata is a cosmopolitan species and has often been reported to inhabit the structures of substomatic chambers (18,39). It is well known that the *P. viticola* mycelium develops first in the substomatic air space, which subsequently becomes

almost completely invaded by the growing hyphae (22). Thus, both the pathogen and the endophytic fungus have substomatic development.

In conclusion, our results demonstrate that the grapevine endophyte *A. alternata* induces ultrastructural alterations in *P. viticola* mycelium, inhibiting sporulation. Furthermore, DKPs extracted from *A. alternata* liquid culture showed a marked ability to prevent *P. viticola* sporulation, thus representing a promising means to control the pathogen. Last but not least, because of their low molecular weight, DKPs are easy and inexpensive to synthesize, since there are many different strategies by which these dipeptides may be obtained (7). Further investigations are in progress to verify the effectiveness of DKPs at reducing *P. viticola* sporula-

Fig. 6. Greenhouse grapevine leaf treated with 6 drops of the three compounds belonging to the diketopiperazines family (DKP mixture) at a concentration of 0.33 mM before infection with *Plasmopara viticola*. Arrows indicate the absence of sporulation in treated areas.

tion in grapevines grown in the field and to clarify the mechanism by which *A. alternata* and these molecules affect *P. viticola* on grapevine leaves.

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