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# Clove Buds (*Eugenia caryophyllata*) and Rosemary (*Rosmarinus officinalis*) Essential Oils Effects on Control of Grapes Gray Mold *in-vitro*

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**SUMMARY**

*The use of natural compounds such as essential oils is a newly growing idea in post harvest technology. In this study, effect of clove buds and rosemary essential oils on grapes gray mold was studied in two different experiments. First, the effect of different concentrations of essential oils on inhibition of mycelium growth of Botrytis cinerea was evaluated on PDA media, in vapor (0, 50, 100, 150, 300, 450 and 600 ppm) and contact (0, 150, 300, 450 and 600 ppm) method. In the second experiment, the effect of essential oil in vapor and dipping method on control of disease severity was investigated by spraying Botrytis spores on berries and keeping them at 15° C, for 7 days. All concentrations inhibited the growth of B. cinerea on PDA depending on essential oil concentration. 300 ppm of clove essential oil was determined as minimum inhibitory concentration (MIC) in contact method while no concentration of rosemary essential oil showed perfect inhibition in this method. But rosemary essential oil in vapor phase was more effective than contact method and inhibited mycelium growth in 300 ppm. 450 ppm of clove essential oil in vapor phase completely controlled gray mold on inoculated berries with keeping their appearance as normal, while 450 ppm of rosemary essential oil in vapor phase with complete control of gray mold had phytotoxic effects on grapes. These results suggest that clove essential oil in vapor phase could be used as an innovative tool to control fungal decay during table grapes storage.*

**Key words**: *Botrytis cinerea*, non-chemical control, Thompson Seedless.

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## INTRODUCTION

*Botrytis cinerea* is a major cause of pre and post-harvest loss in table grapes even at low temperature [1]. This fungus could easily grow at -0.5 °C and rapidly expand in berries [2] and cause considerable damage to the berries [3]. In Iran *Penicillium* and *Botrytis* are two important fungi that mainly cause damage to table grapes [4, 5].

Until now several pre and post-harvest techniques have been used to control post-harvest fungi decay of table grapes. Use of sulphur dioxide in the cold storage as a comfortable and economical method of control currently has many applications [6]. The adverse effects of sulfur dioxide include: over sulphite residue during the food chain is an important consumer problem that causing extreme sensitivity in some of them [7], the hair crack of berries skin [8], bleaching berries induce depressed areas and cause accelerated loss of water [9]. Development of resistance to common fungicides in post-harvest pathogens [10] and lack of new and recommended fungicides [7] is also considerable. Therefore, introducing a new way of increasing storage life and maintaining product quality with minimum safety risks is necessary. In recent decades, use of essential oils and plant extracts on control of horticultural crops post-harvest decay was introduced as a new and safe strategy. Essential oils have long been known as having superior antifungal compounds [11], but they have not been developed into products for post-harvest treatments, since newly synthesized compounds seem to be preferred by industry because of their easier use and protect than natural plant products. Antifungal essential oils are shown to be useful in controlling latent

infections which are residing in an inactive state within the host tissue such as *Botrytis*, compared to synthetic fungicides [12; 13; 14].

Since Iran is one of the rich resources of medicinal plants, evaluation of the effects of herbal essential oils on various products such as grapes seems to be a necessary task. The purpose of this study was investigation of clove and rosemary essential oils effects on *in-vitro* control of *B. cinerea*, with regarding appearance and quality preservation on Thompson Seedless table grapes.

## MATERIALS AND METHODS

### Plant materials

Head branches of *Rosmarinus officinalis* (fresh herb) in flowering stage were collected from the Hamedan Medicinal Plants Garden in late August. Clove buds (dried herbs) were bought from local medicinal plants market. Clove bud and rosemary essential oils were prepared by Clevenger with steam distillation for three hours. Essential oils were stored in a closed dark glass containers at 4 °C until use.

### Experimental design

Two separate experiments were performed to study the effect of essential oils on *B. cinerea*. First; on Potato-Dextrose-Agar (PDA) *in-vitro* and the second; on inoculated grape berries, using completely randomized design.

### *In-vitro* antifungal assay

*B. cinerea* was isolated from infected grape berries. Two methods were used to determine the effect of essential oils on mycelia growth of *B. cinerea*. In the first method as food poisoning or contact method, PDA plates containing; 0 (sterile distilled water), 150, 300, 450 and 600 ppm concentrations of essential oils with 0.5 ml of 0.1% Tween 80 [13] were used. Antifungal agents at above mention concentrations were added separately to PDA at 60 °C, mixed rapidly and poured into Petri dishes. After the agar had cooled, a mycelium disc from the edge of 7-day-old culture of the *B. cinerea* was added to each plate. In the second method to characterize the antifungal activity in fumigation method, a parallel study carried out using two plates, one containing sterile gauze with essential oils at 0 (sterile distilled water), 50, 100, 150, 300, 450 and 600 ppm concentrations and other plate containing PDA, prepared as described in first method. When above mentioned concentrations poured on the gauze two plates were immediately coherent to each other. After incubation at 24 °C the diameter of the mycelia colony was measured every day. Three plates were used in each replicate for each treatment and also the experiment was performed twice. Percentage of mycelia inhibition was calculated by the following formula:

$$\text{Percentage of mycelia inhibition} = (d_c - d_t / d_t) \times 100$$

$d_c$ is mean colony diameter of control sets and $d_t$ is mean colony diameter of treatments sets.

### Fungistatic or fungicidal properties of essential oils

For this purpose the mycelia discs from the treatments with no fungal growth, were subcultured on new PDA medium and fungal growth or no growth was recorded after a week.

### On berry antifungal assay

Fruits of grapes, (*Vitis vinifera* L. cv. Thompson Seedless) were harvested at commercial ripening stage from a local vineyard and immediately transferred to the laboratory. Homogenous size, shape, color and healthy berries were cut from the rachis with pedicel intact. The detached berries were then sprayed with the spore suspension $1.0\times 10^5$ spores/ml [3].The spore suspension was prepared by flooding plates with a small volume of sterile distilled water and spores were removed by gently scraping with a glass rod. The resulting spore suspension was filtered through four layers of cheesecloth to remove mycelia fragments and diluted with sterile water to obtain $1.0\times 10^5$ spores/ml by counting with hemocytometeric method. A volume of 50ml of inoculum per 900 berries was applied with a sprayer. Inoculated berries were kept in a closed container, at 15° C. In dipping method, after 24 hours, inoculated berries were immersed separately for 1min in 0, 150, 300, 450 and 600 ppm concentrations of rosemary and clove essential oils with 0.05% (w/v) Tween 80. All treated berries were allowed to air-dry for 30 minutes at 25° C. In essential oil fumigation section, glass jars with 400 ml of volume with 30 inoculated berries in each, were used. A piece of sterile gauze (6 × 12 cm) was paste to the center of each jar's lid and 0 (sterile distilled water), 50, 100, 150, 300, 450 and 600 ppm of rosemary and clove essential oils were poured into sterile gauze. Four replicates of every treatment were maintained at 15 ° C for 7 days.

Infection severity of single berry was evaluated and scaled as follows: 0: healthy berry; 1: one lesion lower than 3 mm in diameter; 2: one lesion lower than 10 mm in diameter; 3: several lesions or up to 25% of berry surface

infected and 4: more than 25% of the berry surface infected and/or sporulation can be seen. The decay index (DI) was calculated by the formula, DI= Σ (d*f*)/ ND, where d is the degrees of rot severity scored on the berry and f is its respective quantity; N is the total number of berries examined and D is the highest degree of disease severity occurring on the scale [15]. Berry appearance was evaluated to scales as follows**:** excellent (1), good (2), slightly dull (3), <50% brownish and soft berries (4) and>50% brownish and soft berries (5) [3].

**Statistical analysis**

All data were analyzed by analysis of variance using SAS software (version 9.1). Mean separations were performed by Duncan's multiple range test.

## RESULTS AND DISCUSSION

**Antifungal activity of essential oils on control of *B. cinerea in vitro***

Diameter of fungus mycelia growth in all treatments, measured every day until the average mycelia diameter of control sets reached to 8.93 cm on the fifth day, which was considered as the last measurement day. The results observed on the last day due to their significances, were expressed. Both contact and fumigation methods of applying essential oil showed significant effect on mycelia growth inhibition ($p<0.01$) (Table 1). There was no difference in mycelia growth between the two sets of controls, one treated with distilled water and the other without it. The results showed that antifungal activity of these compounds was depended on plant type, concentration and the application method. Percentage of mycelia inhibition was assessed effective in clove than rosemary product and essential oils in vapor phase than contact method ($p<0.01$) (Table 2).

When essential oils were applied in food poisoning method (contact), Different concentrations of rosemary essential oil showed significant differences in terms of mycelia growth inhibition, compared to each other and also to the control ($p<0.01$) (Fig 1). It is evident from these results that rosemary essential oil capability on biological control of *B. cinerea* amplified at a certain concentration. So that the 150, 300 and 450 ppm concentrations indicated gradual increase to 30.94% of inhibition, while inhibitory effect of 600 ppm increased up to 66.40%. Clove essential oil in concentrations of 300, 450 and 600 ppm completely inhibited the fungal growth. The mycelia disc sampled from the plates treated with clove essential oil in all three concentrations and cultured in new medium, but none of them showed mycelia growth, therefore 300 ppm was determined as minimum inhibitory concentration (MIC) of clove essential oil in contact method for this experiment. In this section antifungal activity of clove essential oil was demonstrated more effective than rosemary essential oil.

**Table 1- Antifungal activity of essential oils on control of *B. cinerea in-vitro* condition**

| Treatment | Concentration (ppm) | mean colony diameter cm | Mycelia growth inhibition % |
|---|---|---|---|
| Control (dry) | 0 | $8/933^{a}$ | $0^{a}$ |
| Control + sterile distilled water | 0 | $8/933^{a}$ | $0^{a}$ |
| Rosemary essential oil (contact) | 150 | $8/283^{bcd}$ | $7/242^{bcd}$ |
| | 300 | $6/466^{f}$ | $27/585^{f}$ |
| | 450 | $6/166^{f}$ | $30/944^{f}$ |
| | 600 | $3/00^{h}$ | $66/405^{h}$ |
| Clove essential oil (contact) | 150 | $2/800^{h}$ | $68/645^{h}$ |
| | 300 | $0^{l}$ | $100^{l}$ |
| | 450 | $0^{l}$ | $100^{l}$ |
| | 600 | $0^{l}$ | $100^{l}$ |
| Rosemary essential oil (vapor) | 50 | $5/466^{g}$ | $38/783^{g}$ |
| | 100 | $1/333^{j}$ | $85/069^{j}$ |
| | 150 | $0/4667^{kl}$ | $94/774^{kl}$ |
| | 300 | $0^{l}$ | $100^{l}$ |
| | 450 | $0^{l}$ | $100^{l}$ |
| | 600 | $0^{l}$ | $100^{l}$ |
| Clove essential oil (vapor) | 50 | $2/050^{i}$ | $77/044^{i}$ |
| | 100 | $1/266^{j}$ | $85/816^{j}$ |
| | 150 | $0/660^{k}$ | $92/609^{k}$ |
| | 300 | $0/633^{k}$ | $92/90^{k}$ |
| | 450 | $0^{l}$ | $100^{l}$ |
| | 600 | $0^{l}$ | $100^{l}$ |

*Mean values followed by different letters within the column are significantly different according to Duncan multiple range test (P< 0.01).*

**Table 2- Orthogonal contrast treatments on control of *B. cinerea in vitro* condition**

| contrast | Degree of Freedom | Mean square of colony size |
|---|---|---|
| Clove and rosemary essential oil (contact) | 1 | 167/21** |
| Clove and rosemary essential oil (vapor) | 1 | 1/76** |
| Vapor and contact method | 1 | 116/78** |

***Means significant differences at (P< 0.01)*

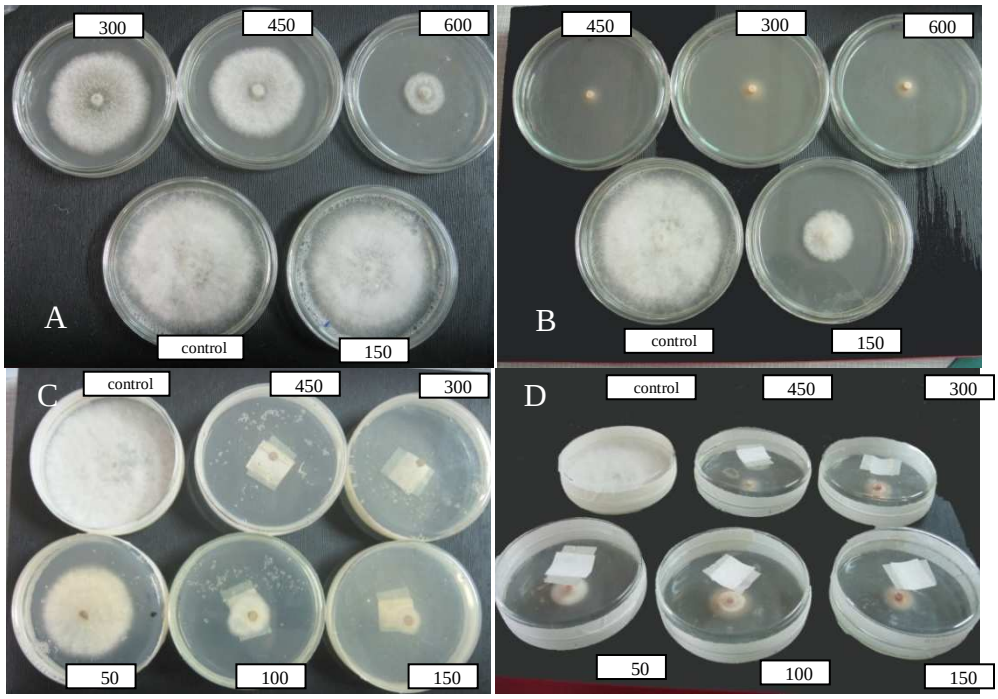


**Fig. 1- Antifungal activity of rosemary essential oil (A), clove essential oil (B) in contact method, rosemary essential oil (C), clove essential oil (D) in fumigation method against *B. cinerea in-vitro* condition**

In application of essential oils in fumigant method, results showed the percentage of rosemary essential oil inhibition from 50 to 100 ppm was highly increased to more than double (Fig.1) in a way that 300, 450 and 600 ppm of rosemary oil completely stopped the fungus growth. The mycelia discs affected in above mentioned treatment were removed and cultured in normal PDA medium, fungus growth resumed in the new medium for all samples. Results suggest that activity of rosemary essential oil is fungistatic not fungicidal. Antifungal effect of essential oils perfectly depends on method used in the experiment [16]. Increasing the antifungal activity of rosemary essential oil in vapor phase compared to contact method is reported by [17] which is in consistent with the results of our study. The researchers believe antifungal activity of vapor phase is result of indirect effects of essential oils on mycelium and lipophilic properties of essential oils provide the opportunity to be absorbed by the mycelium. After subculture of mycelia disc from 450 and 600 ppm of clove essential oil, no mycelium was observed in one of three replicates of 450 ppm and in all replicates of 600 ppm. This could be due to fungistatic activity occurrence in low concentrations and fungicidal effects at higher concentrations [18] which is considered the obvious reason for this view. It seems that fungicidal activity of clove essential oil in contact method took place better than in vapor phase. Antifungal activity of plant product such as essential oils and extracts is proven in various researches including for clove [14, 19, 20, 21, 22, 23, 24, 25] and rosemary [5, 17, 26, 27, 28, 29, 30, 31].

Antifungal activity of clove essential oil was reported strong among many essential oils [14, 32], better performance than the rosemary essential oil in the prevention of *B. cinerea* spore germination [19] and control of *Alternaria porri* [17]. These reports are in accordance with the results of our study. The antimicrobial activity of essential oils is strictly connected to their chemical composition[32].Although, the antimicrobial activity of an essential oil is attributed mainly to its major compounds but each component has its own contribution on biological activity and synergistic or antagonistic effect of one compound in minor percentage in the mixture has to be considered. Also essential oils due to the large number of compounds may have more than one site of action [33, 34]. The essential oils containing phenolic group as their major component, indicate higher activity against microorganisms and clove essential oil belongs to this group; while the rosemary essential oil is rich in 1, 8-Cineol ethers, with weaker

antifungal activity than phenols [32]. This can somehow explain different performance of clove and rosemary oils observed in this study. Morphological changes due to essential oils effect were observed in microscopic level such as compact and highly branched mycelia mass and also on the colony appearance (data not shown) which is in accordance with [32] and [17] reports.

Although these effects may be responsible for reducing mycelia growth rate, however, the mechanism of action of these compounds is not well known. Essential oils can cause damage to proteins and lipids [34] also prevent making DNAs and RNAs and polysaccharides in the fungal cells [32].

**Effect of essential oils on the severity of gray mold and keeping appearance quality of inoculated berries**

There was significant differences between control inoculated berries and treatments ($p<0.01$), (Table 3). No significant differences were observed between four types of controls set in this experiment. But, it was obvious that the control berries immersed in distilled water are showing lower infection than dry controls which could be due to washing spores off the berries.

**Table 3- The antifungal activity of essential oils on severity of gray mold growth on inoculated berries and their appearance at 15 $^0$C**

| Treatments | Concentration (ppm) | Disease severity | appearance |
|---|---|---|---|
| Control 1 (dry) | 0 | 0/103$^{i}$ | 2/00$^{f}$ |
| Control 2 (dipped in distilled water) | 0 | 0/090$^{i}$ | 2/250$^{f}$ |
| Control 3 (dry inoculated) | 0 | 0/659$^{a}$ | 4/750$^{a}$ |
| Control 4 (inoculated and dipped in distilled water) | 0 | 0/637$^{a}$ | 4/750$^{a}$ |
| Rosemary essential oil (dipping) | 150 | 0/515$^{b}$ | 4/250$^{b}$ |
| | 300 | 0/443$^{cd}$ | 4/00$^{b}$ |
| | 450 | 0/362$^{ef}$ | 3/50$^{c}$ |
| | 600 | 0/359$^{ef}$ | 3/50$^{c}$ |
| Clove essential oil (dipping) | 150 | 0/434$^{cd}$ | 4/250$^{b}$ |
| | 300 | 0/256$^{g}$ | 4/00$^{b}$ |
| | 450 | 0/246$^{g}$ | 3/50$^{c}$ |
| | 600 | 0/418$^{d}$ | 4/00$^{b}$ |
| Rosemary essential oil (vapor) | 50 | 0/256$^{g}$ | 4/00$^{b}$ |
| | 100 | 0/156$^{h}$ | 4/00$^{b}$ |
| | 150 | 0/096$^{i}$ | 5/00$^{a}$ |
| | 300 | 0/053$^{ijk}$ | 5/00$^{a}$ |
| | 450 | 0/009$^{jk}$ | 5/00$^{a}$ |
| | 600 | 0$^{k}$ | 5/00$^{a}$ |
| Clove essential oil (vapor) | 50 | 0/090$^{i}$ | 2/750$^{e}$ |
| | 100 | 0/059$^{ij}$ | 2/750$^{e}$ |
| | 150 | 0/015$^{jk}$ | 3/0$^{de}$ |
| | 300 | 0/003$^{k}$ | 3/500$^{c}$ |
| | 450 | 0$^{k}$ | 4/00$^{b}$ |
| | 600 | 0$^{k}$ | 4/00$^{b}$ |

*Mean values followed by different letters within the column are significantly different according to Duncan multiple range test (P< 0.01).*

The gray mold severity control was associated with essential oils source, concentration, application method and their effect on sporulation. The berries appearance was influenced by severity of gray mold and also by the effect of essential oils used on them. In this part of experiment, clove essential oil performed more effective than rosemary essential oil and fumigation method worked better than dipping method (Table 4).

**Table 4- Orthogonal contrast treatments on control of gray mold on inoculated berry**

| Contrast | Degree of freedom | Mean of square Disease severity | Mean of square appearance |
|---|---|---|---|
| Clove and rosemary essential oil (dipping) | 1 | 0/052$^{**}$ | 0/031$^{**}$ |
| Clove and rosemary essential oil (vapor) | 1 | 0/054$^{**}$ | 21/333$^{**}$ |
| Vapor and dipping method | 1 | 2/044$^{**}$ | 3/515$^{**}$ |

$^{**}$ *Means significant differences at (P< 0.01)*

Rosemary essential oil in all concentrations gradually decreased disease expansion on berries. Clove essential oil also showed gradual effect from 150 to 300 ppm and reduced disease expansion significantly, but there was no significant difference between 300 and 450 ppm treatments.

Contradictory to our expectation, 600 ppm of clove essential oil increased severity of disease. It is likely that the high concentration of clove essential oil may destroy the skin of berries which is considered great protective barrier

against pathogens, and thus the severity of gray mold was increased. Also appearance index of berries in immersion treatments showed significant difference with control. View appearance of berries in this experiment showed brown spots in some areas in addition to the other areas that mycelium development occurred (same as in control sets). Appearance of berry was outcome of disease severity and immersion of berries in oil treatment.

Essential oils in fumigant method, caused great reduction in gray mold disease severity ($p<0.01$). In 600 ppm concentration of rosemary essential oil and 450 and 600 ppm concentrations of clove essential oil, no mycelium growth of *B. cinerea* was observed. In these treatments no sporulation occurred. Appearance of mycelia mass in low concentrations of clove and especially rosemary essential oils treatments was different than control. Pressed mycelia mass was observed in light brown under clove essential oil and white in rosemary essential oil (Fig.2).

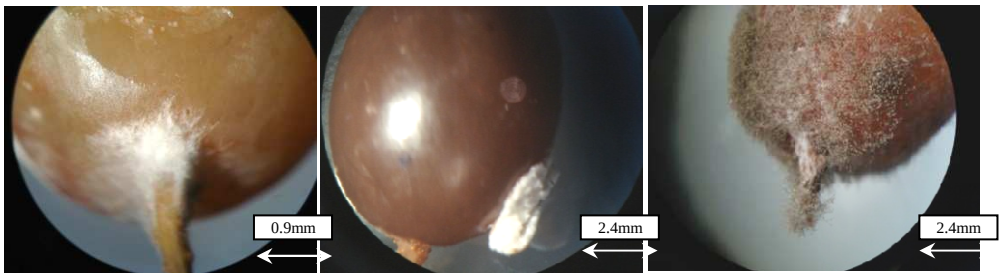


**Fig.2-The effect of clove and rosemary essential oils in vapor phase on control of *B. cinerea* on inoculated berries (respectively from right: control, rosemary and clove essential oils)**

Appearance index enhanced with increasing concentration of essential oils, showing the phytotoxic effect of these compounds in high concentrations at 15 °C. Rosemary essential oil in vapor phase, in addition of skin, pulp was also affected and turned to browning. Jobling (2000) believes the use of essential oils via the vapor phase would make them more effective than dipping. The results of our study also confirmed that So far some researchers have been performed in this field. For example; use of carvacrol [35], grape seed extract [3], ginger, peach and holy basil essential oils [13] and eugenol and thymol [36] on grapes, use of thyme, rosemary and basil essential oils on control of gray mold in pears [5] and English Violet on tomatoes [37]. Regarding the results of this study, application of clove essential oils in vapor phase was shown to have effective performance on control of gray mold, so its use for fumigation in cold storage could be an interesting investigation field for active packing of table grapes.

## CONCLUSION

In conclusion the results of this study showed that essential oils derived from clove and rosemary may be used as alternative for the control of gray mould on table grapes at post-harvest as a natural fumigant in closed container or packaging. These oils under *in-vitro* and *in-vivo* conditions showed a good but with some adverse effect on quality parameters under some treatments of oils. Further study especially under *in-vivo* conditions is recommended to plan and confirm the preservative capacity of essential oils, which may be used for preservation and/or extension of self-life of table grapes and also other fruits and vegetables.

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