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Prolongation of the Shelf-Life of Strawberry Fruits by the Reduction of the Gray Mold; *Botrytis cinerea* using Gamma Irradiation and/or Chitosan

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ABSTRACT

Chitosan plays a central role as an antifungal agent, its impact depends mainly on the concentration. In this respect, chitosan (0.4%) decreased the radial growth of *Botrytis cinerea* by 77.8 % under *in vitro* conditions. *In vivo*, the infection severity significantly reduced after three weeks of storage (13°C). Fruit coating by chitosan (0.4%) significantly increased fruit firmness, whereas reduced the total soluble solids, this effect was obvious with the progress of the storage time. Vitamin C content of the fruit gave fluctuated trend with the progression of storage time. The severity of the infected strawberry fruits was reduced from 100% to 49.9% as a result of gamma irradiation (2.5KGy) whereas, in the healthy strawberry fruits, the severity was reduced from 100% to 29.1% after 3 weeks of storage period. Combination of chitosan and gamma irradiation led to a significant increment of peroxidase activity. Scanning electron microscopy of the pathogen, that was treated by chitosan, showed damages in cell structure and changes in surface morphology, the same effect was observed by gamma irradiation. The same combination was found to alter the pathogen morphology and caused damage to the cell structure. So, it is recommended using such combination for extending the shelf-life of strawberries fruits.

Keywords: Gamma irradiation, chitosan coating, strawberry fruits, shelf-life, *Botrytis cinerea*.

INTRODUCTION

Strawberries (*Fragaria ananassa* Duch.) is a highly perishable fruit in the postharvest stage due to fungal infections. The shelf-life of fresh fruits at the refrigerator was about 5 days. Braun and Sutton (1987) reported that during storage and shipment of fruits major losses occurred in the horticultural industry, mainly by *Rhizopus stolonifera* and *B. cinerea*, which cause soft rot and gray mold diseases, respectively. Fungicides are effective chemicals for regulation the postharvest diseases. This procedure faces various health and environmental problems. In addition to the reports of fungicide-resistant fungal strains. Therefore, there is an ultimate need to alternative save strategy by involving safe control procedures (Tarek, 2004).

The polysaccharide; chitosan has been shown to have fungicidal activity against various fungi (El-Ghaouth *et al*., 1990). Treatment of strawberry fruits by chitosan reduced the occurrence of the infectious fungi compared with the untreated ones, which decayed faster and the decay was detected directly after storage (Vargas *et al*., 2006). Another investigation used commercial chitosan reported obvious effect in reduction of gray mold and rot of strawberries when fruits were treated with the chitosan solution and stored for 96 h at room temperature (Romanazzi *et al.* 2013).

Generally, it was previously established that besides being active in maintaining the quality and expanding the shelf-life of fresh fruits, chitosan also prevent microbial damage, healthy safe and effective coating agent, (Shiekh *et al.* 2013)

Moreover coating fruits by chitosan reduces water loss and maintains the quality of the strawberry such as color, titratable acidity, and vitamin C. Such study reported enhancements in the antioxidant activity of some enzymes, as well as stopping in the flesh browning and reduction of fruit damage as a result of chitosan coating (Petriccione *et al.* 2015)

The *in vitro* and *in vivo* assessment of gamma irradiation as an antifungal agent against *B. cinereal*, revealed that 99kGy reduced the fungal intensity by 90%. At 4.0kGy of gamma irradiation completely inhibited the germination of spores and the growth of mycelia (Chu *et al.* 2015). Hussain *et al*., (2013) studied the combinatory treatment as a synergistic effect to maximize the GI action, in this connection, the modified atmosphere, heat, and washing effectively extended the shelf-life of the fruits.

The present work aimed to increase the shelf-life of strawberries fruits by using gamma irradiation and/or chitosan.

MATERIALS AND METHODS

Strawberry fruits

The fruits were obtained from various farms of El-Sharkia governorate. Then, divided into two groups; non-infected and infected fruits. The infected ones were investigated after 3 days of storage (13°C).

Botrytis cinerea

Botrytis cinerea as a recorded causative agent of gray mold was kindly obtained from Mycological Research and Plant Diseases Survey Department, Plant Pathology Research Institute, Agricultural Research Center, Giza, Egypt.

***In vitro* activity of chitosan**

The anti-activity of chitosan against *B. cinerea* was carried out on plates of Potato dextrose agar (PDA) supplemented with 0.1, 0.2 and 0.4% of chitosan. The plates were inoculated with 3-mm disks of taken form a 7-days-old culture of *B. cinerea*. When control fungus (0 chitosan) reached full growth, the experiment was stopped and the linear growth of the treated fungus was measured.

Inoculum preparation

The concentration of the conidial suspension used in this study was adjusted to 2×10^5 conidia/mL. *B. cinerea* was grown for 2 weeks on PDA, then the conidia were obtained by filtering the mycelial suspension through 3 layers of sterile cheesecloth.

Gamma irradiation (GI)

Indian Co^{60} gamma cell was used at doses of 1.0, 1.5 and 2.5 KGy/hr to treat strawberry fruits. Three

replicates were used, each of 15 fruit. The treatments were packed in perforated plastic containers before storage. During which the fruits were assessed for diseases severity (%) development along 3 weeks at 13°C.

Chitosan solution

Chitosan was prepared by dissolving 0.1، 0.2 and 0.4% of chitosan in 2% acetic acid, heated with constant agitation for 24 h. Then the pH was adjusted to 5.5 by 0.1N NaOH; then 0.1 mL of tween 80 was added (El-Ghaouth *et al*., 1990). The fruits were coated by different chitosan concentrations before stored.

Studied parameters

The method of Kader (1991) was used to determine (1) the total soluble solids (TSS%) using ago (Japan) NI refractometer and expressed in Brix, and (2) the firmness (g/cm^2) of the fruit (the highest penetration force directly before tissue breaking) using hand penetrometer.

The method of Lucoss (1994) was applied for the determination of ascorbic acid (vitamin C) content and expressed as mg/100 mL of juice.

Determination of peroxidase activity

Fruits treated with chitosan (0.4%), GI (2.5kGy) and their combination were evaluated for peroxidase after 10 days storage. The enzyme was extracted by crushing the fruits in sodium phosphate buffer at pH (7.1). The supernatant containing the crude enzyme extract was used for enzyme assay as the variation in absorbance (425 nm) per min (Allam and Hollis (1972).

Scanning electron microscopy

Treated and non-treated *B. cinerea* grown in PD broth were prepared as described by Harley and Fergusen (1990). The examination was performed utilizing Joel Scanning Electron Microscope (JSM-1200 EX).

Design and statistical analysis of the experiments

The experimental design was one-way randomized blocks. The analysis of variance was performed to verify the difference between treatments. Means of the date were compared based on Duncan's mutable range test at probability (*P*) level of ≤ 0.05, all statistical analysis was performed with the aid of CoStat software (CoHort Software, U.S.A) version 6.4.

RESULTS

The anti- *B. cinerea* activity of chitosan

Table (1) shows that higher chitosan concentration caused a marked reduction in the linear growth of *B. cinerea*. As could be seen at 0.4%, chitosan inhibited the linear growth of the fubgal pathogen by 77.8% compared with control.

Table 1. The radial growth of *B. cinerea* as affected by the various chitosan concentrations

Chitosan, %	Linear growth (cm)	Inhibition, %
0.0	9.0 a	0.0 d
0.1	7.0 b	22.2 c
0.2	5.0 c	44.4 b
0.4	2.0 d	77.8 a

Means followed by various letters in each column are varied significantly at 5%.

The effect of different doses of GI (1, 1.5 and 2.5KGy) on the severity of strawberry fruits stored at 13°C for 1, 2, 3 weeks are presented in Table (2). As the storage period increases the disease severity (%) increases. The opposite trend was observed with the various doses of GI. However, the 2.5KGy was effective which decreased severity along the storage period.

Table 2. Reduction of gray mold severity of strawberry fruits by gamma irradiation at different storage periods.

Storage periods (weeks)	Gamma doses (kGy)	Severity, %	
		Infected	Non-infected
1	0	55.5a	48.9a
	1	45.3b	40.1b
	1.5	38.4c	29.8c
	2.5	31.7d	23.3d
2	0	100.0a	100.0a
	1	73.8b	45.5b
	1.5	65.6c	41.7c
	2.5	45.9d	25.1d
3	0	100.0a	100.0a
	1	80.8b	54.4b
	1.5	69.7c	46.3c
	2.5	49.9d	29.1d

Means followed by various letters in each column are varied significantly at 5%.

Data in Table (3) show the effect of different chitosan concentrations (0, 0.1, 0.2 and 0.4%) on the severity of strawberry caused by *B. cinerea* stored at 13°C for 1, 2, 3 weeks. As the concentration of chitosan increases the severity of the disease markedly decreased. The lowest severity % was recorded at 0.4%, Generally, with the prolongation of storage period the severity % increases. The optimum treatments obtained from the above trials were chosen to be applied in combination in the following trial.

Table 3. Reduction of gray mold severity of strawberry fruits by chitosan at different storage periods.

Storage periods (weeks)	Chitosan, %	Severity, %	
		Infected	Non-infected
1	0	59.8a	42.4a
	0.1	31.1b	21.6b
	0.2	20.1c	7.2c
	0.4	9.7d	2.4d
2	0	89.4a	77.66a
	0.1	57.3b	30.1b
	0.2	39.9c	20.4c
	0.4	33.8d	16.9d
3	0	100.0a	100.0a
	0.1	62.5b	40.4b
	0.2	53.4c	28.1c
	0.4	40.1d	19.2d

Means followed by various letters in each column are varied significantly at 5%.

The combined effect of GI and chitosan

The combined effect of GI and chitosan on severity (%) of gray mold development on strawberry fruits is presented in Table (4). The combination between GI (2.5KGy) and chitosan (0.4mg%) was more pronounced in reducing the severity compared with sole application of chitosan or GI. The combined application reduced the severity of gray mold development. In which by the third week of storage, the severity of infected and healthy strawberry decreased from 100 and 100 to 24.7 and 18.9 %, respectively.

Table 4. Combined effect of GI (2.5KGy) and chitosan (0.4%) on strawberry gray mold development at various storage intervals

Period (week)	Treatment	Severity %	
		Infected	Non-infected
1	Control	55.5a	48.9a
	Chitosan	10.8c	4.5c
	GI	31.7b	26.3b
	GI+Chitosan	8.5d	2.1d
2	Control	100.0a	100.0a
	Chitosan	33.8c	16.9c
	GI	48.9b	25.1b
	GI+Chitosan	19.9d	8.7d
3	Control	100.0a	100.0a
	Chitosan	40.1c	19.2c
	GI	49.9b	29.1b
	GI+Chitosan	24.7d	18.9c

Means followed by various letters in each column are varied significantly at 5%.

Quality parameters of strawberry

As shown in Table (5), the quality features of strawberry fruits varied as a result of the interface between storage and chitosan coating. In which, TSS of the treated strawberries with chitosan only significantly reduced along the 3-weeks of storage. The firmness exhibited a similar trend, but it was amplified by chitosan coating at 0.4%, giving the highest values of TSS, firmness and vitamin C at the different storage periods.

Table 5. Quality parameters of strawberry as affected by chitosan concentrations and storage weeks under *B. cinerea* infection.

Storage period (week)	Chitosan %	TSS (Brix)		Firmness (g/cm^2)		Vitamin C (mg)	
		Infected	Non-infected	Infected	Non-infected	Infected	Non-infected
1	0.0	5.90d	6.73d	400.0b	422,5c	0.024d	0.028c
	0.1	6.88c	6.87c	404.1b	423.3c	0.025c	0.029b
	0.2	7.01b	7.10b	453.7a	448.7b	0.027b	0.029b
	0.4	7.21a	8.21a	457.6a	450.1a	0.030a	0.031a
2	0.0	5.35d	5.91d	299.1c	342.7d	0.021d	0.019c
	0.1	5.68c	6.12c	301.8c	345.8c	0.022c	0.019c
	0.2	5.79b	6.33b	330.9b	352.1b	0.025b	0.020b
	0.4	6.13a	7.10a	345.5a	359.3a	0.030a	0.027a
3	0.0	5.09d	4.13d	198.01d	225.8d	0.015d	0.019c
	0.1	5.40c	5.01c	200.0c	235.3c	0.018c	0.019c
	0.2	5.70b	5.93b	214.8b	240.2b	0.023b	0.020b
	0.4	6.01a	6.01a	220.6a	245.7a	0.025a	0.023a

Means having the same letters in each column are statistically insignificant at 5% level

Peroxidase (POD) activity of the infected strawberry

Fig. (1) shows that the combined impact of chitosan (0.4%) and/or GI (2.5kGy) on the infected strawberry inoculated with B. cinereal after one-week of storage. The combination treatment induced the highest (POD) activity, followed by chitosan (0.4%) then GI (2.5 kGy). The fruits of the control treatment came later.

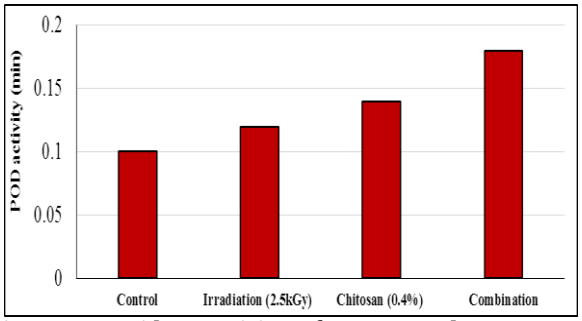

Fig. 1. Peroxidase activity after one-week storage of strawberry fruits as affected by GI (2.5kGy) and/or chitosan (0.4%) under infection by *B. cinerea*

Microstructural variation of *B. cinerea*

Fig. (2) shows the photography of the scanning electron microscopy of the microstructure variation of *B. cinerea* treated with chitosan (0.4%) and/or GI (2.5kGy). as could be seen morphological changes occurred in hyphae and conidiophores of the fungal pathogen.

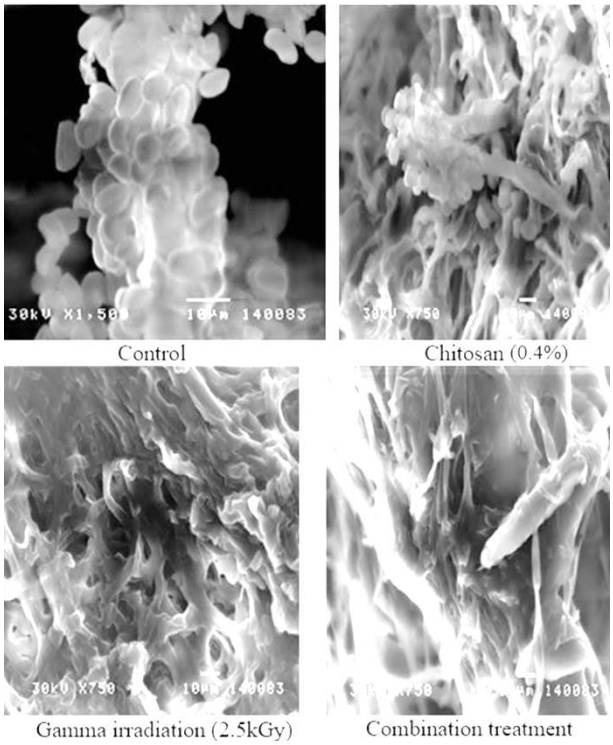

Fig. 2. Microstructural variation of *B. cinerea* recovered by the scanning electron microscopy as affected by chitosan and/or GI.

The non-treated *B. cinerea* has usual organs and cell wall including conidia, hyphae, sporangium, and sporangiophore. Treatment by chitosan at 0.4% changed the surface morphology, leading to marked damage in the fungal cell structure and sporangiophore. No effect was observed on the fungal spores. Regarding GI, there were marked changes in the surface morphology structure, and damage to the hypha and sporangiophore were also noticed. The combination of both chitosan and GI on *B. cinerea* displays an extra destructive influence on especially on the morphology of the surface and more real damage to cell structure, wavy surface. Additionally, no spore was detected.

DISCUSSION

Although strawberry is non-climacteric fruits, it has a high postharvest respiration rate, leading to a rapid deterioration at room temperature (Ribeiro *et al.* 2007). Several methods rather than fungicide chemicals were performed to increase the shelf-life of strawberry fruits since the accumulation of fungicide residues in the fruits cause several health problems and also lead to emerging of the resistant pathogens to chemical pesticides. In this connection, chitosan has been shown to be a promising safe biofungicidal agent against several pathogens (El-Ghaouth *et al*., 1990; Peng and Sutton, 1991 and Bakkali *et al*., 2008).

The present results reported that the severity of gray mold was reduced by 0.4% chitosan along the storage period. earlier work reported that dipping strawberries fruits in chitosan decreased the gray mold infection along with the 10-days of storage at 0 °C, followed by 4 days of shelf-life (Romanazzi *et al*., 2013). Li and Yu (2000) concluded similar results, they established the potential role of chitosan as an antifungal against the brown rot of peach, they added that its mode of action is by the reduction of the incidence of the pathogen that was correlated with the induction of defense response of the plant.

Casariego (2004) confirmed the antifungal activity of chitosan, which its film on was also stated to hinder the growth of fungi and yeasts in the contact area, forming an inhibition zone on the inoculated plates. There are main suggested mode of actions through which chitosan retard the decay of strawberries fruits which are (1) the fungistatic feature, (2) its inducibility of defense-related enzymes such as chitinase, chitosanase and β-1,3-glucanase and (3) its ability to induce plant-defense mechanisms such as phenolics (Benhamou, 1996; Atia *et al*., 2005; Aziz *et al.*, 2006 and Bautista-Bonas *et al*., 2006). So, chitosan could be considered as a novel class of plant protection agents (Atia *et al*., 2005). Ribeiro *et al.* (2007) and Romanazzi (2010) reported a reduction in pre-harvest and postharvest decay of strawberries by chitosan as well as reduction of the pathogen growth.

It seems that chitosan plays the same role in various plants, in this connection, Ben-Shalom *et al*. (2003) reported that in cucumber coated with chitosan, an increased resistance was observed against *B. cinerea* and that was correlated with POD activity. Also, Li and Yu (2001) found retards in the deterioration of peaches coated by chitosan, and that may be due to the decrease in respiration rate, which reduces the malondialdehyde production, stimulation superoxide dismutase activity and maintaining membrane integrity. That is why chitosan can maintain the quality during the shelf-life,

In regard to the mode of action of chitosan on the fungal cell, it was supposed that chitosan molecules penetrate the extracellular structure and damage the intracellular structure. This damage includes the serious harmful effect to the fungal cell, as well as the ability to form a resistant layer around the cell, causing death similar to that occurs by synthetic fungicides (Hernandez-Lauzardo *et al.*, 2011; Junior *et al.*, 2014).

The present data revealed that GI reduced the development of disease severity of strawberry fruits especially at 2.5kGy. Similarly, Shadi and Ehab (2011) found a noticeable reduction in the infected strawberry fruits inoculated with *B. cinerea* as a result of GI at 2.5 KGy compare with control.

The present results concluded reduction in TSS, firmness and vitamin C with the prolongation of storage, whereas the three parameters increased by increasing chitosan concentration. Dam and Nguyen (2011) reported enhancement in the firmness of strawberries fruits when coated with chitosan. El-Ghaouth *et al*. (1990) and Luna *et al*. (2001) reported similar results, in which coating with chitosan led to greater firmness of fruits of strawberries, tomatoes, and peaches.

The pictures of the scanning electron microscopy showed the damages of cell structure, surface morphology, sporangiophore and hypha of *B. cinerea* caused by chitosan and/or GI. The combination of chitosan and GI caused the most destructive effect. Previously, Swelim (2004) concluded that scanning electron microscope confirmed that decrease in sporulation and morphology abnormalities of *Fusarium solani* occurred after GI at high dose (10 kGy), meanwhile, at a low dose (1.0 kGy) caused malformation and compactness of mycelia accompanied with the absence of sporulation.

CONCLUSION

Chitosan at 0.4% and GI at 2.5KGy showed antifungal activity and reduced the disease severity caused by *B. cinerea*, as well as, significantly extended the shelf-life and improved fruit quality (TSS, firmness and vitamin C). Chitosan and GI were found to be more effective in shifting fungus morphology and cell structure. The results recommended using a combination of chitosan and gamma radiation in order to reduce disease development and extend the shelf-life of strawberry.

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إطالة عمر فترة التخزين لثمار الفراولة عن طريق الحد من العفن الرمادي؛ *Botrytis cinerea* باستخدام إشعاع جاما و/أو الشيتوزان

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يمكن أن يلعب الشيتوزان دوراً مهمًا باعتباره مضادًا للفطريات وخصوصاً فطر *Botrytis cinerea*، حيث أن تأثيره يعتمد على التركيز. في هذا الصدد، أحدث الشيتوزان (0.4 %) أنخفاضا فى النمو الميسليومى لفطر *B. cinerea* بنسبة 77.8 % في ظل ظروف المختبر. وفي تجارب التخزين، انخفضت شدة العدوى من 59.8، 89.4 و 100 إلى 9.7، 33.8 و 40.1 بعد الأسبوع الأول والثاني والثالث من التخزين عند 13 درجة مئوية، على التوالي. أيضاً أدى تغليف ثمار الفراولة بالشيتوزان (0.4%) إلى زيادة صلابة الثمار بشكل كبير، في حين إنخفضت المواد الصلبة الذائبة الكلية، كان هذا التأثير واضحًا مع تقدم وقت التخزين. أعطى محتوى فيتامين C فى الثمار اتجاه متقلب مع تقدم وقت التخزين. كما قللت المعاملة بأشعة جاما (2.5 KGy) من شدة إصابة الثمار المعداه من 55.5 و 100 و 100 % إلى 31.7 و 45.9 و 49.9 % وفي الثمار الغير معداه انخفضت شدة الاصابة من 48.9 و 100 و 100 % إلى 23.3 و 25.1 و 29.1 % خلال 1 و 2 و 3 أسابيع من فترات التخزين، على التوالي. تسببت المعاملة بكل من إشعاع جاما والشيتوزان معاً في زيادة ملحوظة في نشاط البيروكسيديز. كما أظهر الفحص بالميكرسكوب الاليكترونى للمسبب المرضى، الذي تمت المعاملة له بالشيتوزان، أضراراً في بنية الخلية والتغيرات في التشكل السطحي، وقد لوحظ نفس التأثير من خلال المعاملة باشعة جاما. كما أن المعاملة بكل من إشعاع جاما والشيتوزان معاً كان أكثر فعالية في تغيير الشكل السطحى للفطريات وتلف بنية الخلية. لذلك، يوصى باستخدام هذا المزيج لتمديد فترة صلاحية ثمار الفراولة.