Interaction of Monoterpenoids, Methyl Jasmonate, and Ca²⁺ in Controlling Postharvest Brown Rot of Sweet Cherry

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Abstract. The banning of synthetic fungicides for postharvest use on fruits in Canada has prompted a search for alternative control strategies for postharvest brown rot caused by *Monilinia fructicola* (Wint.) Honey on sweet cherry (*Prunus avium* L.). Thymol and carvacrol were the two most potent fungicides among the monoterpenoids tested. The brown rot incidences of *M. fructicola*-inoculated cherry dipped in 1000 µg·mL⁻¹ thymol and carvacrol were 24% and 23%, respectively, compared with 81% for the control. The effects of thymol and carvacrol were not significantly enhanced by the addition of CaCl₂ or CaB²y[®], a foliar calcium fertilizer. Decco[®] 282 significantly reduced the activity of thymol. Methyl jasmonate, an elicitor of plant defense mechanisms, did not reduce brown rot by itself, and did not increase the efficacy of thymol and carvacrol when used as an additive in dipping or fumigation experiments. Thymol and carvacrol caused stem browning of cherry fruits in the fumigation experiment, however, 69% and 73%, respectively, of the browning was prevented when methyl jasmonate was used as a co-fumigant. Chemical names used: 5-methyl-2-(1-methylethyl)phenol (thymol); 2-methyl-5-(1-methylethyl)phenol (carvacrol); methyl 3-oxo-2-(2-pentenyl)cyclopentane acetate (methyl jasmonate).

Postharvest diseases are among the most important causes of losses and reduction of quality in fruits and vegetables. Fresh cherry fruit is susceptible to infection by pathogenic fungi after harvest, particularly by *Monilinia* spp. (Eckert and Ogawa, 1988). Brown rot of sweet cherry and other stone fruits can be successfully controlled by preharvest treatments with a number of fungicides. However, many key fungicides, such as captan {N-[(trichloromethyl)thio]-4-cyclohexene-1,2-dicarboximide}, benomyl [methyl-1-(butylcarbamoyl)-2-benzimidazole carbamate], and iprodione [3-(3,5-dichlorophenyl)-N-(1-methylethyl)-2,4-dioxo-1-imidazolidinecarboxamide] have been withdrawn from some or all postharvest uses in Canada and/or the United States because of concerns over the safety of synthetic fungicides in the food supply and in the environment (Wilson et al., 1994). In fact, no fungicide is currently available in Canada for effective postharvest control of brown rot in fruits (Wilson et al.,

1994). In addition, the number of fungicide-resistant fungi is increasing (Wisniewski and Wilson, 1992). All of these factors have created an urgent need for effective alternative control strategies for postharvest diseases in fruits and vegetables.

Some essential oil components, mostly the monoterpenoids, inhibit spore germination and mycelial growth of common postharvest pathogens such as *Botrytis cinerea* Pers., *Monilinia* sp., *Mucor piriformis* E. Fisch., *Aspergillus flavus* Link., and *Penicillium* sp. (Arras et al., 1995; Caccioni and Guizzardi, 1994; Mishra and Dubey, 1994; Shimoni et al., 1993; Tsao and Zhou, 2000). Chu et al. (1999) and Tsao and Zhou (2000) reported that carvacrol, thymol, citronellol, geraniol, citral, (-)-perillaldehyde, citronellal, (-)-perillyl alcohol, and (-)-menthol were among the most active monoterpene compounds against *B. cinerea* and *M. fructicola*. Many of these compounds are generally recognized as safe by the Food and Drug Administration (FDA), and thus meet the new requirements for safe and effective control agents for use in postharvest treatment of fruits.

Another natural product, methyl jasmonate, although not a monoterpene, reportedly modulates a wide variety of plant responses (Koda, 1992; Semadeni and Parthier, 1993). It accelerated chlorophyll degradation and β-carotene accumulation in tomatoes (*Lycopersicon esculentum* Mill.) and apples (*Malus ×domestica* Borkh.), and therefore degreened fruit (Fan et al., 1998; Perez et al., 1993; Saniewski et al., 1987). Methyl jasmonate itself does not possess an-

tifungal activity; however, when used at a moderate concentration, it elicited induction of phytoalexins against postharvest diseases and compounds in living plants that make them more resistant to temperature changes (Stanley, 1998).

Calcium is considered an important mediating agent in the control of cell metabolism, and is associated with the regulation of ripening processes and postharvest storage life of fruits. Maintenance of relatively high Ca²⁺ concentrations in the fruit tissue resulted in reduced CO₂, soluble pectin and ethylene production (Basiouny and Woods, 1992; Glenn and Poovaiah, 1990; Tingwa and Young, 1974). Pressure-infiltration with solutions of 2% or 4% CaCl₂ reduced decay of peach [*Prunus persica* (L.) Batsch.] fruit by 40% and 60%, respectively (Conway et al., 1987). Calcium was also reported to increase the efficacy of some biological control agents (Janisiewicz et al., 1998; McLaughlin and Wilson, 1992; Wilson et al., 1994; Wisniewski et al., 1995; Zhou et al., 1999). However, this was not observed by Kampp (1994).

Additives have been studied for their efficacy as carriers of biocontrol agents in postharvest treatment of fruits. Pusey et al. (1986) found that *Bacillus subtilis* used to control peach brown rot was compatible with commercial "peach wax" when the latter was used as a carrier for the bacterium. Catz et al. (1993), however, found that the application of an antagonistic yeast in water resulted in better control of blue mold (*Penicillium expansum* Link.) on apple than did a simple application in wax. Zhou et al. (1999) noted an increase of brown rot incidence when peach fruits were treated with "peach wax" (Decco[®] 282) as compared with treatments without "peach wax."

In this paper, we report the results of our further investigation on the most potent monoterpenoids found in the previous study and their effect on postharvest brown rot of sweet cherry fruits. We also studied the effects of methyl jasmonate, calcium, and a wax-based additive on the efficacy of monoterpenoids against the disease. A preliminary report has been published (Tsao and Zhou, 1999).

Materials and Methods

Sweet cherry. Sweet cherry fruits, cv. Viva and Vista, were harvested when commercially ripe from research orchards at Jordan Station, Ont. Rovral[®] (iprodione, 50WP, 1.5 kg·ha⁻¹; Rhône-Poulenc Canada, Mississauga, Ont.) was sprayed twice, at 5% and 100% bloom. The harvested fruits were stored at 1 °C for 1–7 d prior to treatment.

Pathogen. Four isolates of *Monilinia fructicola*, Loring-1, M2M, BR-7, and BR-1, were obtained locally from typical brown rot lesions on peach fruits. The isolates were cultured on potato dextrose agar (PDA; Difco, Detroit) slants and stored at 4 °C. Spores were produced on V-8 agar (Dhingra and Sinclair, 1995) at 22 °C and collected from 7- to 10-d-old cultures. A mixture of spores

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from at least two isolates was used in each experiment.

Chemicals. Monoterpenoid compounds were purchased from Aldrich Chemicals (Milwaukee), CaB'y®, a foliar fertilizer containing 10% calcium and 0.5% boron was donated by Stoller Canada (Burlington, Ont.); "peach wax" (Decco® 282, peach, nectarine, and plum luster) was obtained from ELF Atochem North American (Monrovia, Calif.), Tween® 20 was from Fisher Scientific (Nepean, Ont.), and iprodione (Rovral®, 50 WP) was from Rhône-Poulenc. Solutions of monoterpenoids were prepared in 0.01% Tween® 20, and heat (70 °C water bath) was used to assist the dissolution of some of the less water-soluble compounds.

Inoculation. Cherries were surface-sterilized with 0.5% sodium hypochlorite for 4 min, rinsed well with tap water, and air-dried; then, except for the control, they were inoculated with a conidial suspension of *M. fructicola*. The cherries were then wounded on the side with a paper clip (5 mm in depth, one hole/cherry), and inoculated by spraying with a conidial suspension (10⁵ conidia/mL) until the solution ran off the fruits. The inoculated fruits were air-dried at room temperature for 2 h before being treated with the chemical solutions.

Dipping treatment. Inoculated cherries were placed in a wire-mesh cylinder, and dipped in a solution of an individual compound or a mixture of two compounds for 4 min. The treated fruits were laid individually on a wire screen (mesh size 1 × 1 cm) in an aluminum tray (40 × 30 × 5 cm) lined with moist paper towels. The trays were enclosed in a plastic bag to maintain high humidity and incubated at 20 °C in the dark for 5–6 d. A factorial design was used with four replications of 20 cherries per treatment. The numbers of cherries with brown rot were recorded and percentage of incidence of decay was used in assessing the efficacy of control.

Fumigation treatment. Filter papers (Whatman No. 1, 15 cm i.d.; Whatman, Clifton, N.J.) were treated with 3 mL of an acetone solution (100 mg·mL⁻¹) of each monoterpenoid by spreading evenly on the paper with a pipette. The acetone was evaporated in a fume hood for 5 min, after which the filter paper was fixed to the inner surface of a tightly fitting lid on the plastic container (20 × 20 × 10 cm) holding the inoculated cherries. The cherries were kept in the sealed container for 24 h at room temperature before being transferred onto a wire screen (mesh size 1 × 1 cm) in an aluminum tray (40 × 30 × 5 cm) lined with moist paper towels. The trays were enclosed in a plastic bag and incubated at 20 °C in the dark for 5–6 d. Each 20-cherry replicate was in a separate tray and bag. There were three replicates per treatment. In addition to the number of cherries with brown rot, the number of cherries with brown stems was also recorded.

Effect of methyl jasmonate on thymol volatility. Solutions of thymol and of methyl jasmonate (0.5 mL, 1000 µg·mL⁻¹ in acetone), were spread over filter papers (7 cm i.d.) in a

fume hood. Following evaporation of the acetone (1 min), the filter papers were inserted into 250-mL brown bottles that were then sealed with a septum cap. Mixtures of the two compounds, both at 1000 µg·mL⁻¹ were also tested. There were three replicates per experiment. The bottles were kept at room temperature for 24 h, then a sample of the headspace gas was drawn with a gas-tight syringe and injected into a GC-MS system (MD 800; Fisons Instruments, West Sussex, U.K.), using a BD-5 20 m × 0.25 mm (i.d.) fused silica capillary column with a film thickness of 0.1 µm. Operating conditions were: oven temperature, 80 °C (2 min) then 20 °C·min⁻¹ to 250 °C; inlet temperature, 250 °C; ion source temperature, 250 °C; ionization, EI; ionization energy, 70 eV; emission current, 300 µA; carrier gas, He; flow rate, 2 mL·min⁻¹; injection method, splitless; injection volume, 10 µL. The instrument was scanned from m/z 50 to m/z 550 in 0.6 s. The retention times for thymol and methyl jasmonate were 6.41 and 9.01 min, respectively. The headspace concentration of thymol in a bottle containing both compounds was then compared with that in a bottle containing thymol alone.

Data analysis. All data were analyzed using SAS/STAT 6.12 (SAS Institute, Cary, N.C.). The General Linear Model procedure was used for the analysis of variance and mean separations. Differences between treatments were determined by Fisher's protected LSD test.

Results and Discussion

Six day post treatment incidence of brown rot on sweet cherries treated (wounded and dipped) with 1000 µg·mL⁻¹ monoterpenoid solutions was between 23% and 47% vs. 81% for the control (Table 1). The effects of three most active compounds were not statistically different from that of the synthetic fungicide iprodione (16%) at the same concentration of active ingredient (Table 1). This in vivo result was consistent with that found previously in an in vitro experiment on conidial germination and mycelial growth, in which thymol and carvacrol were also the most potent inhibitors of *M. fructicola* (Tsao and Zhou, 2000). Variation in efficacy among the different types of monoterpenoids could be affected by many factors, such as the lipophilicity that controls membrane permeability, and their different affinity with enzymes at specific binding sites.

In a separate dipping experiment with wound-inoculated cherries incubated for 6 d at 20 °C, thymol and carvacrol (500 µg·mL⁻¹) were again the two most effective monoterpenoids, but they were less effective than iprodione (Table 1). The addition of calcium did not significantly affect the activity of thymol or carvacrol. The incidence of brown rot in the control was 100% (Table 1).

A commercial product, CaB'y®, although developed as a foliar fertilizer, contains high levels of calcium (10%). This product was diluted to match the Ca²⁺ level as used in the

Table 1. Effects of selected monoterpenoid compounds and calcium chloride on the percentage of incidence of decay of sweet cherry fruits inoculated with *Monilinia fructicola*.²

Treatment	Concn (µg·mL ⁻¹)	
	500 (Expt. 1)	1000 (Expt. 2)
Control	100.0 a ^y	80.6 a
Terpineol	97.5 a	46.9 b
Perillyl alcohol	95.0 ab	26.9 cde
Eugenol	91.3 ab	31.9 cd
Perillaldehyde	---	34.4 cd
Citral	85.0 ab	28.8 cd
Carvacrol	75.0 bc	23.1 de
Citronellol	---	29.4 cd
Thymol	60.0 c	23.8 de
Thymol/Calcium ^x	58.8 c	---
Carvacrol/Calcium ^x	57.5 c	---
Iprodione	11.3 d	15.6 e
Blank	0.0 d	38.8 bc

²Values are the means of four replications.

^yMean separated within columns by Fisher's protected LSD test ($P \leq 0.05$).

^xThe concentration of calcium chloride was 0.05 M.

above initial study with pure CaCl₂. Addition of CaB'y® did not affect incidences of brown rot of cherries treated (wounded and dipped) with 500 µg·mL⁻¹ thymol (Fig. 1). Decco® 282, a wax-based product developed for preventing water loss in peach fruit and as carrier for postharvest fungicide treatment, not only did not enhance the efficacy of thymol, but increased the brown rot incidence from 36% with thymol alone to 54% with Decco® 282 plus thymol (Fig. 1). This reduction of the thymol effect might be a result of reduced affinity between thymol molecules and the fungus, or of the creation by the wax coating of a more favorable environment for brown rot to initiate decay. A similar phenomenon was observed on peach (Zhou et al., 1999).

In another dipping experiment, in which cherries were treated with methyl jasmonate alone, or with a mixture of methyl jasmonate with either thymol or carvacrol, methyl jasmonate had no effect, nor did it affect the activity of thymol or carvacrol when used in mixtures (Fig. 2). This supports earlier findings that methyl jasmonate serves as a chemical elicitor of defense mechanisms rather than being antimicrobial itself (Stanley, 1998).

Fumigation of *M. fructicola*-inoculated cherries with thymol and carvacrol, however, reduced infection rates from 97% to 42% and 63%, respectively, whereas methyl jasmonate alone had no effect (Fig. 3). Methyl jasmonate did, however, counteract the effects of thymol and carvacrol when used in mixtures. The reason(s) for this are not clear. Methyl jasmonate can increase the resistance of fruits to physical/chemical damages (Stanley, 1998). Such changes in the fruit surface may have affected the penetration or absorption of thymol or carvacrol. The addition of methyl jasmonate might also have affected their volatility, since the negative effect of methyl jasmonate on the response to thymol was not evident in the dipping experiment. In the headspace experiment, the concentration of thymol in the headspace was reduced 60% by

Fig. 1. Effect of a calcium-containing product CaB'y® (Ca²⁺ content 0.05 M) and 1% peach wax (Decco® 282) on the activity of thymol (TH) against brown rot of sweet cherry. Cherries were dipped in thymol solution (500 µg·mL⁻¹). Values are means for four replications. Bars with the same letter are not significantly different from one another by Fisher's protected LSD test ($P \leq 0.05$). All fruits were wounded and inoculated with *Monilinia fructicola* conidia prior to treatment.

Fig. 2. Effect of methyl jasmonate (MJ; 500 µg·mL⁻¹) on the activity of thymol (TH) and carvacrol (CA) against brown rot of sweet cherry. Cherries were dipped in monoterpenoid solutions (500 µg·mL⁻¹). Values are means for four replications. Bars with the same letter are not significantly different from one another by Fisher's protected LSD test ($P \leq 0.05$). Control: inoculated control; blank: noninoculated control.

the pressure of methyl jasmonate, indicating a reduction in volatility. This effect was reciprocal, because thymol reduced the volatility of methyl jasmonate in a similar fashion (61% reduction).

Either hypothesis could also be used to explain another interesting phenomenon that was observed in the fumigation experiment. Although methyl jasmonate failed to enhance

the fungicidal effect of thymol or carvacrol, it reduced their phytotoxicity as indicated by stem browning (Fig. 3). This reduction may have important implications if methyl jasmonate can reduce phytotoxicity without reducing activity of other fumigants, such as acetic acid, which are phytotoxic when used to extend fruit shelf-life (Chu et al., 1999). High concentrations of methyl jasmonate (10

mmol·L⁻¹ = 2.24 mg·mL⁻¹) are phytotoxic (Fan et al., 1998; Stanley, 1998), but the concentrations used in this study caused no injury to cherry fruits.

Monoterpenoids such as thymol and carvacrol are found in numerous plant species, many of which have been used as spices and flavorings. Thymol and carvacrol, although less effective than the synthetic fungicide iprodione, reduced the incidence of brown rot, indicating their potential for use as naturally occurring fungicides. The major problems with using high concentrations of thymol and carvacrol are the strong residual scent and taste in treated fruits and phytotoxicity. However, these disadvantages may be overcome by optimizing treatment conditions, such as concentration of compound and storage temperature. Although all the tests in this study were conducted at 20 °C, our earlier study showed that thymol fumigation, coupled with modified atmosphere packaging, was very effective against postharvest gray mold (*Botrytis cinerea* Pers.) at 2 °C (Chu et al., 1999). Preliminary results from our ongoing investigation showed that thymol was an effective fumigant against brown rot of cherry at 1 µg·mL⁻¹ at 2 °C, and off-flavors and phytotoxicity were negligible at this level (data not shown).

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Fig. 3. Effect of methyl jasmonate (MJ) on activity of thymol (TH) and carvacrol (CA) on (A) brown rot and (B) stem browning of sweet cherry. Cherries were fumigated with either thymol or carvacrol, or a mixture of methyl jasmonate with thymol or carvacrol (300 mg of each), in a 4-L sealed container. Values are means for three replications. Bars with the same letter are not significantly different from one another by Fisher's protected LSD test ($P \leq 0.05$). Control: inoculated control.

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