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Antifungal activity of diketopiperazines extracted from *Alternaria alternata* against *Plasmopara viticola*: An ultrastructural study

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Abstract

Three dipeptides, belonging to the family of diketopiperazines (DKPs), were extracted from broth culture of the grapevine endophyte *Alternaria alternata*, and were tested against *Plasmopara viticola* on leaves of grapevine plants grown in greenhouse. DKPs, used at different concentrations (10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} M) both singularly and in mixtures, demonstrated real effectiveness in inhibiting *P. viticola* sporulation when applied 2 or 24 h after pathogen inoculation. Moreover, no necrotic lesions or other phytotoxicity symptoms were observed on DKP-treated grapevine leaf tissues.

Ultrastructural analysis performed on grapevine leaf tissues revealed that the DKPs used singularly and in mixture, at above reported concentrations, did not cause leaf tissue damages. By contrast, hyphae of *P. viticola* exhibited marked structural changes, similar to those induced by the endophyte *A. alternata*. This demonstrates the involvement of these metabolites in the relationship of *P. viticola* and the endophyte. Further experimental trials will be carried out in the next future in order to test the effectiveness of these molecules also under field conditions, and to better understand the mechanism of action involved in the pathogen inhibition.
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Keywords: *Alternaria alternata*; Antifungal peptides; Biocontrol; Diketopiperazines; Grapevine; *Plasmopara viticola*

1. Introduction

Grapevine downy mildew, caused by the fungus *Plasmopara viticola* (B. et C.) Berl. et De Toni, is one of the most destructive diseases affecting this crop, especially in a warm, wet climate. In organic viticulture *P. viticola* control is based almost exclusively on copper. In the European Union restrictions on the use of copper particularly in organic agriculture have encouraged the search for alternatives and the optimisation of the quantity allowed. In fact, the European Commission Regulation No. 473/2002 of 15 March 2002 has amended Council Regulation No. 2092/91 on the organic production of agricultural products regarding the use of copper compounds as fungicides. Although copper, in the form of hydroxide, oxychloride, (tribasic) sulphate and cuprous oxide employed as a fungicide is very common in organic farming practices, it may have long-term consequences due to its accumulation in the soil, which appear to be incompatible with the objectives of organic farming.

Research on natural products, that could be alternatives to synthetic fungicides, for example, plant extracts and essential oils, has greatly increased during recent years (Wilson et al., 1997; Pradhanang et al., 2003; Cohen et al., 2006). Antimicrobial (antibacterial and antifungal) proteins and peptides are a particular class of natural products with interesting activities against plant pathogens; in fact, it has been shown that they can limit pathogen attacks (Vila et al., 2001; Gonzales et al., 2002; Moreno et al., 2003). These molecules have been found in numerous kinds of organisms such as bacteria, fungi, insects, amphibians, mammals and plants (Moreno et al., 2003).

In particular, dipeptides called diketopiperazines (DKPs) were found to have a wide range of biological functions, i.e. cell

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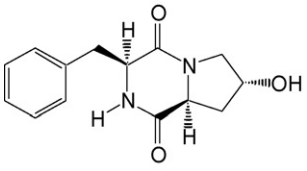
cycle inhibitors, used in medicine as antibiotics, synthetic vaccines and in cancer chemotherapy (Cui et al., 1995); DKPs are also important in agriculture, as herbicides (Brodie and Blakeman, 1975) and germination promoters of rice seeds under low-temperature stress, as well as resistance inducers in rice seedlings against water stress (Horton et al., 2000).

Many of them have an antifungal activity, for example: *Gliocladium* sp. produces a DKP that kills *Pythium* by coagulation of proteins in the cytoplasm (Butt et al., 2001); DKPs extracted from marine fungi are strongly active against *Pyricularia oryzae* (Byun et al., 2003).

DKPs appear to be important, ubiquitous that play fundamental roles, especially as far as organism–organism interactions are concerned; in our case, DKPs could be a promising means of controlling the pathogen.

Recently we reported that both the endophytic fungus *Alternaria alternata* (Fr.) Keissl and three associated DKPs, used in mixed solutions at two different concentrations, inhibited *P. viticola* sporulation in grapevine leaves, whether kept in moisture chambers or in plants maintained in the greenhouse (Musetti et al., 2006). However the sporulation-inhibiting mechanism of these molecules in *P. viticola* infected grapevine leaves is unknown.

As a conseguence, the aims of the present study were: (1) to assess the effectiveness of the DKPs, alone or mixed, at different concentrations, against *P. viticola* in leaves of grapevines maintained in the greenhouse; (2) to study the interactions between these metabolites and *P. viticola* in grapevine leaf tissues, in particular the DKPs-induced ultrastructural modifications.



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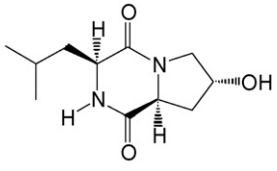

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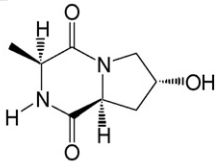

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2. Materials and methods

2.1. DKP preparation

Flasks containing 1000 ml of malt extract broth (MEB) (formula per liter: malt extract 6.0 g; maltose technical 1.8 g; dextrose 6.0 g; yeast extract 1.2 g; distilled water) were autoclaved for 21 min at 120 °C, stored at room temperature and then 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 22 °C for 7 days, and then dehydrated 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 (PLC) on a silica plate (Merck-Kieselgel 60 PF_{254}) 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 (CH_2Cl_2/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 (CH_2Cl_2/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 spectroscopy using a Bruker AV400 (^{1}H and 2D-NMR spectra at 400 MHz) instrument and the residual solvent signals as internal standard (δ in ppm, CHD_2OD = 3.31, $CHCl_3$ = 7.26 and D_2O = 4.90). Mass spectrometry data were obtained on a Kratos MS80 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 (HREIMS) measurements.

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) $C_8H_{12}N_2O_3$, MW 184; **2** = *cyclo*(L-leucine-*trans*-4-hydroxy-L-proline) $C_{11}H_{18}N_2O_3$, MW 226; **3** = *cyclo*(L-alanine-*trans*-4-hydroxy-L-proline) $C_{14}H_{16}N_2O_3$, MW 260.

Aqueous solutions of the three DKPs were prepared at the following concentrations: 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} M. The solutions were also mixed at a ratio of 1:1 and 1:1:1 to obtain the same final concentrations.

2.2. Post-infection effectiveness of the DKPs against *P. viticola* in leaves of grapevine plants grown in the greenhouse

Healthy 2-year-old grapevine plants cv. Pinot grigio, grown in a greenhouse at 21 °C with a 12 h photoperiod, were used. Mature leaves of three grapevines were inoculated with an aqueous suspension of *P. viticola* sporangia at a concentration of 4.25×10^5. The inoculum was applied with a vaporizer. A 2 or 24 h after inoculation, six drops (10 μl) of each different DKP (used alone or in mixtures at the above mentioned combinations and concentrations) were placed on three

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replicates of previously inoculated leaves. Controls consisted of drops of distilled water placed on inoculated leaves; drops of an aqueous solution of $Cu(OH)_2$, at a concentration of 2 g/l, were placed on inoculated leaves as the chemical control.

All the test plants were maintained in a greenhouse at 20 °C, 100% RH, for 7 days. We estimated the induced inhibition of *P. viticola* sporulation by observing, under a stereomicroscope, the downy mildew infected surfaces of the treated leaf areas.

Disease severity was expressed as the percentages of downy mildew infected leaf area and was ''arcsin'' transformed (for homogeneity of variance) before analysis of variance (ANOVA), using SPSS (SPSS, Inc., Chicago, IL). The Duncan test was used to determine significance of differences between the various treatments (Falk et al., 1996).

2.3. Transmission electron microscopy

TEM analyses were performed on leaf tissues inoculated with a *P. viticola* sporangial suspension at a concentration of 4.25×10^5 and treated after 2 h with the solution of the three DKPs, at a final concentration of 10^{-3} M. Control samples were also collected from inoculated untreated or Cu-treated leaf areas.

Small samples (1 mm × 3 mm) 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. (2003). Ultrathin sections were stained with uranyl acetate and lead citrate and observed under a PHILIPS CM 10 transmission electron microscope (TEM) (Philips Scientifics, Eindhoven, The Netherlands), operated at 80 kV.

3. Results

3.1. Post-infection effectiveness of the DKPs against P. viticola in leaves of grapevine plants grown in the greenhouse

After the 7-day-incubation period of the disease, the DKPs, used both alone and in mixtures, demonstrated real effectiveness in inhibiting *P. viticola* sporulation. In fact, within the areas of inoculated leaves that were treated with DKPs 2 h later, pathogen sporangia were, in general, not observed (0% disease severity) (Table 1), with the exception of leaf areas treated with 10^{-6} *cyclo*(L-alanine-*trans*-4-hydroxy-L-proline) (indicated by A) and 10^{-4} *cyclo*(L-phenylalanine-*trans*-4-hydroxy-L-proline) (indicated by P) where, disease severity was, respectively, 6 and 3% (Table 1). In the inoculated leaf areas, treated with the same DKP solutions but 24 h after the fungus inoculation, none or very low values of disease severity were observed (Table 2): leaf areas treated with the metabolites at lower concentrations showed from 1 to 3% disease severity.

In contrast, high disease severity (100% downy mildew infected surface) was observed in the control-water treated areas; in $Cu(OH)_2$ treated zones, few *P. viticola* sporangia were observed (disease severity = 0.33%) compared to the control.

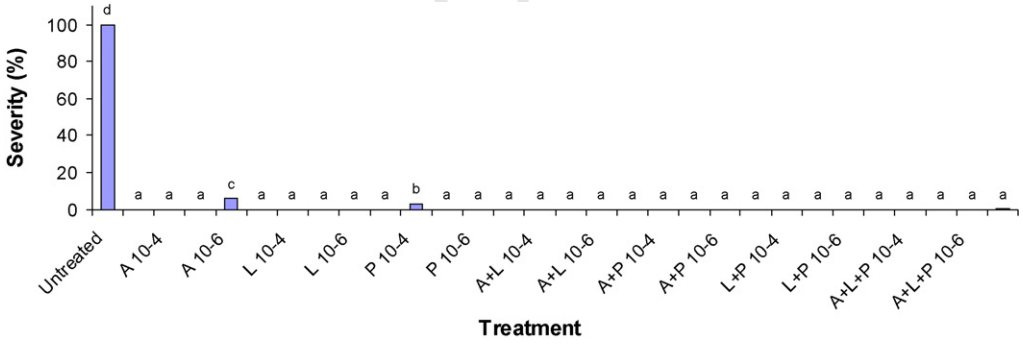
3.2. Transmission electron microscopy

TEM observations performed on inoculated-untreated grapevine leaf tissues demonstrated that *P. viticola* hyphae were well developed and localized in the substomatal zone and in the intercellular spaces of spongy parenchyma; generally they appeared vacuolated (Fig. 1, Hy). Well-structured haustoria were also observed (Fig. 1, ha). Host tissue did not show serious cellular damage.

In leaf tissues inoculated with *P. viticola* and treated after 2 h with the solution containing the three DKPs at a concentration of 10^{-3} M, mycelium developed inside the host in the intercellular spaces of spongy parenchyma. It was still recognizable even if several ultrastructural modifications were evident (Figs. 2–4). In fact, the fungal wall appeared distorted (Fig. 2, arrows), cytoplasm was condensed (Figs. 2 and 3, arrows) and vacuoles contained electron-dense precipitates (Fig. 4, arrows).

A number of haustoria appeared necrotic and irregular in shape (Figs. 5–7, ha); they were often enclosed in plant material consisting of a callose-like substance (Figs. 5–7c) and electron-opaque extrahaustorial matrix (Fig. 7, arrows).

Table 1
Activity of diketopiperazines, applied at different combinations and concentrations, against downy mildew 2 h after *P. viticola* inoculation on grapevine leaves



Severity: % of downy mildew infected surface in leaf treated areas, A = *cyclo*(L-alanine-*trans*-4-hydroxy-L-proline), L = *cyclo*(L-leucine-*trans*-4-hydroxy-L-proline) and P = *cyclo*(L-phenylalanine-*trans*-4-hydroxy-L-proline). Columns are mean value and those followed by the same letter are not significantly different according to Duncan's test at $P \leq 0.05$.

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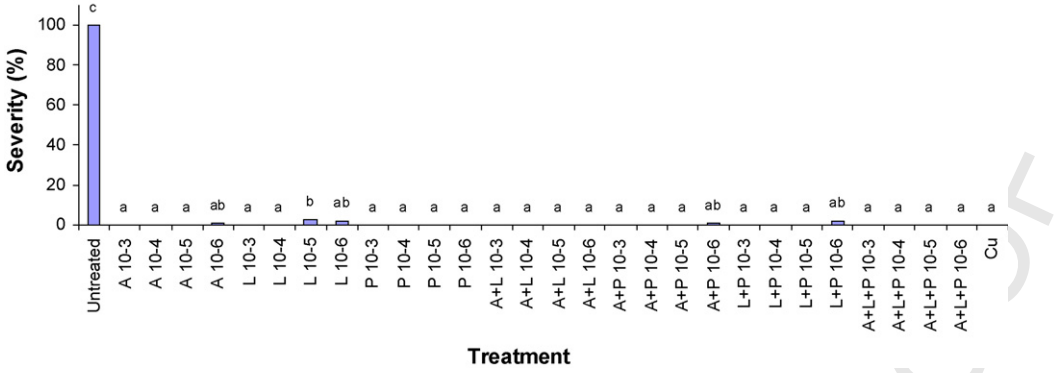
Table 2
Activity of diketopiperazines, applied at different combinations and concentrations, against downy mildew 24 h after *P. viticola* inoculation on grapevine leaves



Severity: % of downy mildew infected surface in leaf treated areas, A = *cyclo*(L-alanine-*trans*-4-hydroxy-L-proline), L = *cyclo*(L-leucine-*trans*-4-hydroxy-L-proline) and P = *cyclo*(L-phenylalanine-*trans*-4-hydroxy-L-proline). Columns are mean value and those followed by the same letter are not significantly different according to Duncan's test at $P \leq 0.05$.

In contrast, treatment with the solution containing the three DKPs at a concentration of 10^{-3} M did not cause ultrastructural modifications in grapevine leaf tissues, cell organelles, and nuclei appeared well preserved (Fig. 8). Cell walls were regular and without distortions, vacuoles contained phenolic accumulations (Fig. 9, Phe). TEM observations of leaf tissues treated with aqueous solution of $Cu(OH)_2$ showed alterations of the cell walls (distortions and thinning) and the plasmalemma (plasmolysis) (Fig. 10, arrows).

4. Discussion

It is known that antimicrobial peptides are key components of the innate immune response in most multicellular organisms (Bulet et al., 1999). The last two decades have witnessed the discovery of an impressive number of antimicrobial structures in both the animal and plant kingdoms. These molecules are considered to be one of the most innovative classes of anti-infective agents, and therefore, possibly a source of novel drug design. Among these, are small cationic amphipathic proteins (comprising 15–50 amino acids), as well as other kinds of peptides, such as DKPs (Hoffaman, 1995).

DKPs are a class of ubiquitous compounds conserved among different kingdoms: in fact they were isolated from marine bacteria (De Rosa et al., 2003), sponges (Adamczeski et al., 1989), lichens (Halama and Van Haluwin, 2004), skin tissues (Ienaga et al., 1987), in addition to deuteromycetous, ascomycetous and basidiomycetous fungi (Trigos et al., 1995; Wang et al., 1999). DKPs were demonstrated to have pharmacological activities (Cui et al., 1995): a lot of them are capable of preventing metastasis, inhibiting tumour growth, and are used as potential antihypertensive agents, as well as antibacterial and antifungal substances (McCleland et al., 2004); moreover fungal diketopiperazines have been shown to possess not only antifungal capacities, but also antibacterial (Arnone et al., 1996) and antiviral properties (Tomassini et al., 1996).

Our experiments have demonstrated the antifungal activity, against *P. viticola*, of three DKPs, that were extracted from the grapevine endophyte *A. alternata*. This activity was generally demonstrated when DKPs, either alone or in mixture were applied, at different concentrations. In fact in leaf areas treated with most of them disease severity was 0%.

A slightly lower effectiveness was observed, in some cases, when lower DKP concentrations were applied. The effectiveness of these metabolites in combinations was not significantly superior to that obtained by the individuals; a synergistic effect could be supposed only by using the mixture of the three DKPs at lower concentrations.

Very slight differences in efficacy were found to depend on the time of DKP application (2 or 24 h after *P. viticola* inoculation).

P. viticola penetrates the host leaf tissues through stomata and, during the first hours (12–15) after penetration, the mycelium is localized in the substomatal air spaces and develops slowly, until the first haustorium is formed (Farina et al., 1976; Langcake and Lovell, 1980). Therefore, this period seems to be the most critical and suitable for the application of DKP treatments. We do not yet know the precise length of the antimicrobial activity of the tested DKPs: however, since a single treatment covers the effect of the entire *P. viticola* inoculation, we assume that the antifungal effect of the DKPs lasts for at least some days.

Under our experimental conditions, stimulation of *P. viticola* sporulation was never observed following DKP treatments of grapevine leaves; no necrotic lesions or other phytotoxicity symptoms were observed in DKP-treated grapevine leaf tissues.

The most effective currently available fungicides against downy mildew permitted by organic standards are based on copper hydroxide and copper sulfate. Copper protects grapevines against downy mildew infections when applied according to preventive control strategies. Copper fungicides do not eradicate existing infections and are not systemic; moreover they are easily washing away by rain (Agrios, 1997). DKPs

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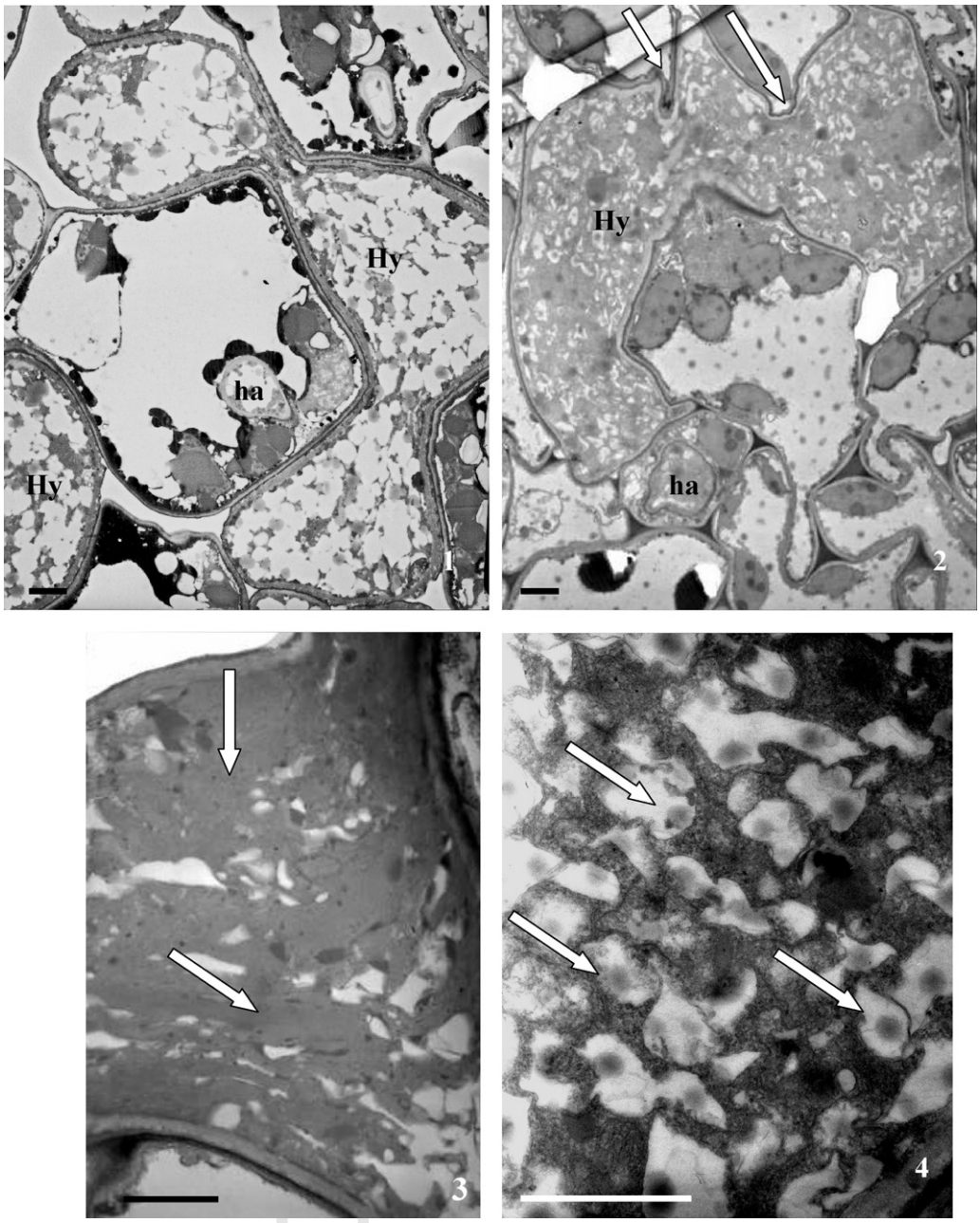

Figs. 1–4. (1) Transmission electron micrograph of *P. viticola* in grapevine leaf tissue: the hyphae (Hy) are localized in the spongy parenchyma and appear typically vacuolated. Haustorium (ha) is visible in a parenchymal cell and it appears well structured. Scale bar = 3 μm. (2) Transmission electron micrograph of *P. viticola* in grapevine leaf tissue treated after 2 h with a solution of the three DKPs, at a final concentration of 10^{-3} M. The typical vacuolisation of the mycelium is no longer present, hyphae (Hy) and haustorium show condensed cytoplasm. Scale bar = 3 μm. (3) Transmission electron micrograph of *P. viticola* in grapevine leaf tissue treated after 2 h with a solution of the three DKPs, at a final concentration of 10^{-3} M. Particular of *P. viticola* mycelium showing condensation of the cytoplasm. Scale bar = 3 μm. (4) Transmission electron micrograph of *P. viticola* in grapevine leaf tissue treated after 2 h with a solution of the three DKPs, at a final concentration of 10^{-3} M. *P. viticola* shows some enlarged vacuoles containing electron-opaque material. Scale bar = 3 μm.

were active against the pathogen even after the start of infection, when the fungus has already penetrated and is inside leaf tissues.

In fact, ultrastructural analysis of infected grapevine leaf tissues revealed that DKPs are active against *P. viticola* inside leaf tissues: *P. viticola* exhibited marked structural changes such as abnormal vacuolization, accumulation of electron-dense material in the vacuoles and necrotic, collapsed or incompletely developed haustoria. In particular, the presence of enlarged vacuoles in *P. viticola* mycelium is reported to be correlated to senescence of the fungus (Langcake and Lovell, 1980), but also with the presence of antagonists and/or toxic metabolites (Hajlaoui et al., 1992; Askary et al., 1997).

In contrast, the same metabolites did not cause leaf tissue damage, suggesting that they, as well as other antifungal peptides (Gonzales et al., 2002), may preferentially compromise fungal rather that plant cell membranes. The ultrastructural modifications were very similar to those reported for *P. viticola* mycelium

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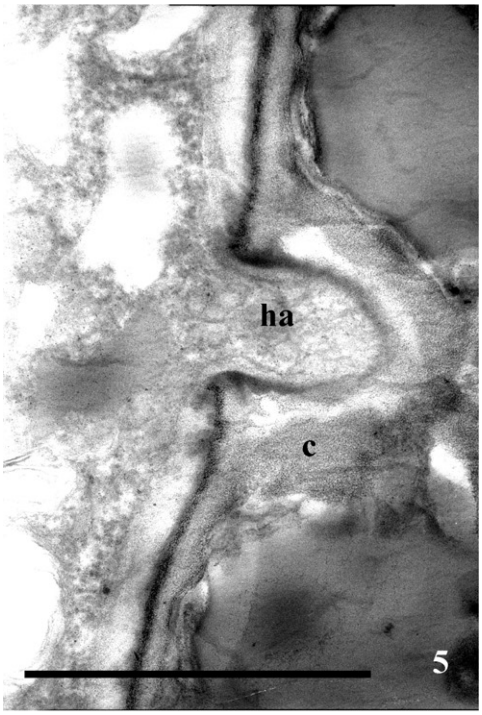

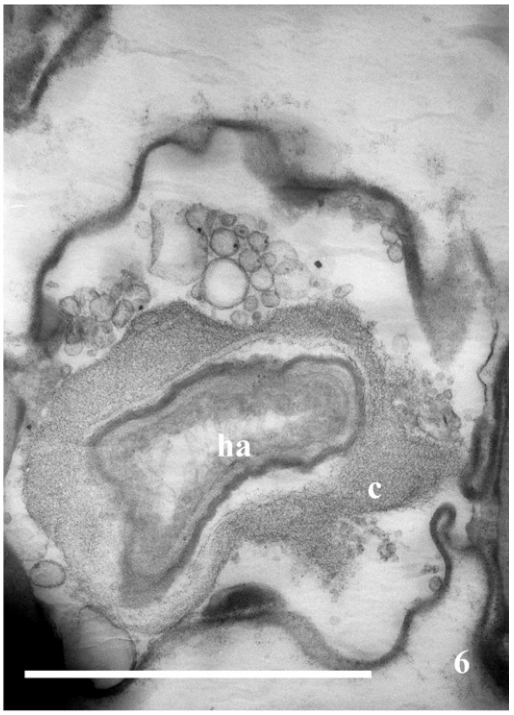

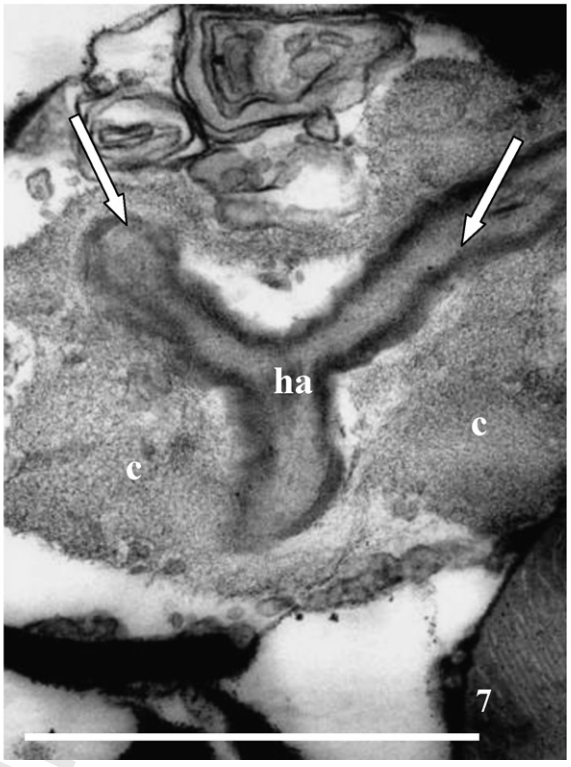

Figs. 5–7. Transmission electron micrograph of *P. viticola* in grapevine leaf tissue treated after 2 h with a solution of the three DKPs, at a final concentration of 10^{-3} M. In the parenchymal cells *P. viticola* haustoria (Ha) are not completely developed, and are surrounded by callose (5 and 6c). Scale bar = 3 μm. (7) Transmission electron micrograph of *P. viticola* in grapevine leaf tissue treated after 2 h with a solution of a three DKPs, at the final concentration of 10^{-3} M. Some haustoria appear necrotic, with an irregular shape or surrounded by callose (c) and electron-opaque extrahaustorial matrix (arrows). Scale bars = 3 μm.

directly treated with the endophyte *A. alternata* (Musetti et al., 2006), thus demonstrating the involvement of these metabolites in the relationship between the two fungi.

Taken together these data show that DKPs extracted from the grapevine endophyte *A. alternata* are effective in controlling *P. viticola* in leaves of grapevines grown in the greenhouse. Further trials will be carried out in the near future in order to test the effectiveness of these molecules also under field conditions, and to better understand the mechanism of action involved in pathogen inhibition.

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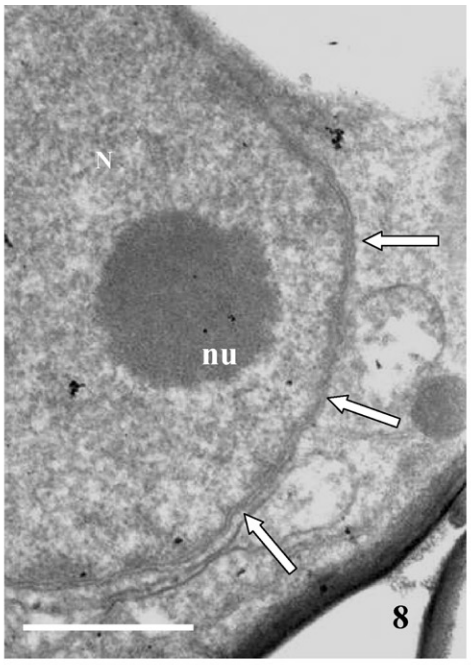

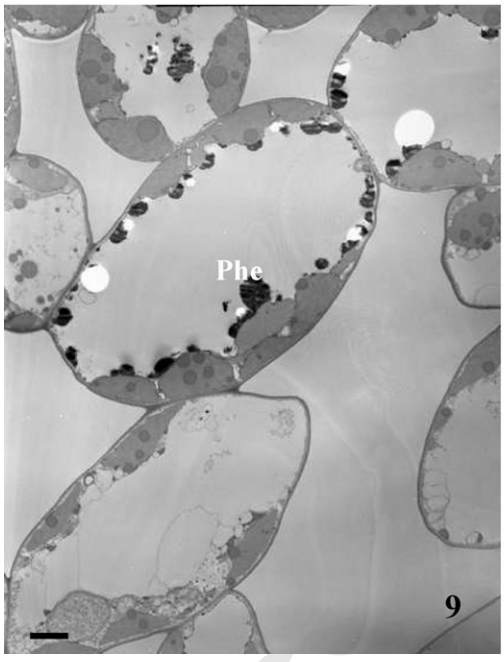

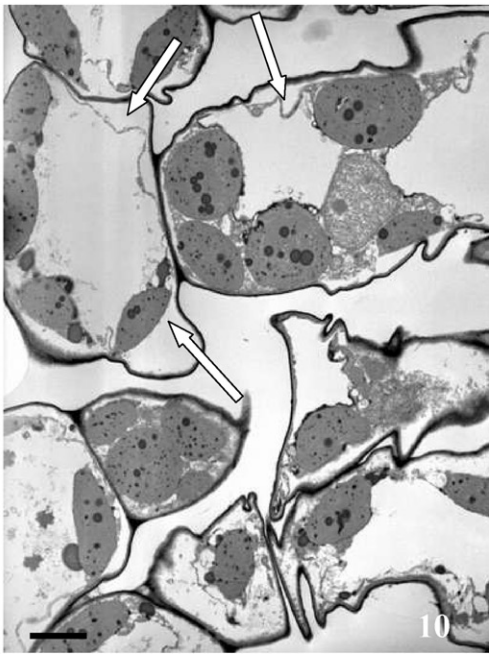

Figs. 8–10. Transmission electron micrograph of grapevine leaf tissue inoculated with *P. viticola* and treated after 2 h with a solution of the three DKPs, at a final concentration of 10^{-3} M. The DKP treatment does not cause damage in grapevine tissues, showing well-preserved nuclei, with intact nuclear membrane (arrows) (8; N; nu = nucleolus) and regular cell walls (9; Phe = phenolics). Scale bars = 3 μm. (10) Transmission electron micrograph of grapevine leaf tissue inoculated with *P. viticola* and treated with an aqueous solution of $Cu(OH)_2$. Cell walls presented distortions, thinning and detachment of plasmalemma (arrows). Scale bar = 3 μm.

If found to be effective and safe, the *A. alternata* metabolites could be a suitable product for combatting downy mildew in grapevines. In particular these products, if obtained by natural extraction, could be used to control *P. viticola* in organic viticulture procedures. Further experiments are in progress to ascertain and define the characteristic of the cited DKPs such as: activity against other grapevine fungal pathogens; way of antimicrobic action; persistence of the antisporulation effect; best formulation (dosage and mixture); possible problems concerning resistance; contingent negative environmental effects.

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Acknowledgements

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