Role of Chitosan Extracted from Shrimp Waste in Controlling Tomato Blackmold Disease Caused by *Alternaria alternata*

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Abstract: Tomato suffers from several diseases at all stages of its life. Blackmold, caused by *Alternaria alternate* (Fr.) Keissler is one of the most important postharvest disease of tomato. The effect of various concentrations of chitosan solution on *A. alternata* the causal agent of blackmold disease of tomato fruits on mycelial growth was studied. The isolate was tested *in vitro* using PDA amended with seven concentrations of chitosan (0, 1, 2, 3, 4, 5 and 6 mg ml^{-}). Chitosan significantly ($P < 0.05$) inhibited the radial mycelial growth of this fungus by 67.4% at 6 mg ml^{1} concentration. Tomato fruits treated with aqueous solution of chitosan compared with the Ipromise® fungicide (Thiophonate-methyl 20% + Iprodione 20%) was artificially inoculated with *A. alternata* and incubated at 8, 18 and 28°C. Lesion diameters and total phenolic contents were recorded 7 and 14-days after inoculation. Chitosan also, significantly ($P < 0.05$) reduced the lesion diameters of tomato fruits which were smaller for all treatments when stored at 8°C compared to the control treatment. Chitosan treatment resulted in the highest increase in total phenolic contents over the untreated control. Whereas a less increase in total phenolic contents was recognized in fungicide treatment. In all treatments, total phenolic contents increased first and declined at the end of storage. The results of this study indicate that chitosan was a alternative safe coating method especially when stored at low temperature degree for prevents tomato fruits blackmold disease which causes economic losses during transportation, marketing and storage.

Keywords: Chitosan, Tomato fruits, Blckmold, *Alternaria alternata.*

INTRODUCTION

Alternaria alternata is a causal agent of blackmold of tomato (*Lycopersicon esculentum* L.) fruit, a disease frequently causing substantial postharvest losses. The occurrence of Alternaria in a wide variety of fruit and vegetables under diverse conditions of cultivation, handling, and storage suggests that the losses caused by Alternaria are comparable to other mold genera such as *Aspergillus* P. Mich ex Link, *Penicillium* Link and *Fusarium* Link (Stinson *et al.,* 1980). Control of blackmold of tomato can be achieved by pre- and postharvest antifungal treatments, since the fungus can infect the fruit in the field and become latent in green tomato, and resume growth as the fruit ripens. The use of fungicides on fruit needs strict control due to potential health risks, and none of them are approved for postharvest use. Hence, there is a need to exploit natural antifungal substances and induced host defenses for the control of postharvest diseases.

As a natural polysaccharide, chitosan (poly β-(1→4) N-acetyl-D-glucosamine) represents a promising alternative treatment for postharvest disease management due to its antifungal activity and elicitation of defense response in the plant host (Terry and Joyce, 2004; Bautista-Baños *et al.,* 2006). Previous reports have indicated that chitosan can inhibit the growth of several postharvest fungal pathogens, including *Alternaria alternata* (Reddy *et al.,* 1997), *Botrytis cinerea* (Chien and Chou, 2006), *Penicillium expansum* (Liu *et al.,* 2007), *Penicillium digitatum* (Pacheco *et al.,* 2008), *Rhizopus stolonifer* (Hernández-Lauzardo *et al.,* 2008). The antimicrobial activity of chitosan has been commonly considered to be closely associated with its molecular weight, degree of deacetylation, pH, and sensitivity of the target microorganism (Xu *et al.,* 2007). Regarding the antimicrobial mechanism of chitosan, it has been proposed that positively charged chitosan reacts with negatively charged molecules on the cell surface of the target organism altering cell permeability which results in material being leaked from the cell and/or material being inhibited from entering the cell. Chitosan induces structural defense barriers in bell pepper fruit [*Capsicum annuum* L. var. *annuum* (Grossum Group)] (El Ghaouth *et al.,* 1994), and elicits the production of phytoalexin in pea pods (*Pisum sativum* L.) Kendra and Hadwiger (1984). Reddy *et al.* (1998) showed that chitosan affects growth, morphology, and toxin production by *A. alternata*. This means that chitosan, in addition to its direct antimicrobial activity, also interferes with pathogenic factors and induces host defenses. We report here in the mechanisms of chitosan action in controlling the progress of blackmold in postharvest tomato fruit.

The objective of this study is to investigate the effectiveness of chitosan activity on controlling tomato postharvest blackmold disease and total phenolic contents.

MATERIALS AND METHODS

1- Isolation and identification of tomato fruit rots:

Tomato fruits with fungal rot symptoms were collected from local markets in Ismailia Governorate and individually placed in a clean plastic bag. Fruits were swabbed in 70% ethanol for 2 min washed with several changes of sterile distilled water and blotted dry with sterile filter papers. Lesions were aseptically cut by using a sterile forceps then wrapped with filter paper for 3–5 minutes and plated on sterile potato dextrose agar PDA medium supplemented with 50 mg streptomycin per liter and incubated at 25±2°C for 5–7 days. The different fungi grown from infected tissues were sub-cultured on separate sterile PDA plates and the resulting fungi were microscopically examined and identified

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according Clipson *et. al.* (2001). All fungal isolates were maintained on PDA at 4±2°C.

2-Chitosan Extraction method:

2.1- Raw materials:

Raw material used was obtained from the skeleton shrimps (*Penaeus monodon*) as a natural source .The pink shrimp shell was collected from seafood restaurants in Ismailia city, Egypt. The sample was washed with warm tap water to remove foreign materials and muscle particles. The samples were oven dried at 60 °c overnight, then were ground by the aid of grinding mill. Sample particles were sieved. Mesh size of 0.841mm and 0.420mm .Dried ground shell was placed in glass bottles and stored at lab temperature until used.

2.2 -Chitin isolation procedures:-

The method of No. *et al.* (1989) and Bolat *et al.* (2010) were employed to extract chitin. The following steps were followed.

2.2.1-Deproteinization:

The dry sample (10gm) of shrimps was individually submitted to deproteinization using 3.5% NaOH solution. The solid sample was poured in 250 ml glass beaker, then NaOH was added at sample ratio of 1:10 (w\v) (a solid to alkali) .The mixture was kept for 2 hr at 65^{o}c with constant stirring, then the mixture was filtered. The solid residue was washed with tap water for 30 minutes and then oven-dried. The obtained product was weighted.

2.2.2-Demineralization:

The dried deproteinized samples were individually poured in 250 ml glass beaker then 1 N HCl was added at ratio of 1:15 (w\v) a solid to acid. The mixture was kept for 30 min at room temperature with constant stirring, then the mixture was filtered. The solid residue was washed with tap water for 30 minutes and then oven-dried. The obtained product was weighted.

2.2.3-Decoloration:

The dried demineralized samples were individually poured in 250 ml glass beaker then 50 ml of acetone were added to each beaker for 10 min, then the mixture was filtered and dried for 2 hr at room temperature .Sodium hypochloride (NaOCL) 0.315 % solution was added at ratio of 1:10 (w\v) solid to acid. The mixture was kept for 5 min at room temperature with constant stirring, then the mixture was filtered. The solid residue was washed with tap water for 30 minutes and then oven-dried. The obtained product was weighted.

2.3-Preparation of chitosan from Chitin:

Preparation of chitosan from the obtained chitin as deacetylation treatment was prepared by using modify method of No *et al.* (2000).

2.3.1-Degree of Deacetylation (DDA):

Degree of Deacetylation was determined by potentiometric titration by using Suitcharit *et al.* (2011) method. The percent degree of deacetylation was calculated using equation:

$$DDA = [203\ Q/(1+ 42\ Q)\ ]\ X\ 100\%$$

Where, Q = NΔV/m, ΔV is the volume of NaOH solution between two inflection points (in L), N is the concentration of NaOH (0.05 M) and m is the dry weight of chitosan samples (gm).

2.3.2-Fourier Transform Infrared spectroscopy, (FTIR)

To be sure that the obtained substance is chitosan, the method of Palpandi *et al.* (2009) is employed. FTIR spectroscopy of solid samples of chitosan relied on a Bio-Rad FTIS - 40 model USA. Sample (100µg) was mixed with 100 µg of dried Potassium Bromide (KBr) and compressed to prepare a salt disc (10 mm diameter) for reading the spectrum further.

3. Characterization of chitosan

3.1- Determination molecular weight:

To determine molecular weight of chitosan the following step were followed:-

3.1.1-Determination of specific viscosity (η_{sp})

The method of Chen and Tsaih (1998), was followed to determine the specific viscosity (η_{sp}). Each of chitosan samples was dissolved in mixture of acetic acid (0.1 M), and sodium chloride (0.2 M). The viscosity was measured by the aid of Ostwald viscometer, using five different concentrations (0.001-0.002-0.003-0.004-0.00 5gm/ml) at a temperature of 25°C.

The capillary tube was filled with 10 mL of each sample that passed through the capillary twice before the running time was measured. Each sample was measured three times. The running times of the sample through the capillary was measured.

The same forgoing procedure was carried out to measure the running time of the solvent (acetic acid and sodium chloride).The density of each of the sample and the solvent were calculated. The relationship between the running time and the density were used to measure the viscosity following equation was used to calculate the specific viscosity (η_{sp}) of the sample.

$$\eta_{sp} = (\eta - \eta_s)/\eta_s$$

Where, (η) is the sample viscosity in poise (ml/gm), (η_s) the solvent viscosity in poise (ml/gm) and (η_{sp}) specific viscosity in poise (ml/gm).

3.1.2-Calculation of intrinsic viscosity [η_{inst}]

To calculate the intrinsic viscosity equation of Alsarra *et al.* (2002). was used as a graph, since

$$[\eta_{inst}] = \lim_{c \to 0} (\eta_{sp}/c)$$

Where, [η_{inst}] is the intrinsic viscosity in poise (mL/gm), (η_{sp}/c) is the reduced viscosity and (c) is the solutions concentrations.

Through the graph (η_{sp}/c) the relation to the solution's concentration (c), supply the intrinsic viscosity of the solution by extrapolation of the straight line obtained by linear regression for c = 0. Excel program version 2003 was used to calculate the point of intersection.

3.1.3-Calculation of molecular weight

The molecular weight of chitosan (M_V) was determined by Mark–Houwink–Sakurada's empirical equation, reported by Roberts and Domszy (1982) that relates the intrinsic viscosity to the polymer's molecular weight, in the following form:

$$M_V = ([\eta_{inst}] / K)^{1/\alpha}$$

Where, M_v = the molecular weight, $[\eta_{inst}]$ = the intrinsic viscosity in mL/gm. (K) = 1.81 $x10^{-3}$ mL/gm, (α) = 0.93 dimensionless.

The constants depend on the polymer system explained by Anthonsen *et al.* (1993) and Kitture *et al.* (1998).

3.2- Solubility:

The method of No *et al.* (2007) was used to estimate the solubility percentage of chitosan samples, by using a glass bottle provided with 100 ml acetic acid 1%, as a solvent. Chitosan distinct weight was added gradually to the bottles. The bottles were immersed in boiling water bath and shaked continuously until saturation. The bottles were left to cool at room temperature. The solution was centrifuged at 10,000 rpm for 10 min. The supernatant was kept as chitosan formulation. The undissolved particles were washed in distilled water (25 ml) then centrifuged a 10,000 rpm for 10 min. The supernatant was removed. The undissolved particles dried at 60°C for 24 hr. The weighed particles were determined the percentage solubility was according to the following equation:

$$\text{Solubility (\%)} = \frac{(\text{Initial weight of tube} + \text{chitosan}) - (\text{Final weight of tube} + \text{chitosan})}{(\text{Initial weight of tube} + \text{chitosan}) - (\text{Initial weight of tube})} \text{ x } 100$$

Solubility percentage presented the concentration of chitosan in the obtained formulation.

4-Effect of chitosan concentrations on the mycelial growth of *A. alternata*:

A laboratory experiment was carried out to study the effect of chitosan at different concentrations on the mycelial growth of *A. alternata*. Extracted chitosan was prepared by dissolving chitosan in 0.25 N HCl by stirring for 8 h at 45°C. Undissolved particles were removed by centrifugation at 10,000 g for 15min. Chitosan was precipitated with 2N NaOH and washed three times in deionized water to remove salts. The purified chitosan was then air-dried and stored at room temperature until required. For incorporation into media, purified chitosan was dissolved in 0.25 N HCl, then adjusted to pH 5.6 with 2 N NaOH (Du *et al.,* 1997). Chitosan was incorporated into potato dextrose agar at concentrations of 0, 1, 2, 3, 4, 5 and 6 mg ml^{-1}.

The chitosan solution and PDA medium were autoclaved separately and combined with the medium after autoclaving. Equal volumes of acid were used for all concentrations of chitosan. A 5-mm-diameter plug from the advancing margin of colony on PDA medium was seeded centrally onto 4 plates of each chitosan concentration. Cultures were incubated at 25±2° C. The diameter of all colonies was measured until the leading edge of the fastest-growing colony had reached the edge of the plate. The highest concentration which gave maximum inhibition of radial growth was chosen to treat tomato fruits.

The average linear growth of tested fungus was measured 7 days after incubation period and reduction in fungal growth was calculated in relative to check treatment according to Fokemma (1973) as the percentage reduction in mycelial growth of the pathogen:

$$\text{Reduction percentage} = (C - T) / C \text{ x } 100$$

Where; C= maximum linear growth in control and T= maximum linear growth in treatment.

5- Effect of chitosan on the development of tomato blackmold disease compared to the Ipromise® fungicide under different temperature degrees:

Experiment was conducted with commercially grown tomatoes from Ismailia Governorate. Tomato fruits castle Rock cv. were selected free from injuries and any infections. Fruits were sterilized with 3% sodium hypochlorite solution followed by immersion in sterile distilled water for two minutes and air-dried before wounding. The fruit were then randomly divided into 10 fruit lots for each replicate and three replicates were used for each treatment. Fruits were dipped in each of chitosan solution (6 $g \cdot L^{-1}$) and Ipromise® fungicide (Thiophonate-methyl 20% + Iprodione 20%) at the rate of 2 ml/1 liter water as recommended and dried under ambient conditions for 1 h. After air drying, fruits were wounded with a sterile stainless steel scalpel where each wound was about 4 mm long and 2 mm deep. Spore suspension was obtained by flooding cultures of 10 days old *A. alternata* with sterile distilled water containing 1 ml·L–1 Tween 80. Spore counts were determined with a haemacytometer, and the spore concentration was adjusted with sterile distilled water to 2.5×10^5 conidia/ml. Twenty µl of spore suspension inoculated into each wound using a micropipette under aseptic conditions then placed in 1.5 L plastic boxes. A layer of water was placed at the bottom of the plastic boxes to maintain high humidity and containers were closed with perforated lids to eliminate any accumulation of CO2. After storage for 7 and 14- days at 8, 18 and 28°C, lesions diameter and total phenolic contents were recorded.

6-Total phenolic contents (TPC):

The total phenolics were determined by the Folin-Cicalteau method as described by Singleton *et al.* (1999), with minor modifications, based on colorimetric oxidation/reduction reaction of phenols. Polyphenols extraction was carried out by adding 10 ml methanol (85%) to 1g of pericarp tissues were sampled from

inoculated fruit by excising the tissue from the inoculation site up to 1 to 2 cm beyond the edge of the expanding lesion. 250 µl of sterile distilled water was added to 250 µl of extract, and then 2.5 ml of diluted Folin Cicalteau reagent (10%) and 2 ml of 7.5% sodium carbonate were added. The samples were shaked for 1.5 to 2 hours. The absorbance of samples was measured at 765 nm by a PG Instruments ltd- T80+ UV/VIS spectrophotometer. Gallic acid was used for calibration curve. Results were expressed as mg gallic acid (GAE)/100 g FW.

7- Statistics

Differences between the concentrations of chitosan on the radial growth of the fungus and the differences between lesion diameters on tomatoes were evaluated by analysis of variance (ANOVA). Duncan's multiple range test at $P < 0.05$ level was used for means separation (Winer, 1971).

RESULTS

1- Isolation and identification of the causal organisms:

Seven different fungi were isolated from tomato diseased fruits collected from Ismailia. Data in Table (1) show that the frequency of fungi associated with diseased tomato rotted fruits varied with different fungi. The isolated fungi can be ranked in descending order as follows: *A. alternata*, *Alternaria solani, Fusarium* spp. *Botrytis cinerea*, *Rhizopus stolonifer*, *Aspergillus niger* and *Geotrichum candedum*. The mean frequency of these fungi was 22.10, 18.0, 15.70, 15.40, 10.90, 10.30 and 7.60% respectively. *A. alternata* was the most isolated fungus by 22.10%.

Table (1): Frequency % of occurrence of fungi associated with tomato rotted fruits.

Fungi	Frequency of occurrence, %
A. alternata	22.10
Botrytis cinerea	15.40
Aspergillus niger	10.30
A. solani	18.0
Fusarium spp.	15.70
Rhizopus stolonifer	10.90
Geotrichum candedum	7.60
Total	100.00

1.1. Chitin yield:

Chitin yield was calculated as the dry weight of chitin obtained from 10 gm of dried raw materials in six replicate in each sample. Chitin compound yielded are tabulated in Tables (2) and (3). Results show that the yield of chitin was 2 gm. The dry shrimp sample weight became 7.8± 0.34 gm after deproteinization, the protein present 22%; whereas the weight became 2.1 ± 0.14 after demineralization, mineral presented 57% of the dry shrimps weight. Color substance were traces , presented only 1% of the dry shrimp sample weight after the three treatment (DP,DM and DC) presented 20% of the dried shrimp sample.

Table (2): Chitin yield of dry shrimps

Treatment *	Weight (gm) After treatment
Deproteinization	7.8 ± 0.34
Demineralization	2.1 ± 0.14
Decoloration:	2 ± 0.1
Chitin	2 ± 0.1

* pre treatment dry weight = 10 gm

Table (3): Chitin yield content

Compound	Content %
Protein	22%
Minerals	57%
color	1%
chitin	20%

1.2. Chitosan yield:

Deacetylation

Conversion of chitin to chitosan was achieved by NaOH to remove the acetyl group from the polymer (Deacetylation treatment). The dry chitin weight (2 ± 0.1 gm) became (1.4 ± 0.014gm) after deacetylation. The percent DDA of chitosan presented in Figure (1) was derived from the differential volume of NaOH between two inflection points obtained from the potentiometrical plots. A set of pH-potentiometric titration curves of chitosan sample three parallel measurements. The curves clarify two inflection points which found to be at 25 ml and 75 ml for NaOH. The difference between the two inflection points (ΔV) found to be 50 ml. According to the equation, the degree of deacetylation was found to be 84% for the sample.

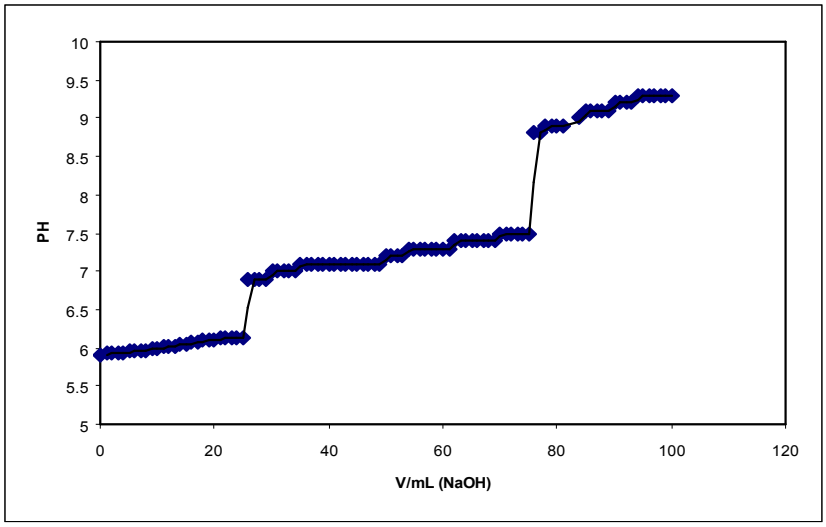

Figure (1): pH-potentiometric titration curves of chitosan from shrimps

3. Fourier Transform Infrared spectroscopy, (FTIR)

Data in Table (4) and Figure (2) show that the obtained substance after deacetylation processes to chitin has 9 bands. The band at 1392.5 cm $^{-1}$ which indicate the presence of primary alcoholic group (-CH2 – OH).The band at 2846.7 cm $^{-1}$ which indicated C–H stretching and another band at 1423.4 cm $^{-1}$ which indicate the C–H deformations. The band at 1033.8 cm $^{-1}$.indicate the presence of free amino groups (-NH_2). The sample of showed band at 1323 cm $^{-1}$ which indicate the presence of acetyl group (C = O).

The sample also showed band centered at 3301.9 cm $^{-1}$ which indicate that the structure is a polymer. The sample of showed band at 910.3 cm $^{-1}$ which act as the finger print for the polymers ring stretching. Fourier Transform Infrared spectroscopy, (FTIR) analysis indicated that the obtained substance is chitosan. As a result of deacetylation the obtained chitosan weight presented 70% of the obtained chitin, whereas it presented 14% of the dry shrimp sample weight (10gm).

Table (4): Wave length of the main bands obtained for the chitosan.

Vibration mode	Length cm $^{-1}$
Primary alcohol (CH_2 OH)	1392.5
C-H Stretching	2846.7
C-H Deformations	1423.4
Amide II band (NH2)	1033.8
C= O(acetyl group)	1323.1
Asymmetric in – phase ring stretching mode	2916.2
Structural unit(polymer)	3301.9
Ring stretching	910.3

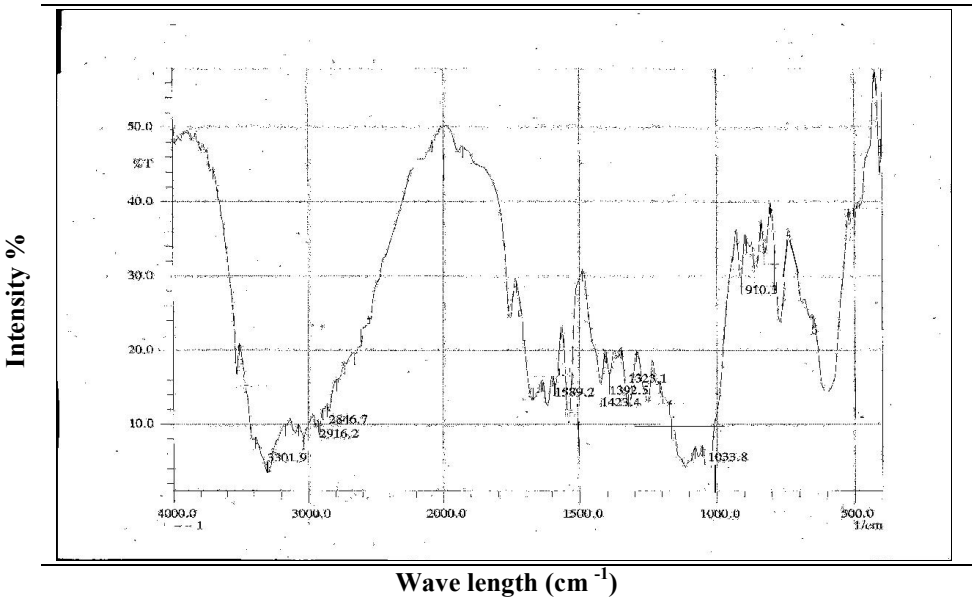

Figure (2): IR spectra of the isolated chitosan from shrimps**.**

4. Characterization of chitosan:

4.1- Molecular weight of chitosan:

Molecular weight of chitosan were measured and presented in Figure (3), the representative graph of the reduced viscosity (ηsp/c) in relation to the five solution concentrations used for the determination of intrinsic viscosity of the chitosan for shrimps shell. By using the equation of Alsarra *et al.* (2002).

$$[\eta_{inst}]=\lim c\rightarrow 0\ (\eta sp/c)$$

Data showed that the intrinsic viscosity of each of the chitosan for every of the sample to be the point of intersection of (Y- axis) when the concentration of the solution equal to zero. Data in Figure (3) and Table (5) show that the intrinsic viscosity [η_{inst}] of shrimps where 189.6 ηsp/c**.**

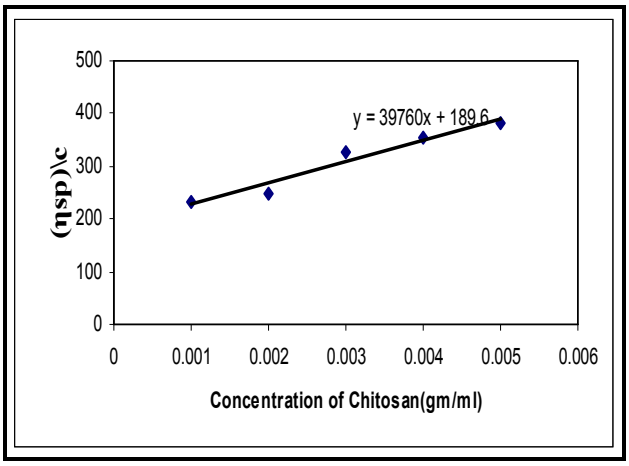

Figure (3): Intrinsic viscosity of the chitosan of shrimps.

Table (5): Specific, reduced viscosity ηsp/c intrinsic viscosity [η_{inst}] and the molecular weight of chitosan from shrimps

Chitosan	Concentration (gm/ml)	η X 10^{-3} * (ml/gm)	η sp	ηsp/c	[η_{inst}]	Mv X 10^3 [kDa]
Shrimps	**Solvent**	7.314	-	-	179.4	249
	0.001	9.66	0.332	225		
	0.002	11.75	0.5	235.4		
	0.003	15.34	0.85	341		
	0.004	18.7	1.34	346		
	0.005	22.41	1.9	387		

* viscosity

The molecular weight of chitosan (MV) was determined by Mark–Houwink Sakurada's empirical equation:

$$MV = ([\eta_{inst}]/K)1/\alpha$$

Found to be 250 X103 kDa for the shrimps

4.2. Solubility

Solubility percentage of chitosan samples were estimated and obtained from shrimp shell in 100 ml acetic acid 1%, as a solvent. The solubility of chitosan which obtained from the shrimps was 80.5 %.

5- Effect of chitosan concentrations on mycelial growth of *A. alternata*:

Antifungal activity of chitosan against *A. alternata* studied *in vitro* and the results presented in Table (6) and Figure (4). Presented data show that, all the tested chitosan concentrations were exhibited a moderate fungicidal activity against the tested fungus compared with the control treatment. Data also indicated that chitosan at 1mg/ml concentration was the least effect on the fungal growth reduction (39.7%). Also, the results indicated that chitosan concentration at 6 mg/ml were the most significant effect in reducing *A. alternata* fungal growth.

Table (6): Effect of chitosan concentrations on mycelial growth of *A. alternata*.

Chitosan concentration (mg ml^{-1})	*A. alternata*	
	linear growth (mm)	Reduction (%)
1.0	54.27 b	39.7
2.0	50.34 c	44.1
3.0	47.35 d	47.4
4.0	39.55 e	56.1
5.0	32.5 f	63.8
6.0	29.3 g	67.4
Control	90.0 a	-
LSD at 5%	2.97	-

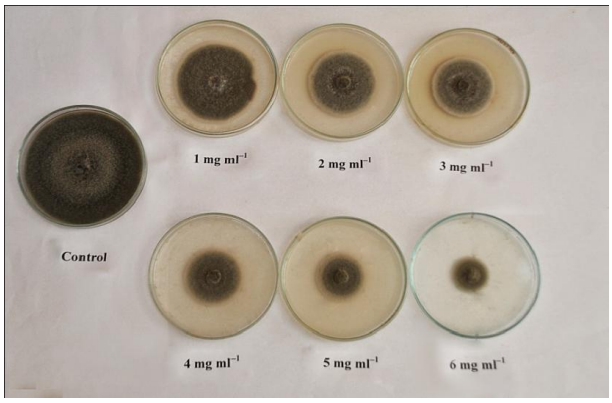

Figure (4): Effect of different concentrations of chitosan on linear growth (mm) of *A. alternata*

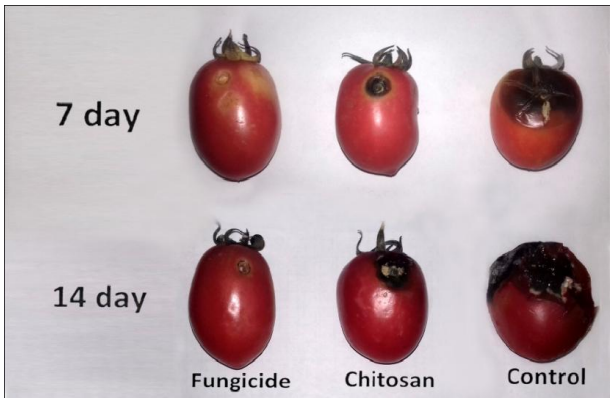

Figure (5): Effect of chitosan compared to the Ipromise® fungicide treatment on the development of tomato fruit blckmold disease after 7 and 14-days of inoculation

6- Effect of chitosan on the development of tomato blackmold disease compared to the Ipromise® fungicide treatment under different temperature degrees:

Effect of chitosan on tomato fruit blackmold severity was obtained in Table (7) and Figures (5 and 6). Data indicated that blackmold were developed at a higher rate in inoculated tomato fruits. In the inoculated fruits, lesions were visible within 7 days after inoculation and increased significantly with increasing storage period under different storage temperature degrees. While in the fungicide -treated fruit, no visual symptoms were observed at 8 and 18°C after 7 and 14-days and lesions were visible only after 7 and 14days when storage at 28°C. Lesion diameter caused by *A. alternata* was decreased significantly with chitosan treatment by 38.23 and 60.90% as compared to the control after 7 and 14 days of incubation at 8°C, respectively. Control of lesion development in chitosan-treated fruit indicated that chitosan had an inhibitory effect against pathogenic fungus under study.

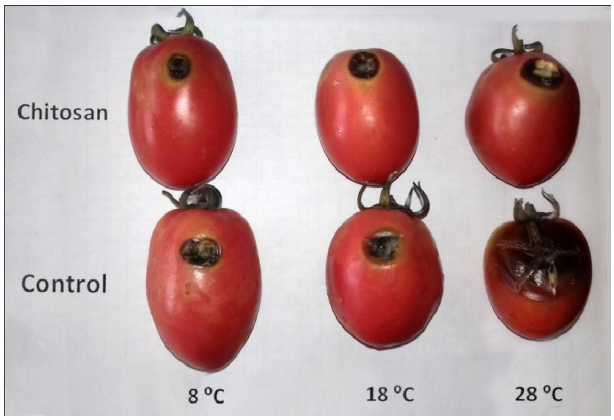

Figure (6): Effect of chitosan on the development of tomato fruit blckmold disease at different temperature degrees

Table (7): Effect of chitosan on the development of tomato blackmold disease after 7 and 14 days of inoculation incubated at different temperature degrees

Treatments	Temp.	Lesion diameter (mm)		Disease reduction (%)	
		7-days	14-days	7-days	14-days
Chitosan	8 °C	6.56	10.68	38.23	60.90
	18 °C	8.62	16.56	27.56	42.22
	28 °C	33.62	41.25	35.92	45.23
Ipromise®	8 °C	0.0	0.0	100	100
	18 °C	0.0	0.0	100	100
	28 °C	22.20	24.20	57.72	67.90
Control	8 °C	10.62	27.32	-	-
	18 °C	11.90	31.37	-	-
	28 °C	52.50	75.32	-	-

L.S.D at 5 %
Incubation periods = 3.46
Temperatures= 4.23
Treatments= 4.23
Incubation periods × Temperatures = 5.99
Incubation periods ×Treatments = 5.99
Temperatures × Treatments = 7.33
Treatments × Temperatures × Incubation periods = 10.37

7- Effect of chitosan on the total phenolic contents of tomato fruit after 7 and 14-days of inoculation incubated at different temperature degrees.

The changes in the total phenolic contents in tomato fruits are shown in Table (8). The total phenolic contents of all coated fruits were significantly higher than that of control. Chitosan treatment resulted in the highest increase in total phenolic contents over the untreated control by (105.89, 94.80 and 94.32%) at 8, 18 and 28°C, respectively after 14- days of storage. In general, the total phenolic contents were found to be higher in fruits treated with chitosan. Whereas, a less increase was recognized in fungicide treatment by (15.05%) at 8°C and 7 days of storage. In all treatments, it increased first and declined at the end of storage.

Table (8): Effect of chitosan on the total phenolic contents of tomato fruit after 7 and 14- days of inoculation incubated at different temperature degrees.

Treatments	Temp.	Total Phenolic contents (mg GAE/100g fresh weight)			
		7-days	Increase over control (%)	14-days	Increase over control (%)
Chitosan	8 °C	77.21	78.85	74.43	105.89
	18 °C	81.24	83.67	76.48	94.80
	28 °C	82.31	76.97	80.14	94.32
Ipromise®	8 °C	49.67	15.05	44.17	22.18
	18 °C	53.12	20.09	48.47	23.45
	28 °C	55.11	18.49	52.35	26.94
Control	8 °C	43.17	-	36.15	-
	18 °C	44.23	-	39.26	-
	28 °C	46.51	-	41.24	-

DISCUSSION

The present study revealed that the use of chitosan coating on fruits is effective in reducing blackmold disease in tomato fruits. *In vitro*, chitosan significantly inhibited fungal growth of *A. alternata* and significant differences were observed in the mean growth rates of tested fungus at all tested concentrations compared to the control. Data also indicated that chitosan at 6 mg/ml concentration has highest effect on the fungal growth reduction (67.4%). This finding is in agreement with Allan and Hadwiger (1979), Saharan *et. al.* (2013) who suggested that chitosan showed the maximum growth inhibitory effects on *in vitro* the mycelial growth of *A. alternata*. In this respect, Reddy *et. al.* (1997) proved that chitosan significantly affected both growth and toxin production of *A. alternata* at higher concentrations. However, at lower concentrations, toxin production was affected more than the growth as evidenced by minimum inhibitory concentrations of chitosan derived for toxin production and mycelial growth. Chitosan has been used in numerous industrial and food applications due to its biological and functional properties (Winterowd and Sanford, 1995; No *et al.,* 2007). The experimental data in this study demonstrates that the antimicrobial characteristics of this substance make it a potential, and moreover, a naturally occurring, food coating material. The effectiveness of chitosan has been reported by numerous authors (El Ghaouth *et al.,* 1997; Reddy *et al.,* 2000; Rhoades and Roller, 2000). Chitosan is nontoxic for humans and has a low environmental impact (Hirano *et al.,* 1990; Li *et al.,* 1992; Shahidi *et al.,* 1999). This is clarified in its recent approval as a food additive in Korea and Japan (Weiner, 1991). The International Commission on Natural Health Products (1995) recognized chitin as a natural product for the 21st century and in 2005, chitosan was considered as generally recognized as safe (GRAS) by the FDA (Food and Drug Administration) based on the scientific procedures for use in foods. Results also showed that chitosan offers a safe alternative to synthetic fungicides in postharvest diseases and could be considered as a potential agrochemical of low environmental impact.

Fourier Transform Infrared spectroscopy, (FTIR) analysis indicated that the obtained substance is chitosan. Our results are agree with that obtained by numerous researches. Saraswathy *et al.* (2001) confirmed that the peak at 1384 cm^{-1} represents the –C-O stretching of primary alcoholic group (-CH2 - OH). De Velde and Kikens, 2004, discussed that absorbance bands of 2878, 1420 cm^{-1} indicated C–H stretching, C–H deformations, respectively. Saraswathy *et al.* (2001) observed the major absorption band between 1020 and 1220 cm^{-1}which represents the free amino group (-NH2) at C2 position of glucosamine, a major group present in chitosan. Palpandi *et al.* (2009) also noted

that the band at 1320 cm^{-1}characteristics of (acetyl group0 C=O, and also evaluated two possibilities, either the large band centered at 3300 cm^{-1} (very near to that at 3450 cm^{-1}) chosen for polymers. Palpandi *et al.* (2009), observed that the band at 897.41 cm^{-1} were characteristic for the ring stretching.

Tomato fruits treated with the highest chitosan solutions showed a significant reduction in lesion diameter compared to control. There were significant differences between the effects of chitosan concentration and incubation temperature on the lesion size of blckmold. Chitosan was the most effective when fruits were stored at 8°C temperature in comparison with controls. Tsai and Su (1999) explained that higher temperatures and acidity in foods increased the bactericidal effect of chitosan. Previous studies demonstrated that the induction of systemic resistance in plants with natural compounds, including chitosan, is a promising approach to disease control (Gozzo, 2003). Also, Faoro *et al.* (2001, 2008) showed that the activity of chitosan was attributed to the accumulation of hydrogen peroxide in treated tissues, which induces a hypersensitive reaction as a consequence of oxidative microburst and phenolic compound deposition. Doares *et al.* (1995) and Howe (2005) indicate that this substance activates jasmonic acid synthesis in treated hosts. In addition, chitosan oligomers of different molecular weight and degree of deacetylation induced an accumulation of phytoalexins in grapevine leaves, which reduced *B. cinerea* and *Plasmopara viticola* infections. Nevertheless, the induction of the defense mechanisms without the antifungal activity was not enough to suppress the disease (Ben-Shalom and Fallik, 2003).

Inhibition of fungal growth as evidenced by reduced lesion size in chitosan treatments showed that chitosan has directly antifungal effect. In addition, chitosan interfered with production of fungal virulence factors such as cell wall degrading enzymes, organic acids, and host specific toxins. Previous studies confirmed direct antifungal action of chitosan (Allan and Hadwiger, 1979; El Ghaouth *et. al.* 1992). Although information is available on the antimicrobial effects of chitosan, it is stable to know the actual mechanism and its impediment in pathogenesis of the causing organism in plant. Several studies have confirmed the antimicrobial effect of chitosan when it is in direct contact with the target organism. Chitosan is unusually susceptible to a variety of enzymes such as proteases, cellulases, pectic enzymes, and lipases (Pantaleone *et. al.*, 1992). At the same trend, the finding of Doares *et al.* (1995) indicated that chitosan oligosaccharides activate plant defense genes by signal transduction involving jasmonic acid similar to wound response, sustains this hypothesis.

The total phenolic contents of all coated fruits were significantly higher than the control. Chitosan treatment resulted in the highest increase in total phenolic contents over the untreated control. Whereas a less increase was recognized in fungicide treatment. In all treatments, it increased first and declined at the end of storage. Besides its antifungal activity, chitosan also has a potential of inducing defense related enzymes (Bautista-Baños *et al.,* 2006) and phenolic contents in plants (Benhamou, 1996). The result is in compatible with Benhamou and Thériault (1992), and Liu *et al.* (2007), who reported that the production of phenolic compounds was induced in tomato plants and fruit treated with chitosan. (Macheix *et al.,* 1990) roported that the decreasing of phenolic compounds at the end of storage might be due to breakdown of cell structure in order to senescence phenomena during storage. Toor *et al.* (2005) reported that high temperatures and light exposure stimulate the production of phenolic acids and other flavonoids and that heat stress in tomato plants increases the activity of phenylalanine ammonia-lyase (PAL) as well as the total phenol and odiphenol contents and induces the accumulation of phenolics by activating their biosynthesis as well as inhibiting their oxidation.

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REFERENCES

Allan, C. R. and L. A. Hadwiger (1979). The fungicidal effect of chitosan on fungi of varying cell wall composition. Expt. Mycol., 3: 285–287.

Alsarra, I. A., S. S Betigeri, H. Zhang, B. A. Evans and S. H. Neau (2002). Molecular weight and degree of deacetylation effects on lipaseloaded chitosan bead characteristics. Journal of Biomaterials, 23: 3637–3644.

Anthonsen, M. W., K. M. Varum and O. Smidstrod (1993). Solution property of chitosan: conformation and chain stiffness of chitosan with different degree of N acetylation .J. Carbohydrate Polymers, 22: 193-201.

Bautista-Baños, S., A. N. Hernández-Lauzardo, M. G. Velázquez-del Valle, M. Hernández-López, E. Ait Barka, E. Bosquez-Molina and C. L. Wilson (2006). Chitosan as a potential natural compound to control pre and post harvest diseases of horticultural commodities. Crop Protection, 25: 108-118.

Benhamou, N. (1996). Elicitor-induced plant defence pathways. Trends Plant Sci., 1: 233–240.

Benhamou, N. and G. Thériault (1992). Treatment with chitosan enhances resistance of tomato plants to the crown and root pathogen *Fusarium oxysporum* f. sp. *radicis lycopersici.* Physiol. Mol. Plant Pathol., 41: 33–52.

Ben-Shalom, N. and F. Fallik (2003). Further suppression of *Botrytis cinerea* disease in cucumber seedlings by chitosan–copper complex as compared with chitosan alone. Phytoparasitica 31: 99–102.

Bolat, Y., Ş. Bilgin, A. Günlü, L. Izci, S. B. Koca, S. Çetinkaya1 and H. U. Koca (2010). Chitin-Chitosan Yield of Fresh water Crab (*Potamon potamios*, Olivier 1804) Shell. J. Pak Vet., 30(4): 227-231.

Chen, R. H. and M. L. Tsaih (1998). Effect of temperature on the intrinsic viscosity and conformation of chitosans in dilute HCl solution. International Journal of Biological Macromolecules, 23: 135–141.

Chien, P. J. and C. C. Chou (2006). Antifungal activity of chitosan and its application to control postharvest quality and fungal rotting of Tankan citrus fruit (*Citrus tankan hayata*). Journal of the Science of Food and Agriculture, 86: 1964-1969.

Clipson, N. J. W., E. T. Landy and M. L. Otte (2001). Fungi. In: Costello, M. J., C. Emblow and R. White (eds), European register of marine species: a check-list of the marine species in Europe and a bibliography of guides to their identification. Collection Patrimoines Naturels, 50: 15–19.

De Velde, K. V. and P. Kikens (2004). Structure analysis and degree of substitution of chitin, chitosan and dibutyrylchitin by FT IR spectroscopy and solid sate 13 CNMR. Journal of Carbohydrate. Polymer, 58: 409- 416.

Doares, S. H., T. Syrovets, E. W. Weiler and C. A. Ryan (1995). Oligogalacturonides and chitosan activate plant defensive genes through the octadecanoid pathway. Proc. Natl. Acad. Sci. USA, 92: 4095–4098.

Du, J., H. Gemma and S. Iwahori (1997). Effects of chitosan coating on the storage of peach, Japanese pear and kiwi fruit. J. Japanese Soc. Hortic. Sci., 66(1): 15-22.

El Ghaouth, A., J. Arul, A. Asselin and N. Benhamou (1992). Antifungal activity of chitosan on post harvest pathogens: Induction of morphological and cytological alterations in *Rhizopus stolonifer*. Mycol. Res., 96: 769–779.

El Ghaouth, A., J. Arul, C. Wilson and N. Benhamou (1994). Ultrastructural and cytochemical aspects of the effect of chitosan on decay of bell pepper fruit. Physiol. Mol Plant Pathol., 44: 417–432.

El Ghaouth, A., C. Wilson and M. Wisniewski (1997). Biologically-based alternatives to synthetic fungicides for the control of postharvest diseases. J. Ind. Microbiol. Biotechnol., 19(3): 160–162.

Faoro, F. D. Maffi, D. Cantu and M. Iriti (2008). Chemical-induced resistance against powdery mildew in barley: the effects of chitosan and benzothiadiazole. Biocontrol 53: 387–401.

Faoro, F., S. Sant, M. Iriti and A. Appiano (2001). Chitosan-elicited resistance to plant viruses: a histochemical and cytochemical study. In: Muzzarelli, R.A.A. (Ed.), Chitin Enzymology. Atec, Italy, pp. 57–62.

Fokemma, N. J. (1973). The role of saprophytic fungi in antagonism against *Drechslera sorokiniana* (*Helminthosporium sativum*) on agar plates and on rye leaves with pollen. Physiol. Plant Pathol., 3: 195-205.

Gozzo, F. (2003). Systemic acquired resistance in crop protection: from nature to a chemical approach. J. Agric. Food Chem., 51: 4487–4503.

Hernández-Lauzardo, A. N., S. Bautista-Banõs, M. G. Velázquez-del Valle, M.G. Méndez-Montealvo, M. M. Sánchez-Rivera and L. A. Bello-Pérez (2008). Antifungal effects of chitosan with different molecular weights on *in vitro* development of *Rhizopus stolonifer* (Ehrenb.Fr.) Vuill. Carbohydrate Polymers, 73: 541-547.

Hirano, S., C. Itakura, H. Seino, Y. Akiyama, I. Nonaka, N. Kanbara and T. Kawakami (1990). Chitosan as an ingredient for domestic animal feeds. J. Agric. Food Chem. 38(5): 1214–1217.

Howe, G. A. (2005). Jasmonates as signals in the wound response. J. Plant Growth Regul., 23: 223–237.

International Commission on Natural Health Products (1995). Chitin and Chitosan. Personal communication. Atlanta, GA.

Kendra, D. F. and L. A. Hadwiger (1984). Characterization of the smallest chitosan oligomer that is maximally antifungal to *Fusarium solani* and elicits pisatin formation in *Pisum sativum.* Expt. Mycol., 8: 276–278.

Kittur, F. S., K. R. Kumar and R. N. Tharanathan (1998). Functional packaging properties of chitosan films. Zeitschrift fur lebensmittel – Untersuchung und – Forschung A (206): 44-47.

Liu, J., S. Tian, X. Meng and Y. Xu (2007). Effects of chitosan on control of postharvest diseases and physiological responses of tomato fruit. Postharvest Biology and Technology, 11: 131-140.

Li, Q., E. T. Dunn, E. W. Grandmaison and M. F. A. Goosen (1992). Applications and properties of chitosan. J. Bioact. Compat. Polym., 7: 370–397.

Macheix, J. J., A. Fleuriet and J. Billot (1990). Fruit phenolics. Florida: CRC Press, Inc.

No, H. K., S. P Meyers and K. S. lee (1989). Isolation and characterization of chitin from crawfish shell waste. J. agricultural and food chemistry. 37(3):.575-579.

No, H. K., S. P Meyers, W. Prinyawtwatkul and Z. Xu (2007). Application of chitosan for improvement of quality and shelf life of foods: a review. Journal of Food Science, 72: 87-100.

No, H. K., Y. I. Cho, H. R. kim and S. P. Meyers. (2000). Effective Deacerylation of chitin under conditions of 15 psi\121oC. J. agricultural and food chemistry, 48(6):.2625-2627.

Pacheco, N., C. P. Larralde-Coron, J. Sepulveda, S. Trombottoc, A. Domardc and K. Shirai (2008). Evaluation of chitosans and *Pichia guillermondii* as growth inhibitors of *Penicillium digitatum*. International Journal of Biological Macromolecules, 43: 20-26.

Palpandi, C., V. Shanmugam and A. Shanmugam (2009). Extraction of chitin and chitosan from shell and operculum of mangrove gastropod Nerita International Journal of Medicine and Medical Sciences, 1(5): 198-205.

Pantaleone, D., M. Yalpani and M. Scollar (1992). Unusual susceptibility of chitosan to enzymatic hydrolysis. Carbohyd. Res., 237: 325–332.

Reddy, M. V., J. Arul, E. Ait-Barka, F. Castaigne and J. Arul (1997). Effect of chitosan on growth and toxin production by *Alternaria alternata* f. sp. *lycopersici*. Hort. Science, 32: 467-468.

Reddy, M. V., J. Arul, E. Ait-Barka, P. Angers, C. Richard and F. Castaigne (1998). Effect of chitosan on growth and toxin production by *Alternaria alternata* f. sp. *lycopersici.* Biocontrol Sci. Technol., 8: 33–43.

Reddy, M. V., K. Belkacemi, R. Corcuff, F. Castaigne and J. Arul (2000). Effect of preharvest chitosan sprays on postharvest infection by *Botrytis cinerea* and quality of strawberry fruit. Postharvest Biol. Technol., 20: 39–51.

Rhoades, J. and S. Roller (2000). Antimicrobial actions of degraded and native chitosan against spoilage organisms in laboratory media and foods. Appl. Environ. Microbiol., 66(1): 80–86.

Roberts, G. A. F. and J. G. Domszy (1982). Determination of the viscosimetric constants for chitosan. International Journal of Biological Macromolecules, 4: 374–377.

Saharan, V., A. Mehrotra, R. Khatik, P. Rawal, S. S. Sharma and A. Pal (2013). Synthesis of chitosan based nanoparticles and their *in vitro* evaluation against phytopathogenic fungi. Inter. J. Bio Macromolecules, 62: 677-683.

Saraswathy, G., S. Pal, C. Rose and T. P. Sastry (2001). A novel bio – inorganic bone important containing deglued bone, chitosan and gelatin. Journal of Bull. Mater. Sci. 24(4): 415- 420.

Shahidi, F., J. K. V. Arachchi and Y. J. Jeon (1999). Food applications of chitin and chitosans. Trends Food Sci. Technol. 10, 37–51.

Singleton, V. L., R. Orthofer and R. S. Lamuela Raventós (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin Ciocalteau Reagent. Methods Enzymol., 299: 152–178.

Stinson, E. E., D. D. Bills, S. F. Osman, J. Siciliano, M. J. Ceponis and E. G. Heisler (1980). Mycotoxin production by *Alternaria* species grown on apples, tomatoes, and blueberries. J. Agr. Food Chem., 26: 960–963.

Suitcharit, C., F. Awae, W. Sengmama and K. Srikulkit (2011). Preparation of Depolymerized Chitosan and Its Effect on Dyeability of Mangosteen Dye. Journal. Science Chiang Mai, 38(3): 473-484.

Terry, L. A. and D. C. Joyce (2004). Elicitors of induced disease resistance in postharvest horticultural crops: a brief review. Postharvest Biology and Technology, 32: 1-13.

Toor, R. K., C. E. Lister and G. P. Savage (2005). Antioxidant activities of New Zealand-grown tomatoes. International Journal of Food Sciences and Nutrition, 56(8), 597–605.

Tsai, G. J. and W. H. Su (1999). Antibacterial activity of shrimp chitosan against *Escherichia coli*. J. Food Prot., 62: 239–243.

Weiner, M. L. (1991). An overview of the regulatory status and of the safety of chitin and chitosan as food and pharmaceutical ingredients. In: Brine, C. J., P. A. Sand ford and J. P. Zikakis (Eds.), Advances in Chitin and Chitosan. Proceedings from the 5th International Conference on Chitin and Chitosan, Prince town, New Jersey, USA, pp. 663–672.

Winer, B. J. (1971). Statistical Principles in Experimental Design. 2nd ed. MiGraw-Hil Kogakusha, LTD, 596 pp.

Winterowd, J. G. and P. A. Sanford (1995). Chitin and chitosan. Food polysaccharides and their applications. In: Stephen, A.M. (Ed.). Marcel Dekker Inc., New York, pp. 441–462.

Xu, J. G., X. M. Zhao, X. W. Han and Y. G. Du (2007). Antifungal activity of oligochitosan against *Phytophthora capsici* and other plant pathogenic fungi *in vitro*. Pesticide Biochemistry and Physiology, 87: 220-228.

دور الكيتوزان المستخلص من مخلفات الجمبرى في مقاومة مرض العفن الأسود في ثمار الطماطم المتسبب عن فطر *Alternaria alternata*

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تعانى نباتات الطماطم بالعديد من الأمراض خلال كل مراحل حياتها. ويعد مرض العفن الأسود المتسبب عن فطر *A. alternata* من أهم أمراض ما بعد الحصاد التي تصيب ثمار الطماطم. وقد تم دراسة تأثير بعض تركيزات محلول الكيتوزان على نمو فطر الألترناريا المسبب لمرض العفن الأسود في ثمار الطماطم. وقد أختبرت عزلة الفطر تحت ظروف المعمل على بيئة أجار البطاطس المضاف لها سبع تركيزات من الكيتوزان (صفر، ١، ٢، ٣، ٤، ٥ و ٦ ملليجرام/مللي). حيث ثبط الكيتوزان بشكل معنوي النمو الطولي للفطر بنسبة ٦٧.٤ % عند تركيز ٦ ملليجرام/مللي. وقد تم إجراء العدوى الصناعية بالفطر المختبر لثمار الطماطم المعاملة بمحلول الكيتوزان مقارنة بمعاملة مبيد إبروميس وتحضينها على درجات حرارة ٨، ١٨ و ٢٨ درجة مئوية حيث تم قياس قطر العفن والمحتويات الفينولية الكلية في الثمار بعد ٧ و ١٤ يوم من إجراء المعاملة. وقد وجد أن الكيتوزان اختزل بشكل معنوى شدة المرض على ثمار الطماطم فى كل المعاملات وخاصة عندما تم تخزين الثمار على درجة حرارة ٨ درجة مئوية مقارنة بمعاملة الكنترول. كما أدت معاملة الكيتوزان إلى زيادة المحتويات الفينولية الكلية مقارنة بالكنترول بينما أدت معاملة المبيد لزيادة طفيفة في الفينولات الكلية. وفى كل المعاملات زادت الفينولات في بداية المعاملة ولكنها أخذت في الإنحدار في نهاية فترة التخزين. وتدل نتائج هذه الدراسة على أن الكيتوزان كان بمثابة أحد بدائل المبيدات الآمنة والتي يمكن استخدامها كطريقة حماية وخاصة عندما يتم التخزين على درجات حرارة منخفضة ضد مرض العفن الأسود في ثمار الطماطم والذي يسبب خسائر اقتصادية كبيرة أثناء التداول والتسويق والتخزين.