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In vitro and in vitro activity of eugenol oil (*Eugenia caryophylata*) against four important postharvest apple pathogens

Achour Amiri [a,*], Robert Dugas [b], Anne L. Pichot [a], Gilbert Bompeix [a]

[a] *Laboratoire de Parasitologie Végétale, BP 155, Université Pierre et Marie Curie, 75252 Paris, France*
[b] *Station Fruitière des Coteaux Nantais, place Pierre Desfossé, Les Ajoncs 44120 Vertou, France*

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ABSTRACT

The activity of eugenol oil was evaluated in vitro and in vivo against four apple pathogens namely *Phlyctema vagabunda*, *Penicillium expansum*, *Botrytis cinerea* and *Monilinia fructigena*. The minimum inhibitory concentration (MIC) of eugenol incorporated in malt extract agar medium was found to be 2 mg ml^{-1}. Mycelial growth of the four test pathogens was completely inhibited when treated with 150 µl l^{-1} of volatile eugenol whether at 4 or 20 °C. Conidia of *P. vagabunda*, *P. expansum*, *M. fructigena* and *B. cinerea* suspended for 2 min in eugenol solution at 2 mg ml^{-1} heated to 50 °C germinated at rates of 19, 37, 38 and 39%, respectively. Three different eugenol formulations (Tween 80, ethoxylate and lecithin) were tested for their in vivo efficacy against the tested pathogens on apples. Ethoxylate– and Tween 80–eugenol formulations applied at room temperature were ineffective in reducing disease incidence. When heated to 50 °C, both formulations induced phytotoxicity on apple surface and caused cuticle damages as revealed by scanning electronic microscopic observations. A mixture of eugenol at 2 mg ml^{-1} and soy lecithin at 50 mg ml^{-1} suppressed the phytotoxic symptoms produced by eugenol on apples and reduced the disease incidence of *P. expansum*, *P. vagabunda*, *B. cinerea* and *M. fructigena* to less than 7, 6, 4 and 2% respectively after 6 months of storage at 2 °C. The application of heated lecithin-formulated eugenol could become a successful alternative to the traditional fungicides used in postharvest disease management of apple fruit.

1. Introduction

Postharvest diseases cause heavy losses of fruits during storage. The species reported to damage apples during this period include *Phlyctema vagabunda*, *Penicillium expansum*, *Monilia fructigena* and *Botrytis cinerea*, which represent the major pathogens in Europe and in many arid growing regions of the world. Traditionally, chemical treatments are considered to be the most effective and cheapest method of controlling postharvest pathogens. The main control strategy consists of drench application and/or spray treatment on the packing line using various fungicides. Thiabendazole (TBZ, a benzimidazole), imazalil (IMZ, a sterol demethylation inhibitor), sodium *ortho*-phenylphenate (SOPP, a phenyl phenol) were the most active ingredients used for decades before the recent introduction to the market of pyrimethanil (anilinopyrimidine) and fludioxonil (phenylpyrrole), two fungicides each with different mode of action. However, following intensive use, resistance to TBZ has been reported in *Penicillium* and *Botrytis* populations in France (Leroux and Clerjeau, 1985; Bus et al., 1991; Giraud and Fauré, 2000) and elsewhere (El-Goorani et al., 1984; Prusky et al., 1985), as well as to IMZ and SOPP (Harding, 1962; Eckert, 1987; Dave et al., 1989). Resistance to anilinopyrimidines has been reported in the late 1990s (Chapeland et al., 1999) and more recently in *B. cinerea* (Latorre et al., 2002; Moyano et al., 2004). Field resistance to fludioxonil has not yet been reported. However, laboratory mutants resistant to phenylpyrroles have been selected for *B. cinerea* and other fungal species (Faretra and Pollastro, 1993; Ziogas and Kalamarakis, 2001).

Resistance to traditional fungicides, the limitation of the number of fungicides allowed for postharvest application, and increasing public concern regarding contamination of perishables with fungicidal residues have increased the need for the development of new safe and biodegradable alternatives (i.e. the so-called "natural" fungicides). In this perspective, plant essential oils constitute a large group of the compounds used in food industry over the multiple molecules that have been extracted from plants (Meeker and Linke, 1988). Several essential oils have been reported to inhibit postharvest fungi in vitro such as *Penicillium* spp. (Caccioni and Guizzardi, 1994; Smid et al., 1995; Arras and Usai, 2001), *B. cinerea* and *M. fructicola* (Wilson et al., 1987; 1997). Essential oils and plant extracts which are thought to play a role in plant defense mechanisms against plant pathogens (Mihaliak et al., 1991; Bouchra et al., 2004) have the advantage of being bioactive in their vapor phase, a characteristic that makes them attractive as possible fumigants for the protection of stored products (Dixit et al., 1995).

* Corresponding author. Current address: Department of Entomology, Soils and Plant Sciences, 114 Long Hall, Clemson University, Clemson, SC 29634, USA.
E-mail address: aamiri@clemson.edu (A. Amiri).

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Eugenol ($C_{10}H_{12}O_2$) is a clear to pale yellow oily liquid extracted from buds and leaves of clove (*Eugenia caryophylata* Thumb) and from cinnamons. Eugenol constitutes the most significant active component of clove oil (85 to 95%) in addition to *iso*-eugenol and methyl-eugenol. It is largely used in perfumes and mouthwashes and as dental analgesic. This monoterpene is well studied for its antimicrobial properties in food industry (Mansour et al., 1996; Ouattara et al., 1997; Wilson et al., 1997; Vazquez et al., 2001). However, its in vivo efficacy has not been largely investigated against postharvest pathogens. Recently, Neri et al. (2006) showed that volatile eugenol at 74 and 984 µl l^{-1} were necessary to inhibit mycelial growth and conidial germination, respectively, in *P. expansum*. Drench application of eugenol oil applied at 2 mg ml^{-1} for 3 min at 50 °C effectively controlled *P. vagabunda* but induced phytotoxicity on several apple cultivars (Bompeix and Cholodowski-Faivre, 2000).

The main purpose of this study was to investigate the efficacy of eugenol oil against four important pathogens of apple fruit in postharvest period. Specific objectives were to evaluate (1) in vitro activity of eugenol oil against *B. cinerea*, *M. fructigena*, *P. expansum* and *P. vagabunda*, (2) different formulations of eugenol for their in vivo activity against the four test pathogens (3) the possibility of integrating hot treatment with eugenol to reduce postharvest decay of apples.

2. Materials and methods

2.1. Isolates and inoculum preparation

Four pathogens were used in this study to evaluate the activity of eugenol in vitro. *P. expansum* (isolate Pvlr1), *M. fructigena* (Mfvc1), *B. cinerea* (Bclr1) were isolated from rooted apples collected from packinghouses in Villers Cotterêt (Picardy, eastern France), whereas *P. vagabunda* (Pvn1) was isolated on apples from the Côteaux Nantais fruit station (Pays de Loire, western France). All fungal species were maintained in 50% glycerol at −80 °C and grown for each experiment on 2% malt extract agar medium (MEA). *P. vagabunda* isolate was cultured on 2% MEA at 4 °C during 2 months in darkness and then at 20 °C under continuous light to favor sporulation. Conidial suspensions were prepared by suspending conidia harvested from 7 to 10-day-old cultures (except for *P. vagabunda*) in 10 ml of sterile distilled water and filtered through a gradient of cheesecloth. The final concentration was determined using a hematocytometer.

2.2. Eugenol formulations

Two formulations of eugenol containing different adjuvants were used for in vitro tests. The first formulation (eugenol–Tween) consisted of 20% eugenol and 35% Tween 80, and the second formulation (eugenol–ethoxylate) consisted of 30% eugenol and 20% monylphenol ethoxylate in 50% water. The two formulations were furnished by Xeda International (St Andiol, France) while pure eugenol oil was provided by the GRAB group (Avignon, France). Lyophilized soy lecithin powder (standard grad, Fisher-France) was suspended in small volume of ethanol before being dissolved in water and mixed with the pure eugenol.

2.3. Inhibition of mycelial growth by liquid and volatile eugenol

Eugenol formulated as eugenol–Tween 80 was dissolved in previously autoclaved 2% MEA at final concentrations of 1 and 2 mg ml^{-1} and immediately poured into 9-cm diameter Petri dishes. Agar plugs (5 mm diameter) were taken from the periphery of 7-day-old cultures of each fungus and transferred to the center of unamended or eugenol-amended MEA plates. Dishes were sealed with parafilm and incubated for 21 days at 4 or 20 °C. Five replicate plates were assessed for each concentration-temperature combination. The experiment was conducted three times. In vitro activity of liquid eugenol was determined by comparison to relative mycelial growth on unamended medium.

To evaluate the volatile activity (vapor phase) of eugenol against the test fungi, 100 ml glass flasks (6 cm diameter) containing 10 ml of solidified MEA were inoculated with 5-mm diameter agar plugs picked up from 7-day-old cultures for each fungus. A filter paper (3 cm diameter) was attached to the inner face of the lid of each flask. Different volumes of pure eugenol oil (50, 100 and 150 µl/l of air volume) were pipetted onto the filters and flasks were immediately sealed and incubated either at 4 or at 20 °C. Five replicate flasks were inoculated for each treatment (fungi/concentration/temperature) and the experiment was conducted three times. In vitro activity of volatile eugenol was determined by comparison to relative mycelial growth in flask without filters. In both liquid and volatile eugenol tests, the lowest concentration of eugenol required to inhibit the growth of the test microorganisms was designated as the minimum inhibitory concentration (MIC).

To check whether eugenol oil has a fungistatic or fungicidal effect on the test pathogens, agar disks of isolates that failed to grow at the MIC concentration were transferred onto new eugenol-free MEA plates. For volatile eugenol test, the filters containing eugenol were removed and flasks were exposed under sterile flow hood for 4 h to evacuate the remaining eugenol vapor. In both experiments, mycelial growth was measured after 7 days of incubation at 20 °C.

2.4. Conidial germination assay with eugenol

Following the results from the mycelial growth assay, the concentration of 2 mg ml^{-1} eugenol was selected for conidial germination test. The activity of pure eugenol alone or in combination with lecithin was evaluated at 18 and 50 °C. For the 18 °C treatment, 2 ml of conidial suspensions (10^6 conidia ml^{-1}) from each fungus were added to 8 ml of eugenol solution (2 mg ml^{-1}) previously diluted in sterile distilled water for a total volume of 10 ml. Treatments were contained in 15-ml sterile plastic tubes and were incubated at 18 °C (room temperature). To determine the effect of elevated temperature on conidial germination, tubes containing the eugenol solution were first placed in water bath at 50 °C and allowed to equilibrate for 20 min then 2 ml of conidial suspension were added to the treatment solution as explained above. In a third treatment, lecithin at 50 and 100 mg ml^{-1} was dissolved in pure eugenol solution and tested at 18 °C and 50 °C as described above. For the control, 2 ml of conidial suspension were mixed with 8 ml of distilled water. After 2 min of incubation in all treatments, tubes were placed on ice and the 10 ml-suspension was filtered under vacuum through a 0.8-µm nitrocellulose membrane (Fisher Scientific, Illkirch, France). The membrane was washed twice with 10 ml of sterile distilled water to eliminate the remaining eugenol and agitated (100 rpm) in 10 ml of sterile water for 10 min to detach the attached conidia. Aliquots of 100 µl from each wash were plated on MEA medium and incubated at 20 °C for 24 h. The percentage of spore germination was determined microscopically by counting 100 spores. Five replicate plates were inoculated for each treatment (fungus–temperature–lecithin concentration) and the tests were conducted three times.

2.5. Toxicity of different eugenol formulations on apple fruit

Three different formulations of eugenol i.e. Tween 80–, Ethoxylate– and lecithin–eugenol were tested for their phytotoxicity on apple cuticle. Phytotoxicity is defined as the extent of apparent burning damages (discoloration) induced on the cuticle. The final concentration of eugenol for each formulation was 2 mg ml^{-1}. The eugenol–lecithin formulation was prepared in the laboratory by incorporating 50 mg lecithin per ml of pure eugenol solution. Apples (cvs. Elstar and Golden Delicious) were surface washed with tap water, dried and immersed for 2 min in a 30-liters tank containing the treatment solution either at 18 °C or 50 °C. The 50 °C temperature was