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Effects of thymol fumigation on survival and ultrastracture of *Monilinia fructicola*

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Abstract

Postharvest treatment of stone fruit with thymol vapours controls brown rot, caused by *Monilinia fructicola*. While the mechanism of the fungicidal action is not known, the gross effect of the thymol vapours appeared to be on the fungal spores and surface mycelia. Harvested plums were inoculated with conidia of *M. fructicola* and incubated for 4–6 days at 21 °C to allow for sporulation. Sporulating fruit was treated with thymol vapours from 0 to 8 μg mL^{-1}. The viability of the conidia obtained from thymol treated and untreated brown rot lesions was tested with four vital stains. *M. fructicola* conidia were immediately affected with a 50% reduction in viability after treatment with 2 μg mL^{-1} thymol as detected by the vital stains fluorescein diacetate, propidium iodide, fluorescent dye $DiOC_6(3)$ and the commercial product FungoLight™. Conidia treated with 8 μg mL^{-1} thymol exhibited 17–23% survival. One millimeter square sections of the infected fruit skin with the associated surface mycelial mass were prepared for transmission electron microscopy. The electron micrographs display the intra and intercellular ultrastructure of the fungal conidia. Thymol crystallized on the outer surface of the fungal cell wall. The cytoplasm of the thick walled spores did not appear changed from the untreated spores. In contrast, all other fungal fine structures on the surface of the lesion were severely affected by thymol vapours. Sections of germ tubes, appressoria and surface hyphae exposed to thymol vapours were characterised by disrupted and disorganised cytoplasmic organelles. The fine structures of the fungal intra- and intercellular hyphae inside the plum tissue were unaffected by thymol treatments.

Keywords: Brown rot; Thymol vapours; Postharvest

1. Introduction

Postharvest infection of stonefruits by *Monilinia fructicola* (Winter) Honey and the associated attempt at control have been well documented in the literature (Byrde and Willetts, 1977; Zhou et al., 1999; Spotts et al., 2002). In Canada, postharvest applications of fungicides are not allowed and restrictions exist on the pre-harvest pesticides. Regulatory restrictions are implemented on the number of applications within a season for any one single product and depending on the fungicide category, stonefruit cannot be sprayed 7–10 days prior to harvest (Fruit Production Recommendations, 2004–2005).

Fumigants are good candidates for postharvest treatment of fruit since their use entails little added handling of the food product and they can be procured from natural compounds (Mercier and Jiménez, 2003). Postharvest application of acetic acid vapours controlled storage pathogens on apple (Sholberg and Gaunce, 1995), peaches, apricots and cherries (Sholberg and Gaunce, 1996). Formic and propionic acids and aldehydes have been used successfully as vapour fumigants (Mattheis and Roberts, 1993; Sholberg, 1998; Sholberg et al., 2000). Novel methods for the postharvest control of brown rot have included the volatile compounds produced by the fungus *Muscador albus* (Mercier and Jiménez, 2003).

Mishra and Dubey (1994) and Arras et al. (1995) demonstrated that the essential oil from thyme, *Thymus capitatus*, had antifungal properties. The effect of monoterpenoids on the conidial germination and mycelial growth of *Botrytis cinerea* and *M. fructicola* was examined by Tsao and Zhou (2000). Using

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volatile thymol, the authors demonstrated that 0.25 mg/Petri dish had an inhibitory effect on the developing mycelium of *M. fructicola*. When thymol was added to solid media on which conidia of *M. fructicola* and *B. cinerea* were placed, conidial germination was 100% inhibited (Tsao and Zhou, 2000). The mode of action for these compounds is unknown, however, activity may be linked to solubility in water and the ability of the compounds to pass through the fungal cell membrane (Knobloch et al., 1988). Thymol vapours were used as postharvest treatments effective for the control of brown rot in apricots and plums (Liu et al., 2002). Light microscopy of the treated and non-treated conidia indicated that thymol vapours killed the conidia and caused shrinkage and collapse of the cytoplasm when exposed to concentrations of 8 mg L^{-1} thymol. Postharvest use of thymol vapours to control *M. fructicola* and *B. cinerea* have been well documented (Tsao and Zhou, 2000; Chu et al., 2001; Liu et al., 2002). Thymol was effective as a postharvest treatment with no apparent phytotoxicity in the treated apricot and plums with no effect on the desirable fruit attributes (Liu et al., 2002). In addition, the US Environmental Protection Agency (EPA-738-F-93-010) stated that for the re-registration of thymol and thymol essential oil applications, it has waived generic data requirements for their registration, because there are no reports of adverse effects of thymol to humans or the environment.

The objective of this study was to examine the effect of thymol vapours on the viability, morphology and ultrastructure of *M. fructicola* conidia and hyphae obtained from the surface of infected plums. Mature thymol treated conidia were processed with four vital stains and examined by fluorescent microscopy. The vital stains used in this study, detected changes in the integrity of the fungal cell membrane and/or the presence of metabolically active cytoplasm. To determine the effect of the thymol vapours on fungal morphology and ultrastructure tissue samples of *M. fructicola* conidia, hypha and sections of infected plum tissue were processed for transmission electron microscopy.

2. Materials and methods

2.1. Fungal cultures and inoculum preparation

Experiments were carried out with a wild type isolate of *M. fructicola* obtained from a single spore from field infected plums. The isolate was maintained on potato dextrose agar (PDA, Difco Ltd., Detroit) for 7–10 days at room temperature near a natural light source. Young conidia that formed at 5–7 days on PDA were characterized by white-light gray appearance. A glass atomizer and pressurised air were used to spray distilled water onto the surface of the agar and dislodge the conidia from the mycelial matt. Using a haemocytometer (American Optical Corp., Buffalo, NY, USA), the conidial suspension was adjusted to 4×10^5 conidia mL^{-1}.

2.2. Inoculation of plums with conidial suspension

Plums, *Prunus salicina* L., from commercial cultivar Stanley and a numbered cultivar V72511 were obtained from the Victoria Farm, University of Guelph, Vineland Station, ON, Canada. Ripe plums were surface sterilised by an 10 min immersion in 1/10 dilution of household bleach containing sodium hypochlorite. The bleach treatment was followed by two washes with sterile water. The sterile fruit were placed on a metal mesh inside a closed tray. Each experimental treatment consisted of 20 fruit/tray/thymol concentration. To facilitate fungal infection, fruit were inoculated by stabbing the surface with a 3 mm diameter nail to a depth of 5 mm. Each wound received 10 μL of *M. fructicola* conidial suspension. The inoculated fruit was incubated at 21 °C for 4–6 days to allow for fungal sporulation on the fruit surface.

2.3. Thymol treatment of inoculated plums

The method used for the fumigation of the fruit by thymol vapour has been fully described by Liu et al. (2002). Thymol crystals (TO501, Sigma–Aldrich, St. Louis, MO, USA) used in the experiment were at 99.5% purity. Sporulating fruit were placed on metal nets in trays for fumigation in 260 L gas-tight chamber with 0–8 μg mL^{-1} concentrations of thymol vapour. Treatments consisted of 20 fruit /tray/thymol concentration. Controls consisted of surface sterilised inoculated fruit fumigated with air. The experiment was fully repeated three times.

2.4. Viability testing of the conidia

Conidia of *M. fructicola* were taken from the thymol treated fruit surface immediately following fumigation. Conidia were removed from the fruit surface by spraying the surface with a TLC atomiser which contained sterile distilled water. The conidial concentration was adjusted to 2×10^5 conidia mL^{-1} prior to staining with fluorescein diacetate (FDA, Sigma Chemical, St. Louis, MO, USA), propidium iodide (PI, Sigma Chemical), 3,3′-dihexyloxacarbocyanine iodide ($DiO_6(3)$, Sigma Chemical) and LIVE/DEAD® *Fungo*Light Kit™ Viability Kit (Molecular Probes Inc., Eugene, OR, USA). Conidial suspensions were mixed in a 1:1 ratio with the fluorescent dyes and each stain required the use of a new aliquot of spore suspension. A drop of the fluorescent dye and conidial suspension were placed on a glass slide, covered with a cover slip and examined under a Leitz Dialux 20 microscope fitted with epifluorescence optics using a blue excitation (450–490 nm) and 526 nm barrier filters.

FDA and PI were prepared according to the protocol described by Firstencel et al. (1990). FDA stock solution consisted of 1 mg mL^{-1} FDA in acetone, stored at −20 °C. The PI stain was prepared by preparing a 3 mg mL^{-1} stock solution and a working solution of 0.3 mL stock in 50 mL sterile distilled water. FDA stain (5 μL), PI (5 μL) and spore suspensions (10 μL) were mixed and examined within 10 min under a fluorescent microscope. Viable conidia fluoresced bright green-yellow with FDA while dead cells fluoresced red with PI (Firstencel et al., 1990; Williams et al., 1998).

$DiO_6(3)$ was dissolved in ethanol at a concentration of 5 mg mL^{-1}, and the working aqueous solutions were prepared at a concentration of 50 mg mL^{-1} according to the method