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Antifungal activity of eugenol against *Botrytis cinerea*

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ABSTRACT

The antifungal properties of eugenol were tested against ten plant pathogenic fungal species and detailed studies were conducted regarding *in vitro* activity of eugenol on *Botrytis cinerea*. The EC_{50} value of eugenol on mycelial radial growth of *B. cinerea* was 38.6 µg/mL; however, eugenol had no bioactivity against conidia germination. *B. cinerea* hyphae treated with eugenol showed strong propidium iodide fluorescence in the cytosol. Eugenol increased the concentration of potassium ion and cellular materials in the medium. Furthermore, light and scanning electron microscopy observations on hyphae exposed to eugenol revealed considerable morphological alterations in hyphae, such as cytoplasmic coagulation, vacuolation, and hyphal shriveling. Eugenol induced the generation of H_2O_2 and increased free Ca^{2+} in the cytoplasm. These results strongly support the idea that the antifungal activity of eugenol is due to membrane binding and permeability alteration, leading to destabilization and disruption of the plasma membrane.
Keywords: natural compound; fungal diseases; mode of action; plasma membrane.

INTRODUCTION

Botrytis cinerea causes gray mold in a variety of fruits, vegetables, and field crops. The pathogen infects leaves, stems, flowers and fruits, and severe damage can occur due to gray mold epidemics (Soulie et al., 2003; Milena & Evelyn, 2005). The control of *B. cinerea* is still based upon multiple applications of fungicides during the flowering and fruiting periods. Currently, there is a worldwide trend to explore new alternatives to synthetic fungicides in order to minimize the risks associated with the development of populations insensitive to these chemical compounds (Elad, 1991; Yourman & Jeffers, 1999) and also to comply with food safety standards (Liu et al., 2007). Frequent applications of site-specific fungicides can result in the emergence of resistant strains of *B. cinerea* (Elad, 1991). Furthermore, the use of some synthetic chemicals to control fungal diseases is restricted due to their high toxicity, long degradation periods, and environmental pollution.

The use of natural compounds as plant extracts may be an alternative to fungicides to control plant pathogens (Tsair-Bor & Shang-Tzen, 2008). Eugenol (4-allyl-2-methoxyphenol) is a naturally occurring phenolic compound which is used as a food flavor and fragrance agent. It is a major component of clove oil and is also present in the essential oils or extracts of many other plants, including cinnamon, basil, and nutmeg (Ghosh et al., 2005). Eugenol has been reported to inhibit the growth of *Escherichia coli*, *Listeria monocytogenes* and *Lactobacillus sakei* (Blaszyk & Holley, 1998; Gill & Holley, 2004) and also exhibits antifungal activity against wood decay fungi (Tsair-Bor & Shang-Tzen, 2008). It has also been demonstrated that the compound induced morphological alterations in *Candida albicans* and *Saccharomyces cerevisiae* (Dalleau et al., 2007). However, there is little work on the effect of eugenol on plant pathogens and its mode of action is poorly understood.

The objective of this work was to assess the antifungal activity of eugenol against ten plant pathogenic fungi, more specifically how it affects *B. cinerea in vitro*, and to understand its mechanism of action by closely examining the interaction of this compound with fungal cell membrane. The results strongly support the idea that the antifungal activity of eugenol is due to the disruption of the membrane, leading to cell death.

MATERIALS AND METHODS

Fusarium moniliforme, Sclerotinia sclerotiorum, Cercospora beticola, Mycogone perniciosa, Phytophthora capsici, Fusarium graminearum, Macrophoma kawatsukai, Thanatephorus cucumeris, Alternaria alternata, and *B. cinerea* were originally isolated from diseased plants and isolates were maintained on potato dextrose agar (PDA) in the dark at 25°C. A 6-mm diameter agar plug containing actively growing fungus was obtained from the edge of the colony of each isolate and placed on potato dextrose agar medium (PDA) in plates containing different

concentrations of eugenol (0, 25, 50, 100, 150, and 200 µg/mL). Eugenol (99.0 %) used in this study was purchased from Sigma-Aldrich (Shanghai, Trading Co. Ltd. China) and it was prepared as a stock solution at 40 mg/mL in 70 % ethanol, and stored in the dark at 4°C. Plates in three replicates were used for each treatment, and all plates were placed in an incubation chamber at 25°C for 3 to 10 days. When mycelial growth on the control plate reached more than 2/3 of the total diameter of the plate, mycelial radial growth was measured and activity was expressed as EC_{50} (the concentration inhibiting growth by 50%). The EC_{50} value was calculated according to the relationship of eugenol concentrations and inhibition rate of mycelial growth (Taylor et al., 2002). First, the inhibition rate was transformed to probability value (Y), concentrations of the compound were transformed to logarithm (X), then linear regression equation (Y=a+bx) was fit and the coefficient (r) was estimated. The logarithm value of X was calculated according to the regression when Y=5. This logarithm value of X was the EC_{50} value.

To examine the effect of eugenol on mycelial growth, 50 mL of potato dextrose broth (PDB) was inoculated with three 6 mm-diameter mycelial agar discs from 6 to 7 day-old cultures of *B. cinerea.* Eugenol was added to PDB from stock solution to reach final concentrations of 0, 25, 50, 100, 150 and 200 µg/mL. The flasks were kept at 25°C under gentle shaking and after 96 h mycelial growth was checked by measuring the dry weight according to the method of Vicedo et al. (2006).

The effect of pH on eugenol efficacy (100µg/mL) was tested against *B. cinerea* under the same conditions described above except for culture medium pH values from 5 to 8 (with one pH unit interval) that were adjusted with 1 M NaOH or HCl. All experiments were repeated three times. All the treatments were applied in three experimental units. Means and standard errors were calculated for all treatments. *B. cinerea* was grown on PDA and conidia suspensions were prepared from the sporulating edges of 2-week-old cultures. Conidia were gently removed with a bacteriological loop suspended in sterile distilled water containing 0.01% Tween-20, and filtered through four layers of sterile cheesecloth to remove remaining mycelia. The concentration was determined with a hemocytometer and adjusted to 10^6 conidia mL^{-1} (Richard et al., 2002).

Spore germination assay: 50 µL of conidia suspension were transferred to a concave slide and a drop of 50 µL of the stock solution of eugenol was added to yield a final concentration of 4×10^4µg eugenol/mL. Total volumes were increased to 100 µL per slide using sterile distilled water. After 18 h, the percentage of germinated conidia was determined from at least 100 conidia per well in four replicate wells by microscopic examination. The effect of eugenol was compared to pyrimethanil (5µg/mL), a standard fungicide for controlling *B. cinerea* (Vicedo et al., 2006).

Conidia and mycelia plasma membrane integrity assay

Conidia and mycelia from *B.cinerea* were incubated in the presence of eugenol at the concentration of 100 µg/mL. After 15, 30, 60, 120, and 240 min of incubation, conidia and mycelia were harvested by centrifugation at 10,000 ×*g* for 15min, washed in PB buffer (50 mM, pH 7.0), and stained with 10 µg/mL propidium iodide for 15 min. Conidia and mycelia were observed under a fluorescence microscope (Olympus BX-60) (Liu et al., 2007).

Release of cellular material

Three 6 mm-diameter mycelial plugs of *B.cinerea* were taken from the edge of 3-day-old colonies and placed in flasks with 50 mL of PDB. The flasks were incubated at 25°C with gentle shaking (140 rpm), and mycelia was harvested by centrifugation at 10,000 ×*g* for 10 min, washed three times and suspended in 20 mL PBS buffer (0.05 mol/L, pH 7.0). After 1 h incubation with different eugenol concentrations, samples were centrifuged at 10,000 ×*g* at 4°C for 10 min. The UV (260nm) absorbing materials in each supernatant were measured (Christopher & Isao, 2000).

Determination of extracellular K^+ contents of the hypha: Mycelia were incubated for 2 days in 50mL PDB, at 25°C with gentle shaking (140 rpm) and washed three times with Hepes buffer (2.5 mM, pH 7.0) and then suspended in 60 mL of the same buffer. The hyphae were then incubated with 100µg/mL eugenol under room temperature, for predetermined times. Samples (10 mL) were taken at 0, 3, 5, 10 min after treatment and immediately chilled on ice. The samples were centrifuged at 10,000 ×*g* at 4°C for 10 min, and the supernatant was removed and stored for the determination of extracellular K^+. The K^+ concentration in the samples was determined by flame photometry (A Analyst 300 Atomic Absorption Spectrometer) (Olivia et al., 1998).

A mycelial agar disc from a three-day-old culture was placed in the center of plates containing PDA before adding 100 µg/mL eugenol and incubated at 25°C for 3 days under dark. Thin layers (1mm) of agar blocks (≈ 2 to 3 cm^2) containing mycelia were cut off from the growing edges of the colonies for examination by light microscopy. The blocks were placed in water on a microscope slide and covered with coverslip. The microscope slides were examined with a light microscope (Nikon YS100, Japan) to observe recognizable cytological changes that were photographed with a digital camera (Nikon COOLPIX 4500, Japan). The mycelia incubated in the absence of eugenol were used as control (Cristesu et al., 2002)

For scanning electron microscopy (SEM) analysis, mycelial discs (6 mm in diameter) were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 12 h at 4°C. The samples were washed twice, each time for 10 min, in the same buffer, and then dehydrated in a graded ethanol series (30, 50, 70, 80, 90, and three times at 100%) for 15 min in each series. The samples were critical point

dried in a drying apparatus (EMITECH TS-7 K850, UK). The fixed material was mounted on aluminum stubs using double-sided carbon tape and coated with gold in a sputter coater system (EMITECH K575X, UK) for 420 s at 10 mA. Finally, the samples were examined with the scanning electron microscope at an accelerating voltage of 10 kV as described (Agizzio et al., 2006).

Detection of H_2O_2 in hyphae

The generation of H_2O_2 after addition of eugenol was measured by using a previously described method with few modifications (Rosa et al., 2004). The pathogen was treated with eugenol and incubated as described for the measurements of cellular material release. Mycelia (0.5 g) were ground into a fine powder with a mortar and pestle using liquid nitrogen and then resuspended with 4 mL of PBS buffer (0.05 mol/L, pH 7.0). The suspension was centrifuged (10,000 ×g, 4ºC, 10 min) and H_2O_2 concentration was measured in the supernatant. An aliquot of the supernatant (100 µL) was added to 100 µL of assay reagent (500 µM ferrous ammonium sulfate, 50 mM H_2SO_4, 200 µM xylenol orange, and 200 mM sorbitol). After 30 min of incubation under room temperature, the peroxide-mediated oxidation of Fe^{2+} to Fe^{3+} was determined by measuring the A_{570} of the Fe^{3+}-xylenol orange complex.

Cytosolic Ca^{2+} measurements

In order to determine if the different biological effects elicited by eugenol in mycelia are related to changes in internal Ca^{2+} concentration, the concentrations of free cytosolic Ca^{2+} at different incubation times were measured. Cytosolic Ca^{2+} measurement in mycelia was carried out by using the fluorescent Ca^{2+} indicator Fluo3-AM (Giudice et al., 2006). The final concentration of Fluo3-AM was adjusted to 5 µM and prepared from a 5 mM Me_2SO stock solution (DMSO). The buffer used was 10 mM Hepes, pH 7.4 and samples were observed with a Confocal Laser Scanning Microscope (LEICA TCS SP2), and images were processed using Leica Confocal Software Lite Version.

Statistical analysis

All experiments were performed two times with three replicates of each treatment. Data were subjected to analysis of variance (ANOVA) and Duncan's multiple range test was used for multiple comparisons of treatment means.

RESULTS

Effect of eugenol on mycelial growth

The EC_{50} values of eugenol on mycelial radial growth were examined to determine its fungicidal spectrum (Table 1). The mycelial growths of *B. cinerea* and *S. sclerotiorum* were the most sensitive to eugenol and the EC_{50} values were 38.6 and 39.9 µg/mL, respectively. The EC_{50} values for *F. graminearum* and *P. capsici* were above 100 µg/mL, therefore these species were not highly sensitive to eugenol. The other species were moderately sensitive to eugenol with EC_{50} values ranging from 46.7 to 96.9 µg/mL. Mycelial growth of *B. cinerea* was inhibited by eugenol in a concentration-dependent manner (Figure 1).

The medium pH did not change the effects of eugenol on mycelial growth (Figure 2). Mycelial growth of *B. cinerea* in the absence of eugenol was not significantly affected by pH values (data not shown). The germination rate of conidia in untreated control was 98.6%. Conidia germination was not affected by eugenol at the concentrations tested. Pyrimethanil was used as a positive control and there was no germination of *B. cinerea* conidia in this treatment.

Assay of plasma membrane integrity of spores and mycelia

The ability of eugenol to permeabilize conidia plasma membrane and mycelia of *B. cinerea* was examined. When observed with a fluorescence microscope, mycelia of *B. cinerea* showed strong PI fluorescence in the presence of

TABLE 1 - Antifungal activity of eugenol against plant pathogenic fungi

Fungal species [a]	Regression equation	EC_{50} value/(µg/mL)	r [b]
Fusarium moniliforme	Y=2.2199x+0.6079	95.17	0.978
Sclerotinia sclerotiorum	Y=1.9056x+1.9482	39.94	0.999
Cercospora beticola	Y=2.1272x+1.2231	59.63	0.989
Mycogone perniciosa	Y=2.2561x+1.2338	46.70	0.987
Phytophthora capsici	Y=4.1630x-4.3882	179.95	0.963
Fusarium graminearum	Y=2.5205x-0.0612	101.87	0.983
Macrophoma kuwatsukai	Y=2.6068x+0.2729	65.07	0.970
Thanatephorus cucumeris	Y=2.4437x+0.5390	66.91	0.974
Alternaria alternata	Y=4.3230x-3.5878	96.94	0.975
Botrytis cinerea	Y=0.8110X+3.7131	38.62	0.987

[a]All tests were conducted on PDA medium in the dark at 25ºC.
[b]Correlation coefficient between concentration and inhibition.

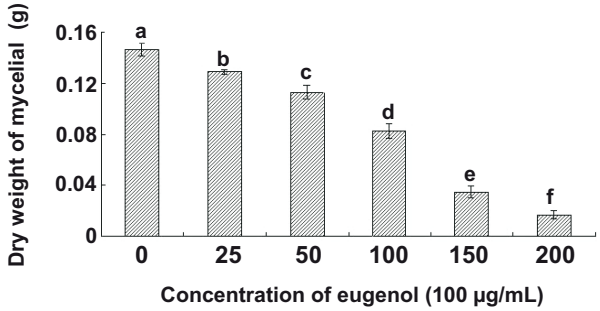

FIGURE 1 - Effect of different eugenol rates on mycelial dry weight of *B. cinerea.* Vertical lines on the graph denote the SE. Vertical bars with different letters are significantly different according to two-tailed unpaired student's t-test (P=0.05).

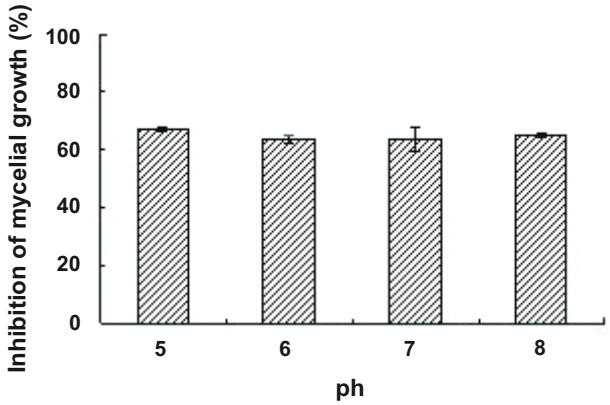

FIGURE 2 - Effect of pH on antifungal activity of eugenol against *Botrystis cinerea*. Measurement of mycelial growth on pH-adjusted PDA amended with 100µg/mL eugenol was performed five days after inoculation with *B. cinerea*.

eugenol (100µg/mL) as compared to the control treatment (Figure 3). Conidia of *B. cinerea* showed no PI fluorescence in the presence of eugenol (data not shown).

Release of cellular material (OD_{260} and K^+)

There was massive leakage of K^+ when mycelia of *B. cinerea* were exposed to eugenol (Figure 4). The level of K^+ reached a maximum after 3 min of treatment with 100 µg/mL eugenol, and remained constant over time.

Effect of eugenol on hyphal morphology

Important morphological damage was detected in the hyphae exposed to eugenol compared to the hyphae in the controls. Hyphae of *B. cinerea* grown in the absence of eugenol showed typical features of the genus. After exposure to eugenol, hyphae appeared degraded and large vesicles were visible (Figure 5). The SEM micrographs showed important morphological damage due to eugenol (Figure 6). Shriveled hyphae were commonly observed in eugenol treated mycelia compared with the normal mycelia.

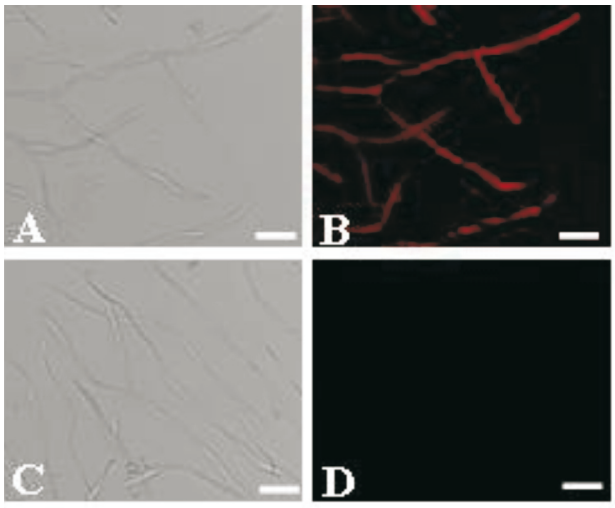

FIGURE 3 - Fluorescence microscopy of *Botrytis cinerea* treated with PI (40× magnification) from the treatments with eugenol (100µg/mL) and **A.** observed with differential interference contrast (DIC); **B.** treated with eugenol (100µg/mL) and observed with fluorescence; **C.** mycelia without eugenol addition and observed with DIC; **D.** and mycelia without eugenol addition and observed with fluorescence (D). Bar=50µm.

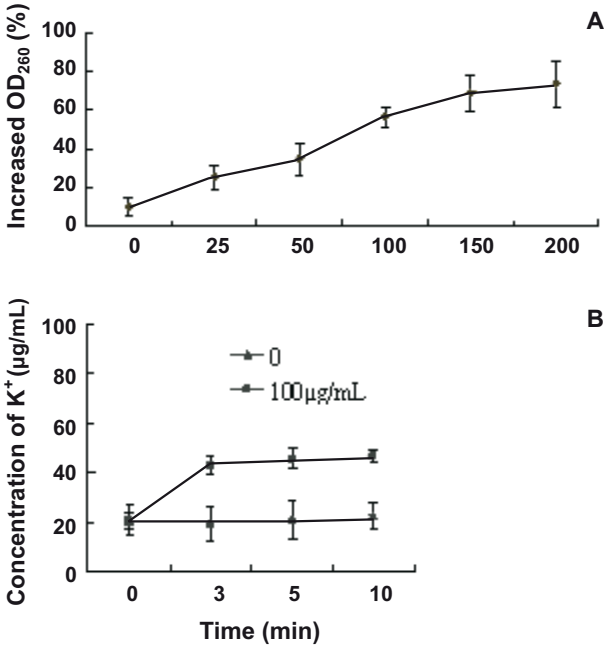

FIGURE 4 - Effect of eugenol on the release of cellular materials from *Botrytis cinerea*. **A.** Release of UV-absorbing materials; **B.** The hyphae of *Botrytis cinerea* were treated with different concentrations of eugenol and the cellular materials were detected. Potassium efflux assay. The hyphae of *Botrytis cinerea* were treated with eugenol (100µg/mL) for the predetermined times, and the relative amounts of K^+ released from the cells were measured. Each of the symbols represents a mean value from three replicate experiments. The error bars show the standard deviations.

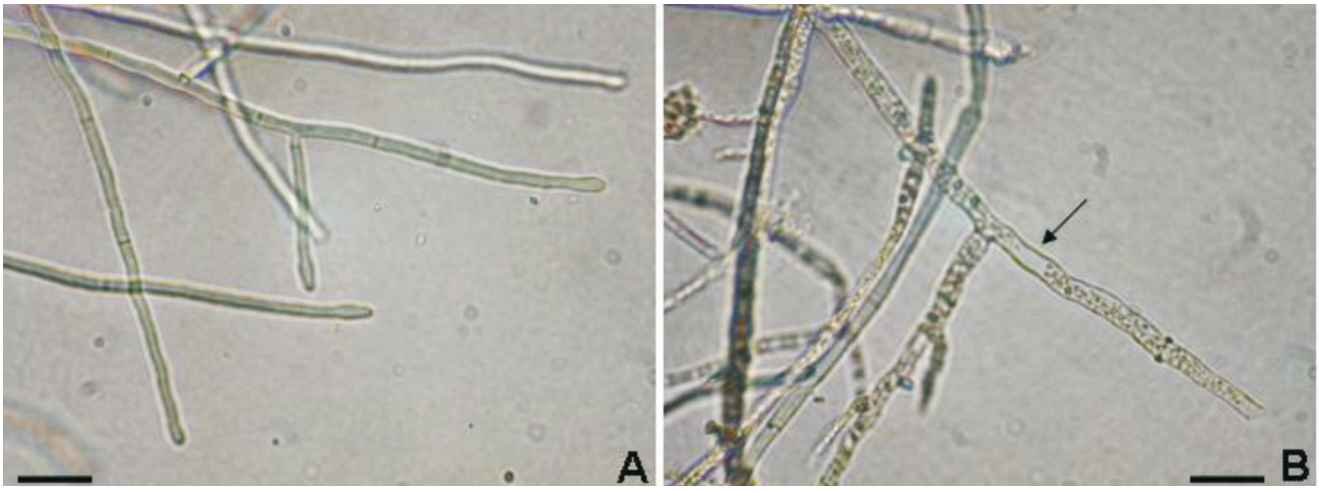

FIGURE 5 - Effect of eugenol on hyphae morphology of *Botrytis cinerea* (40× magnification). Hyphae growing on **A.** regular culture medium or **B.** culture medium supplemented with 100 µg/mL of eugenol. Cytoplasmic coagulation and vesiculation on hyphae are indicated by an arrow. Bar = 50µm.

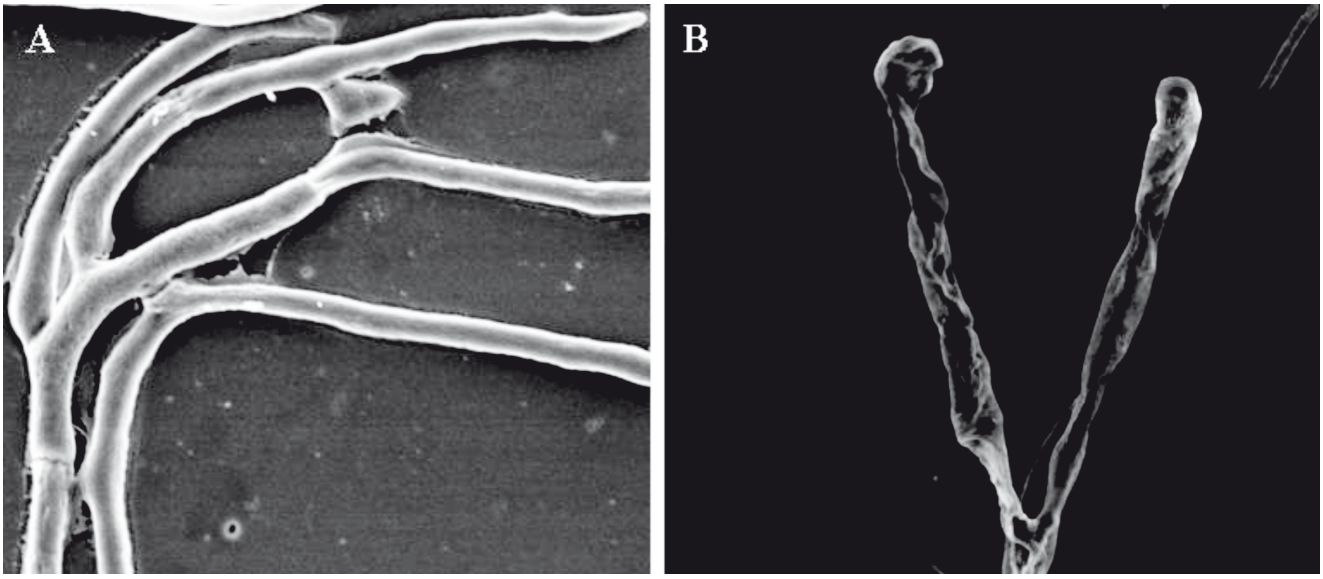

FIGURE 6 - Scanning electronic microscopy of *Botrytis cinerea* in the presence of eugenol. **A.** control without eugenol; **B.** eugenol (100µg/mL). Arrows indicate hyphae malformation. Bar = 10µm.

Detection of H_2O_2 in hyphae

The oxidative burst belongs to the fastest active defense responses. It is defined as a rapid and transient production of large amounts of reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2). The accumulation of H_2O_2 was induced by eugenol and the level of H_2O_2 increased up to 1,000% after 4h of treatment (Figure 7).

Cytosolic Ca^{2+} measurements

The concentration of Ca^{2+} increased at the beginning of the treatment with eugenol and reached the highest level after 30 min, suggesting that either directly or indirectly cytosolic Ca^{2+} is related to the biological effects elicited by eugenol. However, it was reduced from 30 to 120 min of treatment (Figure 8).

DISCUSSION

Eugenol has antimicrobial activity against a variety of food-borne (Rhayour et al., 2003), wood decay fungi, and human pathogens (Vázquez et al., 2001; Gayoso et al., 2005; Ghosh et al., 2005). However, little attention has been paid to its antifungal activity against plant pathogens. It is

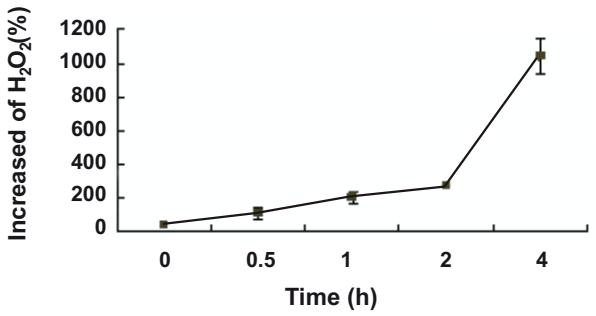

FIGURE. 7 Time-dependent accumulation of H_2O_2 levels in the culture medium after eugenol (100µg/mL) treatment. Values are average of three different experiments.

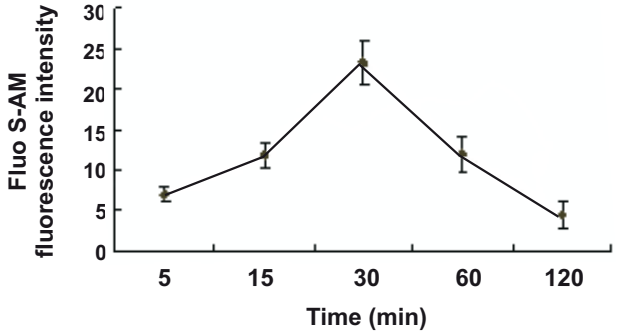

FIGURE 8 - Changes in intracellular free Ca^{2+} in *Botrytis cinerea* hyphae incubated with 100 µg/mL eugenol at different times. Free Ca^{2+} was measured as Fluo3-AM fluorescence intensity.

interesting to point out that eugenol only inhibited the growth of *B. cinerea* mycelia. Conidia germination is the growth stage most sensitive to inhibition by many compounds, but eugenol was not effective in preventing germination of conidia of *B. cinerea*. This suggests that eugenol interferes with processes taking place after the stages of germination. Many fungicides such as carbendazim and N-phenylarbmates have little or no effect on spore germination but strongly inhibit mycelial growth (Sherald et al., 1973; Suzuki et al., 1984). Nevertheless, the reason why eugenol was not effective in reducing conidia germination of *B. cinerea* needs to be further investigated.

Recent investigations about antimicrobial action of eugenol showed disruption of fungal and bacterial membranes (Gill & Holley, 2006). All these reports suggest that this antimicrobial mechanism is due to membrane damage. Eugenol, known to be a lipophilic compound, can enter between the fatty acid chains that make up the membrane lipid bilayers, thus altering the fluidity and permeability of cell membranes (Braga et al., 2007). These findings were supported by the intensive staining of eugenol-treated hyphae of *B. cinerea*, and release of OD_{260nm} absorbing material, although no substantial changes took place in mycelial morphology. *B. cinerea* mycelia treated with eugenol resulted in the leakage of ultraviolet-absorbing materials compared with the controls and the release was concentration dependent. Changes in the membrane permeability occurred simultaneously with cell death, in contrast to ultraviolet-absorbing materials that are released after cell death. However, no staining of conidia was observed. From this experiment, we concluded that Eugenol had no effect on membrane permeability of *B. cinerea* conidia. This might explain the specific composition of the cell wall of conidia.

Few studies of the effects of eugenol on the morphology and ultrastructure of yeast and bacteria have been carried out (Rhayour et al., 2003). In this study, the hyphae of *B. cinerea* grown on PDA with eugenol showed morphological changes, including cytoplasmic coagulation and vesiculation. Shriveled hyphae were commonly observed in eugenol-treated mycelia, compared with the normal mycelia.

Although the order of events leading to cell death provoked by eugenol is not exactly known, membrane permeabilization should be an early effect. Indeed there is a relationship among hyphae viability, H_2O_2 production, eugenol concentration and treated times as shown in the present study, which would indicate that H_2O_2 production and subsequent cell death may be a consequence of membrane changes. It was also demonstrated that eugenol induced an increase in internal Ca^{2+} concentration, and this Ca^{2+} was probably liberated from internal stores (vacuoles, endoplasmic reticulum, etc.). It has been previously shown that the increase in free cytosolic Ca^{2+} concentration induced by the presence of eugenol is related to H_2O_2 production (Marcela et al., 2006). Perhaps eugenol induces either hyperpolarization of the inner mitochondrial membrane or mitochondrial swelling or both. Usually, cells in which mitochondria are destabilized and finally broken down suffer a decrease in the coupling efficiency of the electron-transport chain and therefore can generate ROS intermediates which can lead to oxidative stress (Martindale & Holbrook, 2002). Finally, the present study suggests that eugenol could directly inhibit the growth of *B. cinerea in vitro*. Eugenol can be used in the control of *B. cinerea* and other phytopathogenic fungi and can also be considered as a potential alternative for synthetic fungicides. A further investigation is under progress in our laboratory to find the exact action site of eugenol.

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REFERENCES

Agizzio AP, Cunha MD, Carvalho AO, Oliveira MA, Ribeiro SFF, Gomes VM (2006) The antifungal properties of a 2S albumin-homologous protein from passion fruit seeds involve plasma membrane permeabilization and ultrastructural alterations in yeast cells. Plant Science 171:515-522.

Blaszyk M, Holley RA (1998) Interaction of monolaurin, eugenol and sodium citrate on growth of common meat spoilage and pathogenic organisms. International Journal of Food Microbiology 39:175-183.

Braga PC, Sasso MD, Culici M, Alfieri M (2007) Eugenol and thymol, alone or in combination, induce morphological alterations in the envelope of *Candida albicans*. Fitoterapia 78:396-400.

Buchenauer H (1987) Mechanism of action of triazolyl fungicides and related compounds. In: Lyr H (Ed.) Modern selective fungicides: properties, applications, mechanisms of action. Harlow. Longman Scientific and Technical. pp. 205-231.

Lunde CS, Kubo I (2000) Effect of polygodial on the mitochondrial ATPase of *Saccharomyces cerevisiae*. Antimicrobial Agents and Chemotherapy 44:1943-1953.

Cristescu SM, De Martinis D, Te Lintel Hekkert S, Parker DH, Harren FJ (2002) Ethylene production by *Botrytis cinerea in vitro* and in tomatoes. Applied and Environmental Microbiology 68:5342-5350.

Dalleau S, Cateau E, Berges T, Berjeaud J, Iimbert C (2007) *In vitro* activity of essential oils and their major components against *Candida albicans* yeasts growing planktonically and as biofilms. International Journal of Antimicrobial Agents 29:147.

Elad Y, Yunis, H, Katan, T (1992) Multiple resistance to benzimidazoles, dicarboximides and diethofencarb in field isolates of *Botrytis cinerea* in Israel. Plant Pathology 41:41-46.

Gayoso CW, Lima EO, Oliveira VT, Pereira FO, Souza EL, Lima IO, Navarro DF (2005) Sensitivity of fungi isolated from onychomycosis to *Eugenia cariophyllata* essential oil and eugenol. Fitoterapia 76:247-249.

Ghosh R, Nadiminty N, Fitzpatrick JE, Alworth WL, Slaga TJ, Kumar AP (2005) Eugenol causes melanoma growth suppression through inhibition of E2F1 transcriptional activity. The Journal of Biological Chemistry 280:5812-5819.

Gill AO, Holley RA (2004) Mechanisms of bactericidal action of cinnamaldehyde against *Listeria monocytogenes* and of eugenol against *L. monocytogenes* and *Lactobacillus sakei*. Applied and Environmental Microbiology 70:5750-5755.

Gill AO, Holley RA (2006) Inhibition of membrane bound ATPase of *Escherichia coli* and *Listeria monocytogenes* by plant oil aromatics. International Journal of Food Microbiology 111:170-174.

Liu J, Tian SP, Meng XH, Xu Y (2007) Effects of chitosan on control of postharvest diseases and physiological responses of tomato fruit. Postharvest Biology and Technology 44:300-306.

Giudici M, Poveda JA, Molina ML, De La Canal L, González-Ros JM, Pfüller K, Pfüller U, Villalaín J (2006) Antifungal effects and mechanism of action of viscotoxin A_3. FEBS Journal 273:72-83.

Martindale JL, Holbrook NJ (2002) Cellular response to oxidative stress: signaling for suicide and survival. Journal of Cellular Physiology 192:1-15.

Milena C, Evelyn S (2005) Differences in the initial events of infection of *Botrytis cinerea* strains isolated from tomato and grape. Mycologia 97:485-492.

McAuliffe O, Ryan MP, Ross RP, Hill C, Breeuwer P, Abee T (1998) Lacticin 3147, a broad-spectrum bacteriopcin which selectively dissipates the membrane potential. Applied and Environment Microbiology 64:439-445.

Rhayour K, Bouchikhi T, Antaoui TEA, Sendide K, Remmal A (2003) The mechanism of bactericidal action of oregano and clove essential oils and of their phenolic major components on *Escherichia coli* and *Bacillus subtilis*. Journal of Essential Oil Research 15:286-292.

Richard AS, Eileen PR, David HY (2002) Novel fungitoxicity assays for inhibition of germination-associated adhesion of *Botrytis cinerea* and *Puccinia recondita* spores. Applied and Environmental Microbiology 68:597-601.

Vacca RA, De Pinto MC, Valenti D, Passarella S, Marra E, De Gara L (2004) Production of reactive oxygen species, alteration of cytosolic ascorbate peroxidase, and impairment of mitochondrial metabolism are early events in heat shock-induced programmed cell death in tobacco bright-yellow 2 cells. Plant Physiology 134:1100-1112.

Sherald JL, Ragsdale NN, Sisler HD (1973) Similarities between the systemic fungicides triforine and triarimol. Pesticide Science 4:719-727.

Soulié MC, Piffeteau A, Choquer M, Boccara M, Vidal-Cros A (2003) Disruption of *Botrytis cinerea* class I chitin synthase gene *Bcchs1* results in cell wall weakening and reduced virulence. Fungal Genetics Biology 40:38-46.

Suzuki K, Kato T, Takahashi J, Kamoshita K (1984) Mode of action of methyl N-(3,5-dichlorophenyl)-carbamate in the benzimidazole-resistant isolate of *Botrytis cinerea*. Journal of Pesticide Science 9:497-501.

Taylor RJ, Salas B, Secor GA, Rivera V, Gudmestad NC (2002) Sensitivity of North American isolates of *Phytophthora erythroseptica* and *Pythium ultimum* to mefenoxam (metalaxyl). Plant Disease 86:797-802.

Tsair-Bor Y, Shang-Tzen C (2008) Synergistic effects of cinnamaldehyde in combination with eugenol against wood decay fungi. Bioresource Technology 99:232-236.

Vázquez BI, Fente C, Franco CM, Vázques MJ, Cepeda A (2001) Inhibitory effects of eugenol and thymol on *Penicillium citrinum* strains in culture media and cheese. International Journal of Food Microbiology 67:157-163.

Vicedo B, De La Leyva O M, Flors V, Finiti I, Del Amo G, Walters D, Real MD, Garcia-Agustn P, Gonzalez-Bosch C (2006) Control of the phytopathogen *Botrytis cinerea* using adipic acid monoethyl ester. Archives of Microbiology 184:316-326.

Yourman LF, Jeffers SN (1999) Resistance to benzimidazole and dicarboximide fungicides in greenhouse isolates of *Botrytis cinerea*. Plant Disease 83:569-575.

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