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Study the effect of chitosan, vanillin, and acetic acid on fungal disease control of Rhizopus stolonifer in strawberry fruits *in vitro* and *in vivo*

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ABSTRACT

Fruits such as strawberries are very sensitive to microbial contamination. This study aimed to investigate the inhibitory effect of chitosan treatment, vanillin, and chemical acetic acid and natural on the growth of the fungus Rhizopus stolonifer. Investigating the effect of vanillin, chitosan on the fungi in glass condition showed that Vanillin extract at concentrations greater than 1 g per liter (100% inhibition) and 3 g L chitosan had a significant effect on fungal growth so that considering these results appropriate concentrations were selected for testing in vivo. Selected basis for natural acetic acid concentrations (verjuice) and chemical acetic acid was test panel which its basis was no impact on the fruit of strawberry aroma and taste. Results of fruit inoculated with the suspension of 1×10^5 spore fungi per ml sterile distilled water showed that chemical acetic acid 1% reveal the highest inhibitory effects on growth of Rhizopus and then verjuice (natural acetic acid), 60 and 80 ml control Rhizopus grown considerably.

Key words: chitosan, vanillin, verjuice, strawberries, Rhizopus

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INTRODUCTION

One of the most important factors limiting crop production are plant diseases destroyed each year a significant portion of the production plant and it is when more than 800 million people worldwide do not have adequate food [11]. Botanically strawberry fruit is a complex fruit that will be achieved growth receptacle. The nodes on the receptacle will make it greater and more mature and further growth and will result in larger fruit [8]. The structure of strawberry is very vulnerable and entering damage causing the smooth strawberry, then provides a good place for pathogens attack, fungi of the genus Rhizopus, Aspergillus, Botrytis and Penicillium are of the main microbial factors limiting post-harvest life of strawberries. Given that the time between harvest and consumption of strawberries is very short, therefore, the use of fungicides for disease control and maintain its quality should be done carefully so that no harmful chemical residues remain. Several pre-harvest factors affect the shelf-life of strawberries. Therefore, managing these factors may increase the postharvest life [2]. Rhizopus rot is controlled by rapid and immediately cooling the fruits after harvest and maintain it at the temperature below 60c in warehouse. Evidence suggests that some strawberry genotypes may be resistant against Rhizopus. Safe methods to control postharvest diseases is using of natural ingredients including chitosan, vanillin, natural acetic acid (verjuice). Chitosan is derived from glucan with chitin repeating units; chitin is an abundance mucopolysaccharides are found in exoskeletons of arthropods such as shrimp, crab and in inferior plants such yeasts. This compound was first described in 1811 by Brakonut. It has been proven that chitosan is a substance non-toxic, biodegradable,

biocompatible, and has antimicrobial properties [2]. Properties of chitosan for inhibition of pathogenic bacteria and fungi in antimicrobial films and edible coatings are used. Antimicrobial activity of chitosan resulting from positively charged amino groups. This group responds to negatively charged cell membranes of microorganisms. This reaction leads to the leakage of intracellular protein components and other microorganism components [10].

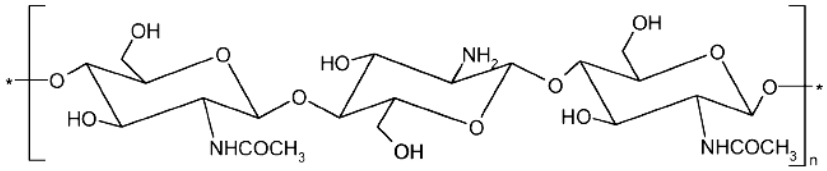

Figure 1 - Chemical structure of chitosan

Chitin polymer applications in the food industry can be noted for keeping food and preventing their bacteria spoilage, production of films and coatings with biodegradable features. Vegetable essential oils include a wide range of secondary metabolites that in most states have represented antimicrobial, allopathic and antioxidant properties and regulatory environment. Chemically, essential oils are a complex mixture which are combined of various chemicals, including hydrocarbons, alcohols, ketones, aldehydes, etc., [7].

Vanillin in the chemical name IUPAC is 4-hydroxy-3-methoxybenzaldehyde. Its molecular formula is (C8H8O3). Vanillin is aldehyde part of the series of methylated (catechol), original perfume of vanilla beans and also there is in the sugar beet and the jewelweed tree (balsam). Vanillin of ancient is used as an aromatic substance in the preparation of various dishes and also has antimicrobial properties. Acetic acid in name of IUPAC etatonic acid or the essence of the grape is among organic acids (carboxylic acid groups) and the formula is CH3COOH. The purpose of this study is to control Rhizopus fungi on strawberries using natural ingredients of chitosan, vanillin and verjuice to increase the storage life and health of consumers.

MATERIALS AND METHODS

Plant material

To investigate above mentioned treatment effect fruits produced from a farm located in the city of Marivan. Healthy fruits (no mechanical damage) and identical as possible in terms of size and color was chosen.

Materials used in the treatment

In this study were used chitosan (Sigma), acetic acid and vanillin (Merck), verjuice (household).

Preparation of suspensions of pathogenic fungi

Initially the fruits infected with fungus Rhizopus were selected then isolated desired fungi and were inoculated on PDA medium into tubes. When fungi initiate spores' creation stage, the spores were isolated and after the counting of spores per unit volume by Lam Newbar and providing appropriate and required concentration of spores (10^5 spores per ml) were used for testing.

Effect of antifungal activity in vitro

Determine the appropriate concentration for used treatments (growth inhibitory effects of treatments on Rhizopus) they were applied on the fruit so that an experiment was conducted in completely randomized design with three replications. To this end, first the PDA medium was prepared then the vanillin in the 500 - 600-700 - 750 - 900-1000 - 1200-1500 - 2000 - 2500-5000 - 7500-10000 mg in 1 ml was added to culture medium and was placed on stirrer for 10 minutes and then for 40 minutes was in an autoclave for sterilizing the medium (solid vanillin oil resistant autoclave temperature). Also, the chitosan on the values 0, 3 and 5 g added to 700 ml of distilled water and then added 12 CC of 1% citric acid to it and was placed on the stirrer heater for 6 hours and it filtered after chitosan was dissolved and the pH was adjusted to 7, then brought to a volume of 1 l and then was added PDA to it and placed in the autoclave. The medium was distributed in sterile conditions and fungal discs in 5 mm diameter that were over 14 days of their culture, was located in the center of each Petri dish. Petri dishes were incubated at a temperature of 24 degrees Celsius. After fungi culture, daily were measured fungus diameter growth until control fungus treatment growth engulfed the entire petri dish.

Effect of antifungal treatment on strawberry fruit

Given that the optimal concentration of vanillin and chitosan were obtained from experiments in vitro (vanillin, 1.5, 2.5 and 5 grams per liter, 3 g of chitosan per liter) and as appropriate concentration of acetic acid, natural (verjuice) with concentrations of 40, 60 and 80 ml per liter of 1% chemical acetic acid were chosen using the testing panel, these treatments were performed for disease control of Rhizopus on fruit in a completely randomized design with three replicates and three experimental replicates on each sample. Thus, healthy fruits after washing and disinfection by 1% sodium hypochlorite solution and rewashed twice in sterile distilled water, dried, then fruits was sprayed with Rhizopus suspension (10^5 spores per ml), and after 2 hours, the spores were stabilized on fruits, was prepared a solution from vanillin essential oil, chemical and natural acetic acid and chitosan. Fruits were treated by dipped method then were packed in polyethylene containers (volume 500 ml) using cellophane and refrigerated at 4 ° C. Fungal growth on fruits at days 4, 6, 9, 12 after SA was measured. As such the surface of each fruit is divided into 8 equal portions and observation of signs of decay or mold in any area were considered equivalent to 12.5% of the infections.

Statistical Analysis

Data analysis was performed using SAS statistical software, and mean comparisons were carried out according to Duncan's multiple range test.

RESULTS AND DISCUSSION

A) The results of the antifungal effect of essential oils in vitro

Inhibitory effect of chitosan

According to the variance analysis table (Table 1), the growth inhibitory effect of treatments on Rhizopus, were significant in probability level at the 1%, the effect of the treatment effect and time also had a significant difference with control group at the 1% probability level.

Table 1 - Analysis of variance and the treatment effect on fungal growth

Source of variation	Degrees of freedom	Rhizopus fungal growth
treatment	4	64.12**
Time	13	26.60**
Time* Treatment	52	5.08**
error	140	0.05
CV		3.73

*** Significant at 1% level*
** Significant at the 5% level.*
ns: no significant difference

According to the comparison results (Fig. 1 and 2), treated with 1% acetic acid had more inhibitory effect on growth of Rhizopus, the next grade were treatments by 3 and 5 g/ L chitosan with acetic acid, chitosan at a concentration of 3 g/ L with no acetic acid showed significant difference in comparing with control group, given that these results can be interpreted such that chitosan may be used without the acid which is a chemical substance. Rhizopus has evolved rapidly up to third day which these results determine its importance that the most effective treatments for control this fungi are those which their inhibitory mechanisms act in such a manner that would have the greatest impact on the early growth stages of the fungus.

The inhibitory effect of acetic acid up to second day is 100% and then also have significant effects on the inhibition of fungal growth, given that the rapid growth of Rhizopus causes the greatest damage in the first 3 days, this is why the best time to eat strawberries is considered to be 3 days therefore it seems acetic acid is more appropriate treatment to control the disease than chitosan. Hessenberg and colleagues [6] reported a 2 mg in 1 liter of acetic acid vapor for 30 minutes every 3 times causes a reduction of 56% Botrytis decay fungi.

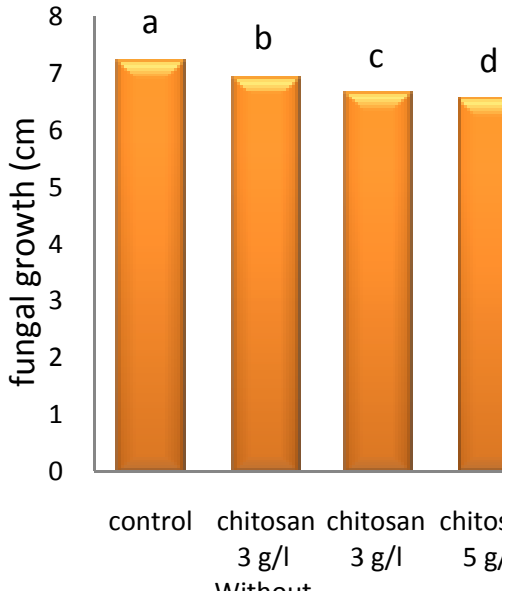

Figure 1 - Effect of treatments on Rhizopus growth (in vitro)

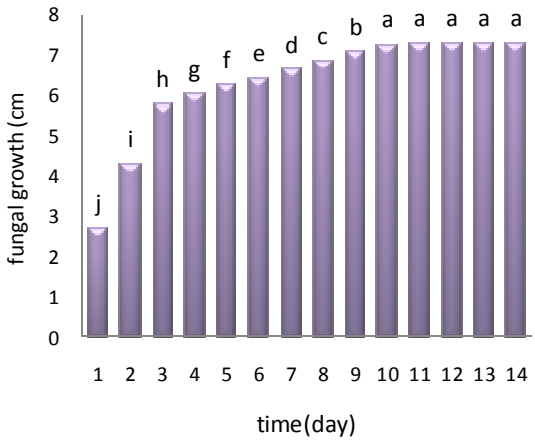

Figure 2 - The effect of time on growth of Rhizopus (in vitro)

Vanillin inhibited (in vitro)

The analysis of variance table showed the effect of treatment, time and treatment * time was significant at the 1% level.

Table 2-4 - Analysis of variance the treatment effect and time on fungal growth

Source of variation	Degrees of freedom	Rhizopus fungal growth
treatment	13	517.12**
Time	13	16.72**
Time* Treatment	169	2.04**
error	392	0.03
CV		5.60

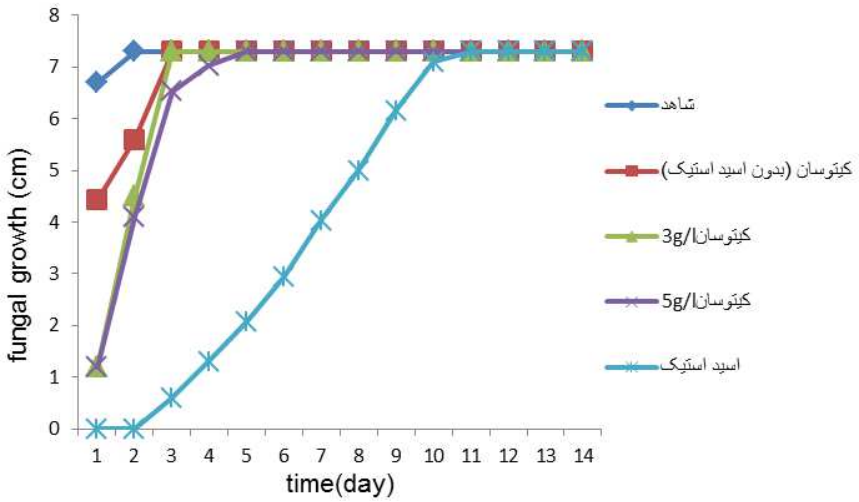

Figure 3 - Effect of time treatments on growth of Rhizopus

The results (Figure 4, 5 and 6) were identified that gradually increase the inhibitory effects with increasing concentrations of vanillin, so that vanillin, at concentrations of 1 and 0.9 g /l up to fourth day greatly reduced the fast growth of fungi however, in concentrations of 1.2 grams and higher has preventive effect of 100%. Growth of the fungus within 6 to 14 days has no significant differences and compared to chitosan treatment is less inhibited over time.

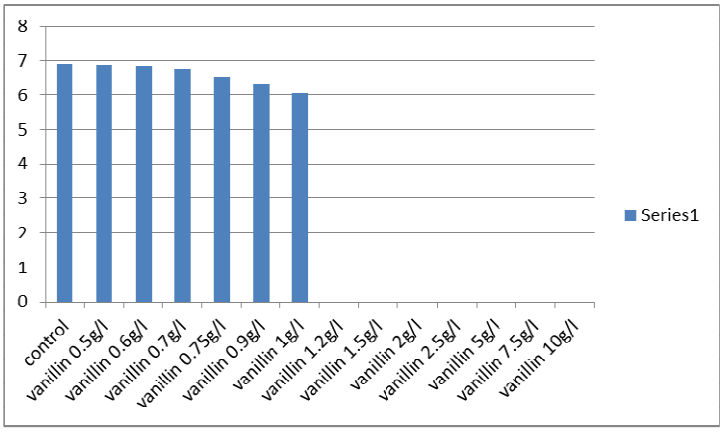

Figure 4 - Simple effect of treatments on growth of Rhizopus

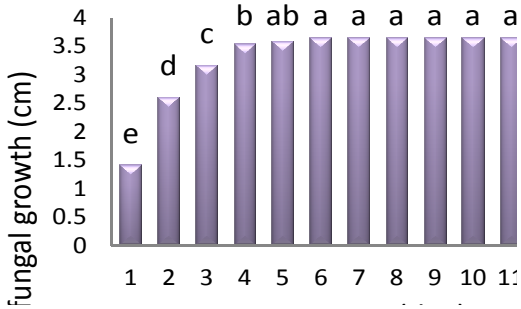

Figure 5 - Simple effect of time on the growth of Rhizopus

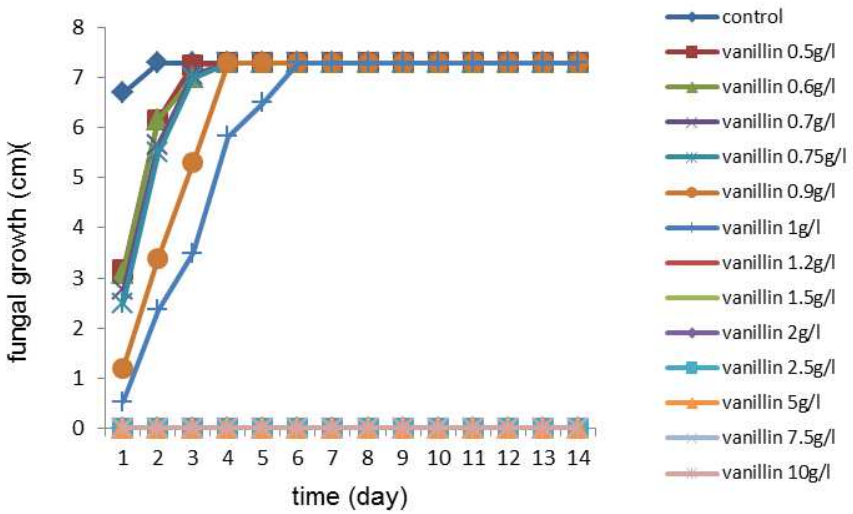

Figure 6 - Effect of treatments*time on growth of Rhizopus

Assessment of decay rate on the fruit

Results of table analysis of variance showed that the effect of treatment and time as well as the treatment in time on fungal growth in fruits was significant at the 1% probability level. Treatments of verjuice 60 and 80 mL/ L had inhibitory effects on growth of Rhizopus, but were much less than 1% acetic acid. Chitosan 3 grams per liter and 2.5 g vanillin not only did not prevent fungal growth but also compared to control group amplified fungal growth. researcher reported that 3 grams per liter chitosan has been reduced the fungal growth rate that appears is not consistent with our results so that there is some reasons to say that the details of these two studies were not identical and this will be a major factor in changing the outcome; some of these factors are : in this study we focused on Rhizopus while the mentioned report was about all fungal diseases in postharvest strawberry, notably, in another study conducted on Botrytis fungi suggests that the inhibitory effect of chitosan was significantly on Botrytis and perhaps this contradiction can be extended to the point that these two factors simultaneously cause disease on strawberry fruit and the results of lowering fungal growth effects was more relevant to Botrytis fungi. Due to the fact that fungi will less grow under acidic conditions, in this experiment to attribute the results entirely to the effects of treatments and not environmental effect therefore, we adjusted pH over 7; while in that study pH were 5.4.

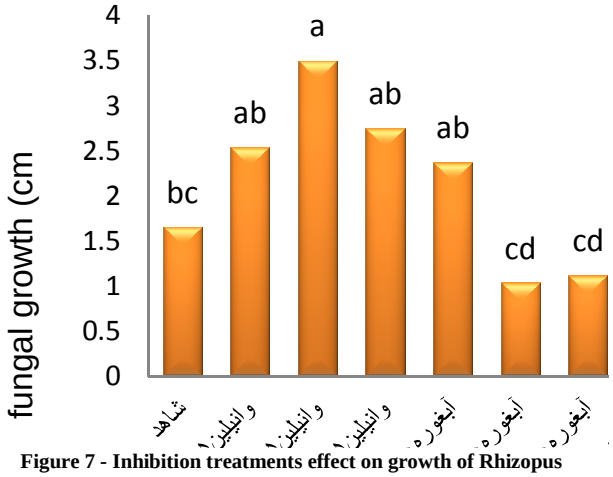

Figure 7 - Inhibition treatments effect on growth of Rhizopus

Table 3 - Analysis of variance of the effect of treatment on fungous growth on fruit

Source of variation	Degrees of freedom	Rhizopus fungal growth
treatment	8	7.22**
Time	1	35.04**
Time* Treatment	17	6.30**
error	36	0.80

CONCLUSION

It was concluded from the results of the first experiment (in vitro) that the treatment by concentration of vanillin oil between 0.12 to 1 g per liter resulted in complete control (100%) of fungal growth which in compared with chitosan and acetic acid treatments were significant. In the second experiment (SA on the fruit) the results showed that, despite vanillin produced 100% inhibit fungal growth in Petri dishes but on strawberry fruit has not been successful as in vitro conditions. Chemical acetic acid 1% treatments and natural acetic acid (verjuice) at concentrations of 60 and 80 ml per liter had a significant inhibitory effect on growth of Rhizopus while chitosan enhances growth of Rhizopus, considering the results of other research that the authors concluded that "treatments of 3 g per liter of chitosan and natural acetic acid (verjuice) at concentrations of 40 and 60 mL/ L had inhibitory effects on the growth of Botrytis fungi" we conclude that although chitosan inhibited the growth Botrytis but is not the best treatment for control fungal disease of postharvest strawberry because the fungus after harvest strawberries (mostly Botrytis and Rhizopus) almost simultaneously causes disease of strawberries fruits and chitosan cannot play an inhibitory role unless it is supplemented with an additional deterrent. A compound may be used alone or in combination with other causing antifungal activity of the essential oil [7]. Treatment by verjuice 60 mL/ L concentration displayed inhibitory effect on both fungous and for this reason it can be considered as better treatment.

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