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Postharvest Application of Chitosan and Thymol For Controlling Grey Mould and Soft Rot diseases of Strawberry Fruits

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Abstract : Evaluation the efficacy of chitosan and Thymol for controlling postharvest diseases of strawberry fruits was studied.
In vitro trails **,** results revealed that all tested concentrations of both chemicals significantly reduced the linear growth of both fungi. Complete inhibition of linear growth was obtained with chitosan and Thymol at concentrations of 5.0 and 6.0 g /L for *B. cinerea and R. stolonifer* respectively. Also, complete inhibition of spore germination was obtained with chitosan and Thymol at concentrations of 5.0 and 6.0 g /L for *B. cinerea and R. stolonifer* respectively. The highest reduction was obtained with chitosan and Thymol at concentrations of 4.0 g /L which reduced spore germination more than 79.4 and 67.4% for *B. cinerea and R. stolonifer* respectively**.** Moreover, in vivo trails , results indicated that all tested concentrations of both chemicals significantly reduced the disease incidence. The most effective treatments are chitosan and Thymol at concentrations of 6.0 g /L which reduced the disease incidence by 88.0 &84.0 and 92.0 & 86.0 % for grey mould and soft rot respectively. The highest reduction was obtained with chitosan and Thymol at concentrations of 5.0 g /L which reduced both diseases more than 76.0 %.
As for disease severity, results indicated that all tested concentrations of both chemicals significantly reduced the disease severity of grey mould and soft rot. The most effective treatments are chitosan and Thymol at concentrations of 6.0 g /L which reduced the disease severity more than 91.0 and 85.5 % for grey mould and soft rot respectively. The highest reduction was obtained with chitosan and Thymol at concentrations of 5.0 g /L which reduced both diseases more than 81.8% . It could be suggested that chitosan and Thymol essential oil are excellent treatments for controlling postharvest diseases of strawberry fruits.
Key words : Strawberry fruits – Postharvest diseases- Chitosan – Thymol - Grey mould- Soft rot.

Introduction

Botrytis cinerea, and *Rhizopus stolonifer* are the microorganisms that most commonly attack strawberries (*Fragaria ananassa* L.)[1]**.** The strawberry fruit rot (grey mould) caused by *Botrytis cinerea* develops in any part of the fruit, but is mostly found on the calyx end or on the sides of fruit in contact with other rotten fruit. *Rhizopus stolonifer* fruit rot is associated with the presence of wounds in the fruit, but may also occur in intact fruit [2,3]**.** Using of chemical fungicides gave satisfactory control against mould infection , but have residual harmful effect to human and environment. Therefore, alternative fungicide treatments are needed for the management of postharvest diseases of fruits[4,5]. Several naturally occurring essential oils have been identified to be effective in inhibiting microbial growth[6,7] and have started to become an effective alternative to synthetic fungicides [8]. Thymol is a natural monoterpene phenol derivative of cymene, found in oil of thyme, as

a white crystalline substance and strong antiseptic properties [9]. Thymol has anti- microbial activity because of its phenolic structure. Thymol is listed by the food and drug administration (FDA) as foods for human consumption. It is considered generally recognized as safe GRAS **[10]**. Treatment of strawberry with Thymol, eugenol, or menthol significantly delayed deterioration of the fruit[(11)] and improved fruit quality and safety in table grapes and sweet cherries [12,13,14]**.**

Chitosan is a linear amino polysaccharide of glucosamine and N-acetylglucosamine units and is obtained by alkaline deacetylation of chitin extracted from the exoskeleton of crustaceans such as shrimps and crabs, as well from the cell walls of some fungi [15]**.** Chitosan is soluble in dilute organic acids, and could theoretically be used as a preservative for coating fruit. The coating is non-toxic and safe [16,17].

Chitosan coating is known to have the potential to prolong the storage life and control the decay of strawberries, tomatoes, peaches, pears, kiwifruit, litchi, apples, and citrus fruits, as examined by [4,18,19,20 ,21]. The aim of this study was to evaluate the efficacy of chirtosan and Thymol for controlling postharvest diseases of strawberry fruits.

Materials and Methods

Fungal isolates and strawberry fruits

Botrytis cinerea, and *Rhizopus stolonifer*, the causal organisms of grey and soft moulds respectively were obtained from Plant Pathol. Dept., (NRC) Egypt and were maintained on potato dextrose agar PDA for further study. While, strawberry fruits were obtained from the Department , of Vegetable Crop Research, Agricultural Research Centre, Giza, Egypt.

Chitosan and Thymol

Chitosan and Thymol (essential oil) were purchased from Sigma chemical Co. and used in the present study.

In vitro trails

Testing of different concentrations of chitosan and Thymol against linear growth of pathogenic fungi

Chitosan and Thymol at different concentrations *i.e* 0.0 ,1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 g /L were tested to study their inhibitory effect on linear growth of *B. cinerea and R. stolonifer* . Chemical solutions were added to conical flasks containing sterilized PDA medium to obtain the proposed concentrations, then mixed gently and dispensed in sterilized Petri plates (9 cm – diameter). Plates were individually inoculated at the center with equal disks (6-mm- diameter) of 10-days old culture of *B. cinerea and R. stolonifer*. Five plates were used as replicates for each particular treatments . Inoculated plates were incubated at 20 ± 2C° .The average linear growth of tested fungi was calculated after 10 days of incubation .

Testing of different concentrations of chitosan and Thymol against spore germination of pathogenic fungi

Chitosan and Thymol at different concentrations *i.e* 0.0 ,1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 g /L were tested to study their inhibitory effect on spore germination of *B. cinerea and R. stolonifer*.. Spores of 10-days- old cultures of each fungus were harvested in sterilized water (containing 0.01% Tween 80) then adjusted to reach concentration of 10^6 spore / ml . One ml of each prepared spore suspension was placed in Petri plates. PDA media containing different chemical concentrations were poured before solidifying into the previous inoculated plates and rotated gently to ensure even distribution of fungal spores. Inoculated plates were incubated at 20°C for 24 h. Germinated spores were counted microscopically and percentage of spore germination was calculated.

Controlling grey mould and soft rot of strawberry fruits in vivo

Testing of different concentrations of chitosan and Thymol against grey mould and soft rot of strawberry fruits in vivo

Healthy and fresh strawberry fruits cv. Camarosa apparently free of physical damages and diseases were used in this experiment. Fruits were immersing in water solutions containing chitosan and Thymol at different concentrations *i.e* 0.0 ,1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 g /L for 30 seconds and then air dried for two hours in laminar flow. Inoculation of fruits was carried out by spraying fruits with spore suspension (10^6 spores/ml) of *B. cinerea and R. stolonifer* individually then air dried. A set of inoculated fruits with *B. cinerea and R. stolonifer*, individually only were left as control. Each treatment as well as the control was performed in triplicate. All treated or un-treated (control) fruits were placed into carton boxes (46 × 23 × 30 cm) at the rate of 50 fruits/box and stored for 10 days at 20±2^{o}C and 90-95% relative humidity for assessment. The fruits were examined regularly to detect mould and regarded as infected if a visible lesion was observed. Results were expressed as percentage of fruit infected. Disease incidence (%) were expressed as percentage of fruit infected, while disease severity (%) were expressed as percentage of rotted part of fruit which was calculated from the following formula:

$$\text{Disease severity \%} = \frac{\text{Rotted part weight of fruit}}{\text{Fruit weight}} \text{ X } 100$$

Statistical analysis

Tukey test for multiple comparison among means was utilized [22].

Results

In vitro trails

Effevt of different concentrations of chitosan and Thymol against linear growth of pathogenic fungi

Chitosan and Thymol at different concentrations *i.e* 0.0 ,1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 g /L were tested to study their inhibitory effect on linear growth of *B. cinerea and R. stolonifer* . Results in Table (1) reveal that all tested concentrations of both chemicals significantly reduced the linear growth of both fungi. Complete inhibition of linear growth was obtained with chitosan and Thymol at concentrations of 5.0 and 6.0 g /L for *B. cinerea and R. stolonifer* respectively. The highest reduction was obtained with chitosan and Thymol at concentrations of 4.0 g /L which reduced linear growth more than 79.8 and 59.6 % for *B. cinerea and R. stolonifer* respectively. Meanwhile, other concentrations were less effective.

Table 1. Linear growth of *Botrytis cinerea,* and *Rhizopus stolonifer* as affected with different concentrations of chitosan and Thymol

Treatment	Conc. (g/L)	Linear growth (mm)			
		Botrytis cinerea		*Rhizopus stolonifer*	
		Linear	Reduction	Linear	Reduction
Chitosan	1.0	64.2 c	28.7	75.0 b	16.7
	2.0	54.0 d	40.0	62.0 c	31.1
	3.0	23.4 f	74.0	51.0 d	43.3
	4.0	12.2 g	86.4	33.5 e	62.8
	5.0	0.0 h	100.0	18.0 f	80.0
	6.0	0.0 h	100.0	0.0 g	100.0
Thymol	1.0	74.0 b	17.8	76.0 b	15.6
	2.0	56.0 d	37.8	64.5 c	28.3
	3.0	32.1 e	64.3	54.0 d	40.0
	4.0	18.2 f	79.8	36.4 e	59.6
	5.0	0.0 h	100.0	22.1 f	75.4
	6.0	0.0 h	100.0	0.0 g	100.0
Control	00.0	90.0 a	00.0	90.0 a	00.0

Figures with the same letter are not significantly different (p=0.05)

Effect of different concentrations of chitosan and Thymol against spore germination of pathogenic fungi

Chitosan and Thymol at different concentrations *i.e* 0.0 ,1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 g /L were tested to study their inhibitory effect on spore germination of *B. cinerea and R. stolonifer*. Results in Table (2) indicate that all tested concentrations of both chemicals significantly reduced the spore germination of both fungi. Complete inhibition of spore germination was obtained with chitosan and Thymol at concentrations of 5.0 and 6.0 g /L for *B. cinerea and R. stolonifer* respectively. The highest reduction was obtained with chitosan and Thymol at concentrations of 4.0 g /L which reduced spore germination more than 79.4 and 67.4 % for *B. cinerea and R. stolonifer* respectively. Meanwhile, other concentrations were less effective.

Table 2. Spore germination of *Botrytis cinerea,* and *Rhizopus stolonifer* as affected with different concentrations of chitosan and Thymol

Treatment	Conc. (g/L)	Spore germination			
		Botrytis cinerea		*Botrytis cinerea*	
		Spore germination	Reduction %	Spore germination	Reduction %
Chitosan	1.0	54.0 c	41.9	76.0 b	17.4
	2.0	42.1 d	53.2	54.2 d	41.1
	3.0	18.0 f	80.6	42.0 e	54.3
	4.0	6.0 g	93.5	17.5 g	81.0
	5.0	0.0 g	100.0	7.0 h	92.4
	6.0	0.0 g	100.0	0.00 h	100.0
Thymol	1.0	67.5 b	24.4	80.0 b	13.0
	2.0	48.2 c	48.2	67.2 c	27.0
	3.0	30.0 e	67.7	44.0 e	52.2
	4.0	19.2 f	79.4	30.0 f	67.4
	5.0	0.00 g	100.0	14.0 g	84.8
	6.0	0.00 g	100.0	00.0 h	100.0
Control	00.0	93.0 a	00.0	92.0 a	00.0

Figures with the same letter are not significantly different (p=0.05)

Controlling grey mould and soft rot of strawberry fruits in vivo

Effect of different concentrations of chitosan and Thymol against grey mould and soft rot of strawberry fruits in vivo

Chitosan and Thymol at different concentrations *i.e* 0.0 ,1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 g /L were tested to study their effect against grey mould and soft rot of strawberry fruits in vivo.

a- Effect on disease incidence

Results in Table (3) indicate that all tested concentrations of both chemicals significantly reduced the disease incidence of both diseases . The most effective treatments are chitosan and Thymol at concentrations of 6.0 g /L which reduced the disease incidence by 88.0 &84.0 and 92.0 & 86.0 % for grey mould and soft rot respectively. The highest reduction was obtained with chitosan and Thymol at concentrations of 5.0 g /L which reduced both diseases more than 76.0 % . O other concentrations were less effective.

Table 3. Grey mould and soft rot incidence of strawberry fruits as affected with different concentrations of chitosan and Thymol

Treatment	Conc. (g/L)	Strawberry fruit rots			
		Grey mould		Soft rot	
		Disease incidence	Reduction%	Disease incidence	Reduction %
Chitosan	1.0	56.2 c	43.8	62.0 c	38.0
	2.0	44.0 d	66.0	51.0 d	49.0
	3.0	32.5 e	67.5	45.3 d	54.7
	4.0	22.5 fg	77.5	32.4 e	67.6
	5.0	18.0g	82.0	24.0 f	76.0
	6.0	12.0 h	88.0	16.0 g	84.0
Thymol	1.0	62.0 b	38.0	71.0 b	29.0
	2.0	51.4 d	48.6	58.0 c	62.0
	3.0	36.2 e	63.8	48.2 d	51.8
	4.0	22.1 fg	77.9	30.1 e	69.9
	5.0	14.0 h	86.0	20.4 f	79.6
	6.0	8.0 h	92.0	14.0 g	86.0
Control	00.0	100.0 a	0.0	100.0 a	00.0

Figures with the same letter are not significantly different (p=0.05)

a- Effect on disease severity

Results in Table (4) indicate that all tested concentrations of both chemicals significantly reduced the disease severity of grey mould and soft rot . The most effective treatments are chitosan and Thymol at concentrations of 6.0 g /L which reduced the disease severity more than 91.0 and 85.5 % for grey mould and soft rot respectively. The highest reduction was obtained with chitosan and Thymol at concentrations of 5.0 g /L which reduced both diseases more than 81.8% . Other concentrations were less effective.

Table 4. Grey mould and soft rot severity of strawberry fruits as affected with different concentrations of chitosan and Thymol

Treatment	Conc. (g/L)	Strawberry fruit rots			
		Grey mould		Soft rot	
		Disease severity	Reduction %	Disease severity	Reduction %
Chitosan	1.0	49.0 b	51.0	55.2 c	44.8
	2.0	40.0 c	60.0	46.4 d	53.6
	3.0	29.2 d	70.8	34.0 e	66.0
	4.0	18.4 e	81.6	28.5 f	71.5
	5.0	12.0 f	88.0	18.2 g	81.8
	6.0	9.0 g	91.0	14.5 gh	85.5
Thymol	1.0	55.0 b	45.0	65.2 b	37.8
	2.0	43.2 c	56.8	55.0 c	45.0
	3.0	29.0 d	71.0	41.2 d	58.8
	4.0	21.0 e	79.0	24.2 f	75.8
	5.0	12.0 f	88.0	18.0 g	82.0
	6.0	8.0 g	92.0	11.4 h	88.6
Control	00.0	100.0 a	0.0	100.0 a	00.0

Figures with the same letter are not significantly different (p=0.05)

Discussion

The strawberry fruit rot (grey mould and soft rot) caused by *Botrytis cinerea* and *Rhizopus stolonifer* respectively are the most important diseases attach strawberry fruits [1,2,3]**.** Using of chemical fungicides gave satisfactory control against mould infection, but have residual harmful effect to human and environment. Therefore, alternative fungicide treatments are needed for the management of postharvest diseases of fruits [4,5].

Thymol is a natural monoterpene phenol derivative of cymene, found in oil of thyme, as a white crystalline substance and strong antiseptic properties[9]**.** Thymol has anti- microbial activity because of its phenolic structure. Thymol is listed by the food and drug administration (FDA) as foods for human consumption. It is considered generally recognized as safe GRAS [10]**.** In the present study, results indicated that in vitro trails **,** Complete inhibition of linear growth and spore germination was obtained with chitosan and Thymol at concentrations of 5.0 and 6.0 g /L for *B. cinerea and R. stolonifer* respectively. Moreover, in vivo trails , results indicated that the most effective treatments are chitosan and Thymol at concentrations of 6.0 g /L which reduced the disease incidence and severity more than 88.0 % for grey mould and soft rot.

Treatment of strawberry with Thymol, eugenol, or menthol significantly delayed deterioration of the fruit [11] and improved fruit quality and safety in table grapes and sweet cherries [12,13,14]**.** Thymol has microbial activity because of its phenolic structure. Thymol is listed by the food and drug administration (FDA) as foods for human consumption. It is considered generally recognized as safe GRAS [10]**.** Moghtader[9] reported that the high percentage antifungal activities of *Thymus* oil are related with thymol as the main compound.

The antifungal effect of thyme oil by paper disk method against *Botrytis cinerea* in strawberry fruit was reported by [23,24]. The microbial mode of action of thymol has been postulated as disruption of cellular membrane functions and interference with active sites of enzymes and cellular metabolism[(25, 26)] and may change the permeability of membranes of the microbes for cations and alter the ion gradients that lead to impairment of vital processes in cells and eventually cell death [27]. Thymol with phenolic group as its major component have shown enhanced the shelf life of strawberry fruits during storage time by protecting them from grey mold. The fungicidal or fungistatic activity of the essential oil of *Thymus vulgaris* can be attributed to thymol, especially the hydroxyl group of this compound [28].

Chitosan is a linear amino polysaccharide of glucosamine and N-acetylglucosamine units and is obtained by alkaline deacetylation of chitin extracted from the exoskeleton of crustaceans such as shrimps and crabs, as well from the cell walls of some fungi[15,16]. In the present study, results indicated that in vitro trails**,** complete inhibition of linear growth and spore germination was obtained with chitosan at concentrations of 5.0 and 6.0 g /L for *B. cinerea and R. stolonifer* respectively. Moreover, in vivo trails , results indicated that the most effective treatments is chitosan at concentration of 6.0 g/L which reduced the disease incidence and severity more than 88.0 % for grey mould and soft rot.

Several hypotheses have been postulated by which chitosan affects the growth of pathogenic fungi [29], first: by its polycationic nature, it is believed that chitosan interferes with negatively charged residues of macromolecules exposed on the fungal cell surface. This interaction leads to the leakage of intracellular electrolytes and proteinaceous constituents. Second: the interaction of diffused hydrolysis products with microbial DNA, which leads to the inhibition of mRNA and protein synthesis, third: the interaction of chitosan with fungal DNA and RNA. Fourth: malformation of fungal mycelial. Chitosan coating is known to have the potential to prolong the storage life and control the decay of strawberries, tomatoes, peaches, pears, kiwifruit, litchi, apples, and citrus fruits, as examined by [4, 18,19,20 ,21,.]

Finally, controlling of plant diseases depends mainly on fungicides application such chemicals are not always desirable due to potential hazards to human beings and the environment. Alternatives approaches of fungicides are needed for controlling plant diseases [30,31,32,33,34,35,36,37,38]. It could be suggested that chitosan and Thymol essential oil are excellent treatments for controlling postharvest diseases of strawberry fruits.

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