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ORIGINAL ARTICLE

Antifungal Activity of Thymol, Salycilic acid (SA) and Methyljasmonate (MeJA) Against Postharvest Pathogen *Botrytis cinerea*

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ABSTRACT
Because of greater consumer awareness and concern regarding synthetic chemical additives, foods preserved with natural additives have become popular.Thymol, SA and MeJA are naturally occurring compounds known as biocides. Therefore invitro antifungal activity of the thymol, SA and MeJA were evaluated against postharvest pathogen Botrytis cinerea.Thymol(0, 125, 250, 500and 1000 µll^{-1}), MeJA (0, 10, 30 and 60µll^{-1}) and SA (0, 25, 50 and 100 µll^{-1}) were applied via three methods (PDM (Paper Disc Method), adding to PDA and spraying) under in vitro condition.The results showed thatthe most effective concentration of thymol was 250 µll^{-1} and 125µll^{-1} was the least effective concentration. The most effective concentration of SA was 100 µll^{-1}where the percentage of mycelia growth inhibition was 61.03 and adding to PDA method produced more profound effects compared to PDM and spraying methods.No differences were found among different MeJA levels and spraying method was the most effective method. As a general result, among all of these treatments thymol in all concentrations (125, 250, 500 and 1000 µll^{-1}) produced more profound effects and the least effect on fungal growth were observed in SA with 25 and 50 µll^{-1} concentrations.
***Keywords**: Natural compounds, Fungal disease, Mycelia inhibition, Invitro*

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INTRODUCTION

Gray mold caused by *Botrytis cinerea* is the most economically significant postharvest pathogen of fruit [23].The use of certain fungicides against gray mold caused by *B. cinerea* increased the frequency of diseases caused by *Mucor spp.* and *Rhizopus stolonifer*[20].Additionally consumer concern over pesticide residues on foods, along with pathogen resistance to many currently used pesticides, has increased the need to find alternative methods for decay control [23].Thymol (also known as 2-isopropyl-5-methylphenol, IPMP) is part of naturally occurring compounds known as biocides, with strong antimicrobial attributes when used alone or with other biocides. Thymol as a natural monoterpenephenol, found in oil of thyme and extracted from *Thymus vulgaris* and various other kinds of plants [2]. Methyl jasmonate (MeJA), as a natural plant regulator compound, plays important roles in plant growth and development, fruit ripening, and responses to environmental stress [16]. Salicylic acid (SA) is a natural phenolic compound present in many plants and is an important component in the signal transduction pathway [30].

BACKGROUND RESEARCHES

Fumigation of sweet cherries with thymol was effective in controlling postharvest gray mold rot caused by *B. cinerea*[14] and brown rot caused by *Monilia fructicola*[15].Liu et al., 2002 also found that thymol was effective for controlling brown rot symptoms on apricots and fumigation of plums with relatively low concentrations such as 2 or 4mgL^{-1} can greatly reduce postharvest decay without causing any phytotoxicity [27].Thymol reduced gray mold rot of *B. cinerea* inoculated cherries from 36 to 0.5 % [27]. MeJA vapor or emulsion inhibits the microbial contamination of fresh-cut celery and peppers [10], inhibits the gray mold infection in strawberries [33], suppresses green mold decay in grapefruit [19], inhibits aflatoxin production by *Aspergillus flavus*[22], and suppresses *B. cinerea* rot in cut rose flowers [31]. Babalar et al., 2007 indicated that the treatment of 'Selva' strawberry which 1 and 2mμ SA at vegetative stage followed by postharvest stage application was the most effective strategy for decreasing ethylene production and control of postharvest diseases [7]. Positive SA effect have also been reported for control of *Fusarium oxysporum* in tomato [29], anthracnose disease caused by *Colletotrichum gloeosporioides* in mango [47], *Penicillium expansum* in sweet cherry fruit [48]. So the main purpose of this study was to evaluate the antifungal potential of thymol, MeJA, SA against important pathogenic fungus *B. cinerea* which can reduce the storability of strawberry fruit.

MATERIALS AND METHODS

Fungal culture

Phytophatogenic fungi including *B. cinerea* was supplied from the plant pathology laboratory, Islamic Azad University of Tehran, Science and Research Branch. The fungi cultures were maintained and grown on Potato Dextrose Agar (PDA)[3].

In vitro antifungal activity evaluation

Thymol, SA and MeJA was dissolved in 95% ethanol. Ethanol was used as a solvent in this study to dissolve and vaporize [27].Three separate experiments were performed to study the effect of treatments on *B. cinerea*. *Method 1*: The antifungal test was carried out in vitro according to the method described by Pitarrokili et al., (2003) using petri dishes 8cm in diameter containing PDA [36]. Treatments was added to PDA immediately before it was filled into the Petri dishes at a temperature of 45-50 ˚C. Thymol concentrations tested were 0, 125, 250, 500 and 1000 μLL^{-1}, SA concentrations tested were 0, 25, 50 and 100μLL^{-1} and MeJA concentrations tested were 0, 10, 30 and 60 μLL^{-1}.The controls included the same quantity of PDA. The phytopathogenic fungi (*B. cinerea*) were inoculated immediately after preparation of the petri dishes by placing in the center of each plate a 5mm diameter disk of the test species cut with a sterile cork borer from the periphery of actively growing cultures on PDA plates. The petri dishes were incubated in the dark at a temperature of 24˚C [34].

Method 2: In vitro antifungal activity test was carried out by the Paper Disc Method (PDM). 10 ml of PDA medium were poured into petri plates (80 mm in diameter). Equal discs (5mm) of actively grown *B. cinerea* culture (7 days old) were centered placed on the other side. An aliquot of thymol (0, 125, 250, 500 and 1000 μLL^{-1}), SA (0, 25, 50 and 100 μLL^{-1}) and MeJA (0, 10, 30 and 60 μLL^{-1}) was added onto a paper disc in a plate and allowed only volatile compounds to be the causative agents for mycelia growth inhibition [20].

Method 3: For antifungal activity test 20 ml of PDA medium were poured into Petri plates (80 mm in diameter). Equal disc (5 mm) of activity grown *B. cinerea* culture (7 days old) were centered placed on the other side. Antifungal agents (thymol, SA and MeJA) at different concentrations were sprayed separately to fungi culture[4].

In all above methods the plates were immediately sealed with Para film after adding thymol, SA and MeJAthen incubated for 4 days at 20 ˚C.The observations were recorded on the fourth day and the percentage mycelia inhibition was calculated by the following formula:

Percentage of Mycelia Growth Inhibition (GI %) = d_c-d_t /d_c*x 100*

Where d_c is mean colony diameter of control sets and d_t is mean colony diameter of treatment sets [41].Mean growth rates were calculated from five replicates of each fungal species every 24h until fungi in the control filled the Petri dishes completely [34].Diameter of fungus mycelia growth in all concentrations, measured every day until the average mycelia diameter of control sets reached to 8 cm on the fourth day, which was considered as the last measurement day [43].

Statistical analysis

Statistical analysis of the data obtained in the present study was carried out using split factorial method in a completely randomized design layout with 3 replications. Data obtained were subjected to analysis of variance (ANOVA).

RESULTS

In vitro antifungal activity of the thymol was evaluated against postharvest pathogen. At all concentrations tested thymol exerted a strong inhibitory effect on fungal growth where the percentage of inhibition was between 91.7 to 92.44. The most effective concentration of thymol was 250 $\mu l l^{-1}$ and 125$\mu l l^{-1}$ was the least effective concentration. These are in agreement with results obtained by Lira–Mota et al., [28]. At all concentrations tested thymol exerted a strong inhibitory effect on the germination process of the sporangiospores of *R. oryzae*, where the percentage of inhibition varied between 94 and 100% [26]. The essential oil of lemongrass and cumin showed under in vitro condition at 1µL concentration, a significant antifungal activity against the mycelia growth of *B. cinerea*, with inhibition rate of 64.62 and 52.16% respectively. Besides application of thyme oil at the rate of 1µL had a slight effect with inhibition rate of 10.77% [38].

The inhibition of the fungi was depending on the concentration of thymol and method of treatment. In all cases, PDM, adding to PDA and spraying the thymol respectively presented noticeable antifungal activity under invitro conditionswhere the percentage of inhibition varied between 89.77 to 93.33%.

Antifungal effect of thymol perfectly depends on method used in the experiment [6]. Percentage of mycelia inhibition was assessed effective in PDM and adding to PDA method than spraying method. Apparently, the former method (PDM) facilitated the vaporization of the volatile compound [44]. PDM method had the most inhibitory effect on fungi growth and spraying method had the least effect. Our results were in agreement with those obtained by Asghari et al., (2009). They suggested that among three methods that were used (PDM, spraying, adding to PDA) the most effective method was PDM, which reduced the mycelia growth [4]. Vesaltalab et al., [43] suggested that fungicidal activity of clove essential oil in contact method (adding to PDA) took place better than in vapor phase.

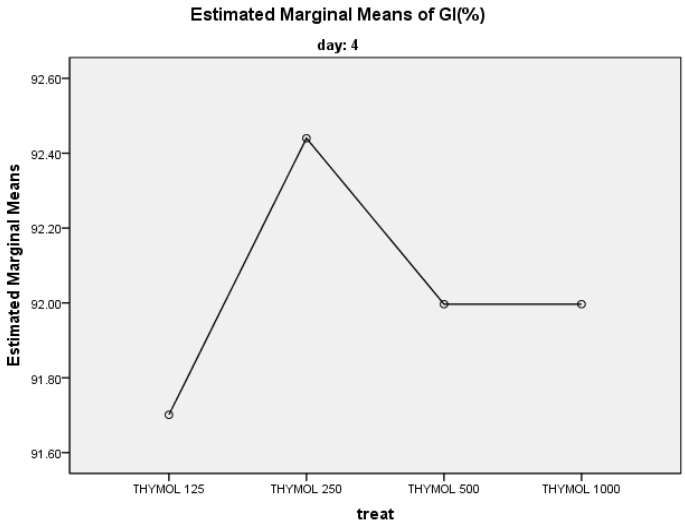

Figure 1The effect of thymol treatment on GI%

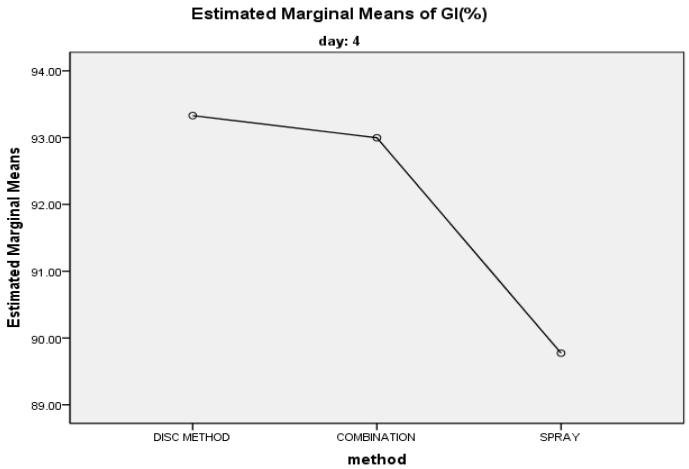

Figure 2The effect of method of treatment on GI%

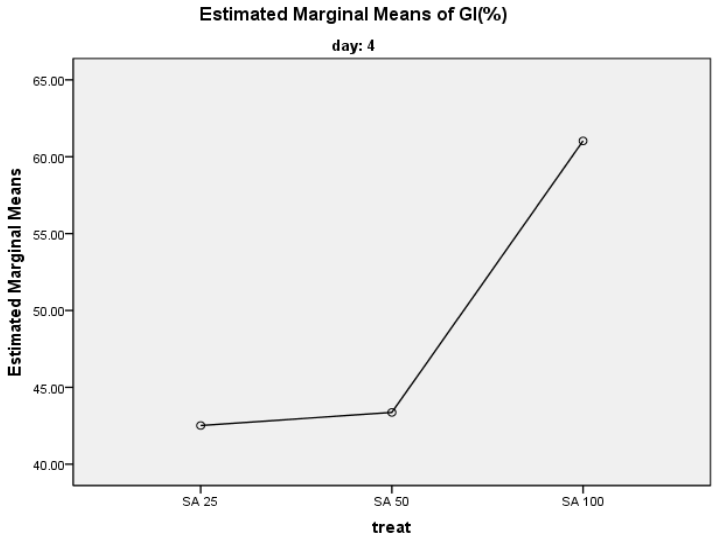

Figure 3The effect of SA treatment on GI%

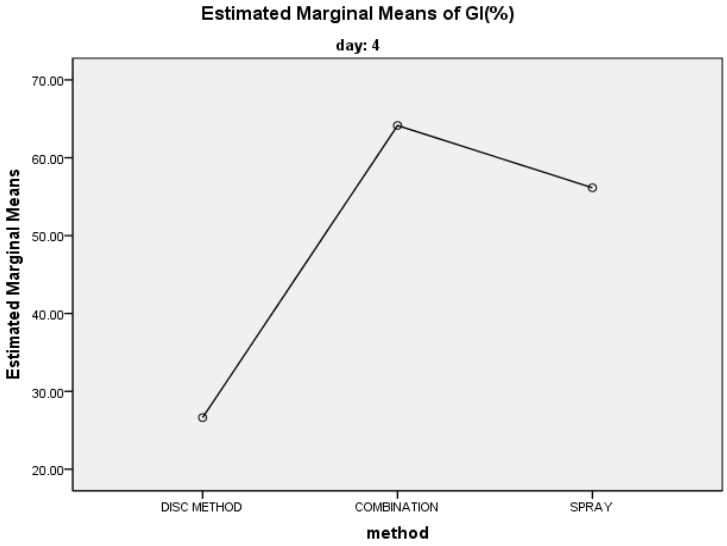

Figure 4The effect of method of treatment on GI%

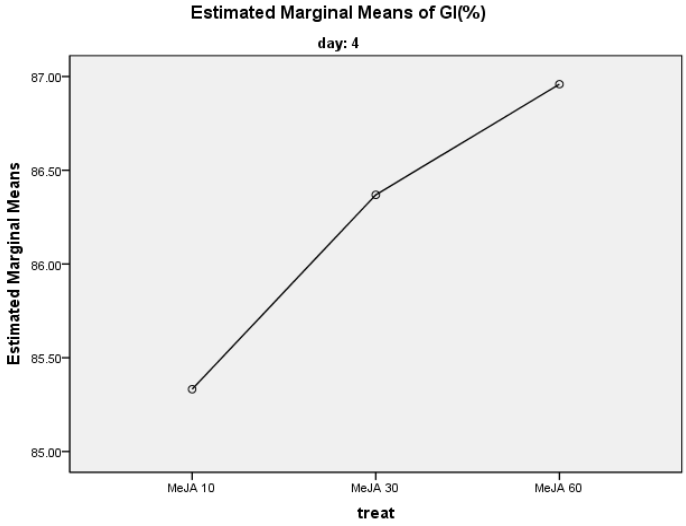

Figure 5Theeffect of MeJA treatment on GI%

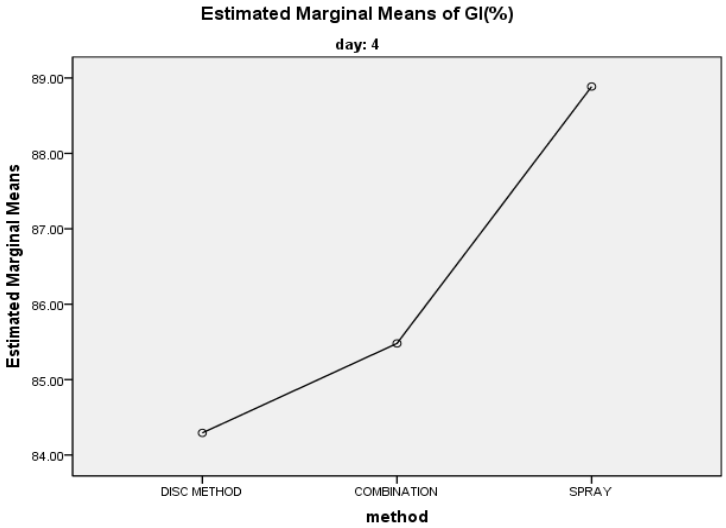

Figure 6The effect of method of treatment on GI%

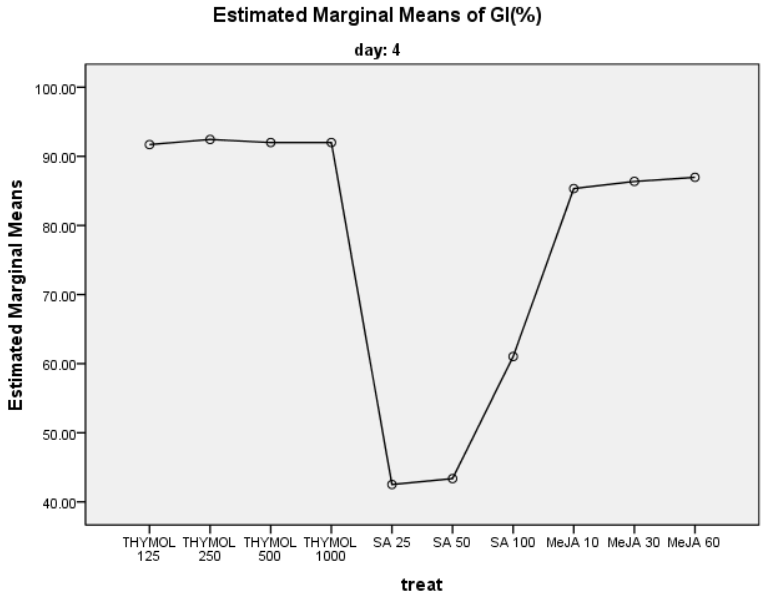

Figure 7The differences between thymol, SA, MeJA treatment effects on GI%

The incidence of gray mold rot and total decay of *B. cinerea* inoculated sweet cherries was significantly reduced by fumigation with thymol [27].Thymol exhibited approximately two to four times more potent fungicidal activity compared to the essential oil of *T. vulgaris*[26].Thymol exhibited strong fungistatic and fungicidal activities against *Aspergillus, Penicillium, Cladosporium, Trichoderma, Mucor and Rhizopus*[26].
The data shows that 25 and 50 $\mu l l^{-1}$ concentrations of SA had the least effect on fungal growth where the percentages of inhibition were 42.51 and 43.36, respectively. The most effective concentration of SA was 100 $\mu l l^{-1}$ where the percentage of mycelia growth inhibition was 61.03. No difference was found between 25 and 50 $\mu l l^{-1}$ concentrations.
Preliminary experiments have shown that adding to PDA method produced more profound effects compared to PDM and spraying methods. A striking difference in the severity of fungal growth between control and SA treatments was observed after 4 days. The efficacy of SA was influenced by many factors such as application concentration, method of treatment, pH of solution [37]. SA treatment at concentration of 0.5mμ could significantly enhance resistance against infection by *Penicillium expansum* [12].SA was effective in inhibitory mycelia growth of *Monilinia fructicola* on PDA [46].
The results showed that at all concentrations tested MeJA exerted inhibitory effect on fungal growth where the percentage of inhibition was between 85.33-86.95 and no differences were found among different MeJA levels . These are in agreement with results obtained by Ghasemnezhad and Javaherdashti [21].

Besides, spraying method was the most effective method where the percentage of mycelia growth inhibition was 88.88. PDM and adding to PDA methods had the least effect in decreasing fungal growth. Our results were in agreement with those obtained by Wang and Buta [44]. MeJA (200µmoll^{-1}) markedly inhibited mycelia growth of *P. expansum In vitro* [44]. However, MeJA had a little inhibition on mycelia growth of *M.fructicola*compared with the control [46]. These data are very similar to those of Tsao and Zhou [42].

As a general result among all of these treatments thymol in all concentrations (125, 250, 500 and 1000 µll^{-1}) produced more profound effects and the least effect on fungal growth were observed in SA with 25 and 50 µll^{-1} concentrations.

DISCUSSION AND CONCLUSION

According to Kalemba and Kunicka, (2003) antifungal activity can be classified in the following decreasing order:

Phenols >Aldehydes >Ketones >Alcohols >Esters > Hydrocarbons [24].

Therefore in agreement with these observations phenolic compounds such as thymol have strong activity.It seems possible that phenol components may interfere with cell wall enzymes like chitin synthase/chitinase as well as with the α- and β- glucanase of the fungus [1].Thymol has very high antifungal activities even higher than the commercial fungicide bifonazole[40].Biologically active natural products have the potential to replace synthetic fungicides [34]. Thymol thought to play in the plant defense mechanism against phytopathogenic microorganisms [32].Ergosterol is the principal sterol present in yeasts and filamentous fungi, where it is necessary for the growth and normal function of the fungal cell membrane. Besides controlling the fluidity, asymmetry and integrity of the membrane, ergosterol contributes to the proper functioning of enzymes bound to the membrane[28].The majority of existing drugs for the treatment of fungal infections target the cell wall or plasma membrane directly or indirectly, particularly ergostrol and its biosynthesis [28, 35].Essential oils and their phytoconstituents with ergosterol of the membrane, which binds directly to ergosterol and forms pores that destabilize the membrane, resulting in eventual loss of intracellular material and cell lysis [8, 35].The researchers believe antifungal activity of vapor phase is result of indirect effects of essential oils and their constituents on mycelium and lipophilic properties of essential oils provide the opportunity to be absorbed by the mycelium [43].SA is a molecule involved in some signal transduction systems, which induce biosynthesis of defense compounds such as polyphenols, alkaloids or pathogenesis-related (PR) proteins [39, 46]. Xu and Tian [45] indicated that SA treatment stimulated the activities of antioxidant enzymes and they concluded that activation of antioxidant defense plays the main role in resistance against *P. expansum*. Resistance to pathogens is based on both constitutive and inducible by biotic or abiotic agents such as SA [46]. SA treatment increased the level of five heat shock proteins (HSP), in addition to antioxidant and pathogen related proteins. HSP are of the major classes of chaperone molecules and play many roles inside the cells. These proteins may act as a primary defense mechanism during oxidative stresses caused by pathogens, thus preventing damage of ROS to cellular membrane [13]. Recent studies have shown that SA can be introduced as a potent alternative to chemicals [5].MeJA has been shown to induce the synthesis and expression of some stress proteins such as heat shock proteins (HSP) and pathogen-related proteins (PR) which lead to the increased resistance of the pathogens and the decreased incidence of the decay [17, 18].MeJA has direct inhibitory effect on pathogen growth because of the induction of natural disease resistance [9, 48, 11,19]. Generally, among all of mentioned treatments thymol in all concentrations (125, 250, 500 and 1000 µll^{-1}) produced more profound effects and the least effect on fungal growth were observed in SA with 25 and 50 µll^{-1} concentrations and the most effective method in thymol was PDM and in SA and MeJA spraying method was more effective than PDM.

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