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Biocontrol of postharvest fungal decay of tomatoes with a combination of thymol and salicylic acid screening from 11 natural agents

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ABSTRACT

To control the decay of fresh vegetables or fruits after harvest and overcome higher costs using a natural agent, synergistic antifungal effects were studied in tomatoes and their main decay fungi *Fusarium solani* and *Rhizopus stolonifer*. After screening 56 groups with a checkerboard method based on 11 natural agents, only one combination of thymol and salicylic acid (S_{TSA}) had a synergistic effect on both fungi. Their average minimal inhibitory concentration (MIC) values decreased significantly to 0.43 fold compared with the single agents tested. Their mycelial growth was completely inhibited; the inhibition rates of spore germination exceeded 96% at 0.5, 1, and 2 MIC. The protective and therapeutic effects were found to be dose-dependent during exposure; the former was always better than the latter against both fungi at any tested concentration in wound-inoculated tomatoes, and phytotoxicity occurred only when the concentration of S_{TSA} reached 2 MIC in naturally stored fruit. Consumer evaluation showed that the natural, non-inoculated tomatoes treated with 1 MIC and the wound-inoculated tomatoes treated with 2 MIC were both acceptable. The in vitro and in vivo results show that a combination of thymol and salicylic acid could be developed as a control measure and could reduce costs.

1. Introduction

Postharvest losses during the storage and transportation of fruits and vegetables are brought about by decay, which is mainly caused by fungal plant pathogens (Eckert & Ogawa, 1988; He, Zheng, Yin, Sun, & Zhang, 2003). For example, tomatoes decay because of their thin peel and juiciness; their rate of loss in New York markets ranges from 11.4% to 14.2% (Ceponis, Cappellini, & Lightner, 1986). A previous study demonstrated that *Fusarium solani* and *Rhizopus stolonifer* are tomatoes' significant spoilage organisms (Battilani, Chiusa, Cervi, Trevisan, & Ghebbioni, 1996). *R. stolonifer* mainly colonizes wound sites during harvest or packing and causes rhizopus rot; its spores spread rapidly from infected fruit to adjacent fruit when the temperature is higher than 5 °C (Bonaterra, Mari, Casalini, & Montesinos, 2003).

Natural agents are preferred to synthetic chemicals to combat fungal decay because of their safety. However, the higher cost of natural agents hinders their wide application, and finding a way to reduce the absolute amount required is one solution to this problem. The antimicrobial activity of essential oils is related to their structure, the scale of their components, and the interactions among them (Dorman & Deans, 2000; Marino, Bersani, & Comi, 2001; Delaquis, Stanich, Girard, & Mazza, 2002). Different effects can be seen among the components of essential oils, including additive, synergistic, indifferent, and antagonistic effects. For example, basil essential oil is significantly more effective than its main component of linalool or methyl chavicol against *Lactobacillus curvatus* and *Saccharomyces cerevisiae* (Lachowicz et al., 1998). A mixture of cinnamaldehyde and eugenol shows significant antimicrobial activity against four microorganisms within 30 days, whereas no antimicrobial effect is seen when either is used alone (Moleyar & Narasimham, 1992). The essential oils from conifers show better activity against *Listeria monocytogenes* than their active components (Mourey & Canillac, 2002). Thymol and carvacrol, as the principle components of oregano essential oil, show an additive effect against *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Lambert, Skandamis, Coote, & Nychas, 2001). The combination of thymol and carvacrol shows a synergistic effect against *Aspergillus*

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niger, with the highest synergism being achieved at a concentration of 50% thymol and 50% carvacrol. The active concentrations used in combination are about 2fold lower than when used alone (Guarda, Rubilar, Miltz, & Galotto, 2011), which attracted our interest to further study the antimicrobial activity of the combined effects of various active agents.

It has been reported that the decay fungi (*R. stolonifer, A. niger*, etc.) of tomatoes and peppers after harvest could be inhibited with cinnamon (*Cinnamomum zeylanicum* L.) oil (Tzortzakis, 2009). The hyphal growth and spore germination of *R. stolonifer* in nectarines and peaches could be directly inhibited by tea polyphenols (Yang & Jiang, 2015). Essential oil (containing 33% thymol) from *Thymus vulgaris* L. and pure thymol have shown strong antifungal activity against a range of fungal genera, including *Aspergillus*, *Penicillium,* and others (Klarić, Kosalec, Mastelić, Piecková, & Pepeljnak, 2006). Thymol is the main active ingredient of *Thymus pectinatus* Fisch. et Mey. Var. *pectinatus* (Lamiaceae) and shows strong antifungal activity against *Candida albicans* and Candida *krusei* (Gülhan et al., 2003). Salicylic acid is a natural phenolic compound found in many plants that could be involved in local and systemic resistance to fungal pathogens (Raskin, 1992). Its application in nontoxic concentrations to susceptible plants could enhance their resistance to fungal pathogens (Murphy, Holcombe, & Carr, 2000) and enhance the biocontrol efficacy of *Cryptococcus laurentii* in pears (Yu et al., 2007), apples (Yu & Zheng, 2006), and cherries (Qin, Tian, Xu, & Wan, 2003).

In this study, some pure natural agents were collected, and their antifungal capacities toward two common fungi (*F. solani* and *R. stolonifer)* that result in the postharvest decay of tomatoes were determined. Based on the antifungal activity of the single components, their synergistic effects were screened by means of a checkerboard method. According to the combinations with synergistic effect, the in vitro and in vivo antifungal effects were evaluated in terms of mycelia growth, spore germination, and protective and therapeutic effects in wound-inoculated or natural tomatoes. The practical effects of application were evaluated by means of a sensory experiment. Our results are expected to improve the safety level of fresh vegetables and fruits during storage and transportation.

2. Materials and methods

2.1. Chemicals

Cinnamaldehyde (CAS-No. 104 -55-2) and n-hexanoic acid (CAS-No. 142-62-1) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Suzhou, China). Geraniol (CAS-No. 106-24-1), carvacrol (CAS-No. 499-75-2), citral (CAS-No. 5392-40-5), eugenol (CAS-No. 93-53-0), thymol (CAS-No. 89-83-8), cinnamic acid (CAS-No. 621-82-9), hexanal (CAS-No. 66-25-1), L-(-)-carvone (CAS-No. 6485-40-1), and salicylic acid (CAS-No. 69-72-7) were purchased from J&K Scientific Co., Ltd. (Shanghai, China). All other chemicals and reagents were of analytical grade.

2.2. Fungal culture

To determine the antifungal characteristics of different fungistats, the following microorganisms were used. *F. solani* (ATCC 36031) was obtained from the China Center of Industrial Culture Collection, and *R. stolonifer* (ATCC 12939) was obtained from Yu Ding Xin Jie Scientific Co., Ltd. (Beijing, China). Potato dextrose agar (PDA) medium was purchased from Hope Bio-Technology Co., Ltd. (Qingdao, China). The organisms were subcultured on PDA slants and stored at 4 °C in a refrigerator.

2.3. In vitro antifungal test

2.3.1. Inhibition zone test

The cylinder diffusion method was used to detect the ability of various fungistats to inhibit the growth of *F. solani* and *R. stolonifer*. Approximately 15 mL sterilized PDA medium was poured into a sterile Petri dish (9 cm in diameter) and allowed to cool and solidify. Thereafter, 100 μL of spore suspension (at a concentration of about 10^6 cfu/mL) was incubated into the fresh PDA media with an even coating. Three evenly placed wells (6 mm in diameter) were put onto the surface of the solidified medium, and 200 μL of the extract was added into the wells with a pipette. Absolute ethyl alcohol was used as a negative control. The containers were transferred to storage at 30 °C and 25 °C for *F. solani* and *R. stolonifer*, respectively, and then cultured for 24 h. A triangle ruler was used to measure the diameter of the inhibition zone. All assays were replicated three times, and the average of the three results was used.

2.3.2. Minimal inhibitory concentration (MIC) test

The 2-fold dilution method was used to determine the minimal inhibitory concentration (MIC). The concrete operation method was performed as follows. 1) A volume of 100 μL of potato dextrose (PD) fluid media was pipetted into each well of the plate. 2) Using a pipette, 100 μL of one type of fungicide was added to the first well of row "A, B, C," and the same volume of another fungicide was added to the first well of row "D, E, F," and 2 fold dilution was performed. 3) A 100 μL volume of spore suspension was then pipetted into each well, resulting in three replications of the same concentration of each MIC value. 4) PD fluid media (100 μL) and antifungal agents (100 μL) were used as a negative control in row "G"; and PD fluid media (100 μL) and spore suspension (100 μL) were used as positive controls in row "H." 5) The plates were placed at 30 °C and 25 °C for *F. solani* and *R. stolonifer,* respectively, and cultured for 24 h. The lowest concentration at which a well had no fungal growth was taken as the MIC value. The average of three values was calculated and used as the MIC for the test agent and the corresponding fungal strain.

2.4. Combined effect of different antifungal agents

The antifungal activities of mixed antifungal agents were determined by a modified checkerboard assay. The antifungal agent dilution was determined according to the MIC value from Section 2.3.2. The maximum concentration was four times the MIC value of each agent, and the fungicide was then diluted with PD fluid medium to obtain final concentrations of 4, 2, 1, 0.5, 0.25, 0.125, and 0.0625 times the MIC. Using the concrete operation method, the first eight columns and rows were selected, and fungicide A was added in the columns (varying the concentrations from low to high) and fungicide B was added in the rows (varying the concentration from low to high) at a volume of 100 μL. The first well of each column or row was used as a control with no fungistat, and fungicide alone without spore suspension was added to the other wells in the first column and row. Finally, 100 μL of spore suspension was then pipetted into each well. The plates were prepared in triplicate, warmed to 30 °C and 25 °C for *F. solani* and *R. stolonifer*, respectively, and cultured for 24 h. The combined effect was determined as proposed by Takashi et al. (2014) as the fractional inhibitory concentration (FIC) with the formula: FIC = (MIC of A in combination/MIC of A) + (MIC of B in combination/MIC of B). The interaction was defined as synergistic if the FIC index was 0.5 or less, as additive if the FIC index was between 0.5 and 1, as no interaction if the FIC index was between 1 and 2, and as antagonistic if the FIC index was greater than 2.