Scientia Horticulturae 257 (2019) 108736

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

Effect of pre-harvest application with some organic acids and plant oils on antioxidant properties and resistance to *Botrytis cinerea* in pepper fruits

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ARTICLE INFO

Keywords:
Pepper fruits
Botrytis cinerea
Plant oils
Salicylic
Citric acid
Antioxidant

ABSTRACT

Pepper is planting widely in plastic houses and the fields; *Botrytis cinerea* infection starts in the field, but is strongly appear during storage. Results revealed that spraying pepper plants under plastic houses condition with salicylic, citric acids and clove or olive oils at different concentration (Salicylic acid at 8 mM and citric acid at 30 Mm, meanwhile clove and olive oils at rate 5 m/l) reduced development of grey mold on fruits during cold storage. The mycelial growth of *B. cinerea* was the most sensitive to salicylic acid, where it completely inhibited the growth of tested fungus at concentrations 4 and 8 Mm. Clove and olive oils at their tested concentrations (2.5 and 5 m/l) reduced the growth of *Botrytis cinerea* to 100%. All tested plant oils and citric acid increased the epidermis and cuticle thickness and decreasing simultaneously the disease infection incited by *Botrytis cinerea* on pepper fruits. Spraying pepper plants with all tested treatments increased peroxidase and polyphenoloxidase activities at 2 and 5 days from storage in naturally infected pepper fruits and at 5 days from storage in infected fruits with *B. cinerea*. The total phenolic compounds (TPC) and antioxidant activity (2,2-Diphenyl-1-picrylhydrazyl DPPH) were determined. Natural infection treated by clove 2.5 m/l had the highest content of TPC. The high antioxidant activities of clove treated pepper fruits were attributed to its high contents of total phenolic compounds.

1. Introduction

Pepper (*Capsicum annuum L.*) is considered one of the most important vegetable crops cultivated in Egypt for both local market and exportation. Pepper occupied an important rank in the Egyptian and world agriculture due to its nutritional value and high profit (El-Hifny et al., 2011). Pepper is exposed to heavy losses in yield and quality due to number of diseases (Zitter, 2011).Gray mould fungus, *Botrytis cinerea* is a significant necrotrophic plant pathogen causing devastating diseases on more than 500 plant species, especially on fresh fruits and vegetables including pepper (Li et al., 2018). *Botrytis cinerea* is a causal agent of gray mould of pepper fruits and causes post-harvest losses in fruit quality (Kamara et al., 2016).

In recent years, significant alternative control strategies have been used to control post-harvest diseases that are economic, effective and safe for human health. Through treatment of pepper fruits with salicylic acid (SA) and abscisic acid as resistance inducers significantly decreased gray mould development under laboratory conditions (Kamara et al., 2016).Salicylic acid (SA) is a phenolic hormone that plays a crucial role in stress resistance in plants. Pre and post-harvest spraying with SA has achieved better control against pathogens in pear (Jiankang et al., 2006), also in sweet cherry through induction of the defense resistance system(Yao et al., 2005) and stimulation of antioxidant enzymes (Xu and Tian, 2008).Spraying *Capsicum frutescens* with SA induce a significant increase in peroxidase activity and keep catalase activities in values near that of negative control (Amin et al., 2009). Also, SA as pre-harvest treatment caused significant increase in polyphenol oxidase and peroxidase activities in pepper (Mahdavian et al., 2007). Citric acid had lower degree of browning and relatively lower pH value than the other treatments (Kanlayanarat, 2003), strongly decreased grey mould (*Botrytis cinerea)* on different plant hosts (Elad, 1992) and showed high effective in controlling *B. cinerea* infection on snap bean pods (Abdel-Rahman, 2015).

The plant oils are known as natural antioxidants due to as antifungal and biodegradable ability and do not leave any residual effect on fresh produce (Kalemba and Kunicka, 2003). Essential oils have strong impact on post-harvest decay caused by *Botrytis cinerea* and fruit quality of peach (Samane and Aminifrad, 2012). Also, clove oil was used as alternative options for the control of gray mould on post-harvest organic fruits (Sirirat et al., 2009). On the other hand, Olive oil Mill Wastewater

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https://doi.org/10.1016/j.scienta.2019.108736
Received 22 April 2019; Received in revised form 28 July 2019; Accepted 31 July 2019
Available online 14 August 2019
0304-4238/

(OMW) inhibits *B. cinerea* linear growth *in vitro* may be due to the activity of the phenolic compounds contained on olive OMW. Furthermore, olive oil significant decreased fungus mold formation on the tested fruits (Vagelas et al., 2009). Peroxidase and polyphenoloxidase activities were increased in pods of bean plants sprayed with Carnation oil (Abdel-Mageed et al., 2014).

Anatomical changes were investigated by (Nour et al., 2012). who found that spraying green bean plants with salicylic acid or citric acid increased thickness of leaflet blade, thickness of palisade and spongy, except salicylic acid at 100 ppm that had the opposite effect on these leaflets anatomical characters. Also, Marhoon and Abbas (2015) studied stem cross sections of sweet pepper sprayed with seaweed extract and found that the thickness of cortex and vascular cylinder and diameter of vascular units increased significantly with increasing concentration of extract.

Plants produce secondary metabolites as a response to non-favorable environmental conditions developmental stages. Pathways of secondary metabolic have stimulated a range of plant defense compounds, which are secondary metabolites. Many accumulate in high levels in some species. In addition, many thousands of molecules have been identified. These compounds can be characteristic for a certain plant family, genus, or even species and therefore may be used as taxonomic tools in classifying plants (Yang et al., 2018).

The present work aimed to determine the role of pre-harvest spraying of pepper plants with some organic acids and plant oils in protecting fruits during storage against gray rot caused by *B. cinerea*. Some anatomical changes in cuticle and epidermis of treated pepper plants with tested treatments were investigated. Also, polyphenoloxidase and peroxidase activity as well as the phenolic compounds and the antioxidant activity in treated pepper fruits were evaluated.

2. Materials and methods

2.1. Source of chemicals and plant oils

Two organic acids *i.e.*citric and salicylic acids and Standards 2,2-Diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Folin-Ciocalteus's phenol reagent was obtained from Merck Chemical Supplies (Darmstadt, Germany). Chemicals reagents and extraction solvents were of analytical grade. Olive (the fruit of *Olea europaea*) and clove (*Syzigium aromaticum* L.*)* were bought from Cairo Company for oils, Cairo Egypt. The chemical composition of olive oil was described by (Ramos-Comenzana et al., 1995; Ethaliotis et al., 1999 and Medina et al., 2013) as well as the chemical composition of clove oil was determined by (Vieira et al., 2018 and Sameza et al., 2016)

2.2. The pathogen

The pathogen *Botrytis cinerea* was isolated from infected pepper fruits obtained from the local market in Qalyoubia and inoculated on potato dextrose agar (PDA) medium under sterilized conditions. The purified isolate was identified depend on their morphological and cultural characters utilizing the descriptions of *B. cenirea* (Raposo et al., 1995). Fungal culture was identified in Mycology Research and Disease Survey Dept., Plant Pathology Research Institute, ARC, Giza governorate, Egypt. The tested fungus (*B. cinerea*) was grown onPDA medium, which are considered to be the most suitable medium for growth and sporulation (Vagelas et al., 2009).

2.3. Effect of some organic acids and plant oils on mycelial growth of B. cinerea in vitro

Two organic acids (salicylic and citric acids) and two plant oils (Olive and clove oils) were tested for their efficiency on growth of *B. cinerea in vitro*. Sterilized 100 ml potato dextrose agar (PDA) in 250 ml conical flasks were used. Each acid was tested *in vitro* at three concentrations, 2, 4 and 8 mM for salicylic acid and 7.5, 15 and 30 mM for citric acid. Each concentration of the tested acids was added to PDA medium before solidification. Meanwhile, olive and clove oils were tested *in vitro* at 1.25, 2.5 and 5 m/l PDA and each concentration was added to PDA medium with 0.5% tween 20.Unamended PDA medium was used as control treatment. The amended or un-amended medium with acids and oils were poured into 3 Petri dishes per each treatment. After medium solidification, 3 mm mycelia-discs cut from the periphery of seven days old cultures of *B. cinerea* were placed in the center on the surface of medium, and then incubated at 22 ± 2 °C for 5 days. The diameter of developed colonies was measured when fungal mycelium covered one plate in control treatment to calculate the inhibition effect.

The percentage of mycelial growth inhibition (I) at each concentration was calculated by the following formula:

$$I = [C\text{-}T]/C \times 100$$

Where: C is the diameter of the control colony and T is the diameter of the treated colony (Pârvu et al., 2008).

2.4. Plastic houses experiment

This experiment was carried out on Khayrat pepper variety under protected cultivation system in commercial plastic houses of Ministry of Agriculture and Soil Reclamation, A.R.E. (Dokki location), at Giza governorate, Egypt, during two successive growing seasons 2017, 2018. The cultivated pepper plants received traditional agriculture practices, *i.e.*irrigation, fertilizers, *etc.* The experimental plastic house consists of 9 rows, each (0.9 x 60 m, width x long) divided into 10 parts 6 m long each. The experimental unit, plot, consisted of three rows of pepper plants, each row is 6 × 0.9 m (18 hill per row and each hill contain two plants). Three plots were used as replicates for each treatment in complete randomized design.

2.4.1. Effect of pre-harvest spraying pepper plant with some organic acids and plant oils on gray mold incidence of stored pepper fruits at 12±1°C and 90–95% RH for 20 days

The growing sweet pepper sprayed with proposed treatments 3 times, at bloom stage and repeated every 20 days as time interval between sprays till 5 days before harvesting. Salicylic acid was sprayed at concentrations of 4 and 8 mM. In case of citric acid was at 15 and 30 mM. While, olive and clove oils at rate of 2.5 and 5 m/l (with 0.5% tween 20) were tested also for their abilities to control pepper fruits gray mold. The sprayed sweet pepper plants with plain water served as control treatment.

Pepper fruits were harvested at mature-green from each treatment and transferred to Post-harvest diseases Department, Plant Pathology Research Institute, ARC. Fresh sample of pepper fruits were graded into uniformity of size. Pepper fruits (Khayrat, cv.) were divided into two groups. First group, pepper fruits surface were sterilized by dipping them into 70% ethyl alcohol for one minute, rinsed four times with sterilized distilled water then wounded by puncturing the peel of each fruit on the equator with a template of 4 sterilized steel rods (2-mm deep by 0.5 mm diameter) in circle area of 5 mm diameter, and left for air-dried onto filter paper prior to use. Punctured fruits were inoculated with the prepared spore suspensions of *B. cinerea* using an atomizer, spore suspension was prepared by brushing the surface of the culture in the presence of 10 ml of sterilized water per 9-cm Petri-plate and then the spore suspensions were filtered through three layers of sterile cheese cloth to get rid of mycelial residue. The required concentration of 10^6 conidia /ml was adjusted by diluting the suspension after counting them using a hemocytometer. Second group, pepper fruits were left without sterilization and puncturing (as natural infection).

Three replicates were used for each treatment. Each replicate

consisted of 15 fruits and put in sterilized punctured carton box (one box for each replicate). Naturally infected and artificially inoculated pepper fruits were stored at12 ± 1 °C and 90–95% relative humidity (RH) for 20 days. Disease severity (%) of infected pepper fruit were recorded according to Vagelas et al. (2009) as follows:

$$\text{Disease Severity}(\%) = \frac{\sum (n\times c)}{D\times N} \times 100$$

Where: n = number of infected fruits in each category, c = numerical values of symptoms category, D = maximum of numerical values of symptoms categories and N = total number of fruits.

The five categories are represented as following:

0 = healthy fruit, 1 = decayed area of the fruit ranged 1–25%, 2 = decayed area of thefruit 26–50%, 3 = decayed area of the fruits 51–75% and 4 = decayed area of the fruit76 to 100%.

2.4.2. Anatomical studies (cuticle and epidermis layers thickness)

The fruit samples (natural infected fruits) were taken 15 days after the third spray with organic acids (salicylic acids was sprayed at concentrations of 8 mM and citric acids at 30 mM) and plant oils (Olive and clove oils were sprayed at rate 5 m/l).The samples contained fruit No 1 from the beginning of newest branch in pepper plant. The taken vegetative specimens were killed and fixed in FAA dehydrated in ethyl alcohol, embedded in paraffin wax and sectioned to 20μ in thickness according to (Sass, 1951). Sections were stained using the double stain method (fast green and safranin), cleared in xylene and mounted in Canada balsam (Johanson, 1940). Selected sections were examined and photographed using microscope (at lens of 100 X – scale bar 20 μm) to determine the anatomical changes in fruits (cuticle and epidermis) responses resulted from such treatment. The histology work was carried out in the Regional Center for Mycology and Biotechnology, AL-Azhar Univ.

2.4.3. Effect of pre-harvest spraying pepper plant with some organic acids and plant oils on peroxidase and polyphenoloxidase activity in pepper fruits

2.4.3.1. Enzyme extraction and activity assay. Pepper fruits under naturally and artificially inoculated with *Botrytis cinerea* were used to measure the oxidative enzymes activities, at 2 and 5 days post inoculation (DPI) according to (Anand et al., 2007).

2.4.3.2. Assay of polyphenol oxidase activity(PPO). The activity of Polyphenol oxidase was measured according to Matta and Dimond (1963).Polyphenol oxidase was expressed as the change in the absorbance of the mixture at 495 nm by Spectrophotometer (Spectronic 601 Milton ROY) every 0.5 min for 3 min period.

2.4.3.3. Assay of peroxidase activity (POD). Peroxidase activity was determined according to the method described by Allan and Hollis (1972). Peroxidase was expressed as a difference in the absorbance of the blend every 0.5 min for 3 min period at 425 nm by Spectrophotometer (Spectronic 601 Milton ROY).

2.4.3.4. Assay of total phenol compounds (TPC) and antioxidant activity (DPPH)

2.4.3.4.1. Preparation of pepper fruits extracts. The samples of treated pepper fruits with all tested treatments under naturally and artificially inoculated with *Botrytis cinerea* were used to estimate the total phenol and antioxidant activity at 2 DPI. treated fruits were washed under tap water running. The fruits were freeze-dried by using a freeze dryer Edwards Modulyofreeze-drier (Edwards Ltd, UK) with total pressure equal to 4.6 Pa and temperature inside a vacuum chamber −40 °C. Average freeze-drying time was approximately 48 h and after drying, a final product reached a final temperature of about 25 °C. The freeze-dried was ground by coffee grinder extracted. The freeze-dried powder was storage at −40 °C until further analysis. Dried fruits were powdered and macerated in methanol for two days, filtered with Whatman filter paper No. 1, concentrated under vacuum at 50 °C. Each dried extract was re-suspended in methanol at concentration (1 mg/ml) as stock solution for further analysis.

2.4.3.5. Determination of total phenolic contents. Total phenolic of pepper fruits extract were determined by using Folin-Ciocalteu's method described by Singleton et al. (1999). The absorbance of the reaction mixtures was measured at 650 nm and results were expressed as milligrams of gallic acid equivalents (GAE) per gram of sample.

2.4.3.6. Determination of antioxidant activity. DPPH radical scavenging assay: Scavenging activity of different extract against DPPH radicals was assessed according to the method of Blois (2002) with a slight modification. One milliliter of DPPH radical solution (0.1 mM) in methanol was added to 3 ml of methanolic of each fruit part and whole fruit at different concentrations (10–150 μg/ml). Then the absorbance was determined at 517 nm against blank (methanol pure). The blend was shaken violently and left at room temperature for 30 min. in the dark. The butylated 4-Hydroxyl toluene (BHT) was used as a positive control; and negative control contained the entire reaction reagent except the extracts. The experiment was conducted in triplicate. The DPPH scavenging percentage effect (%) was calculated utilizing the following equation:

$$\text{DPPH\_ scavenging effect (Inhibition \%)} = [(Ac - AS/Ac)] \times 100$$

Where: Ac was the absorbance of the control reaction and as the absorbance in the presence of the plant methanolic extract.

Tested plant extracts concentration and the ordinate illustrate the average percent of scavenging capacity (Excel program). IC_{50} values denote the concentration of sample required to scavenge 50% of DPPH radical. Results were expressed as IC_{50} values (μg/ml). The IC_{50} values were calculated by sigmoid non-linear regression model using plots, where the abscissa represented the concentration of tested plant extracts and the ordinate represent the average percent of scavenging capacity (Excel program). IC_{50} values denote the concentration of sample required to scavenge 50% of DPPH radical. Decreasing absorbance of the reaction blend showed increasing free radical scavenging activity.

The experiment was designed as a completely randomized design (CRD). ANOVA was performed for the experiment using CoStat© Ver. 6.400 statistical data analytical software. Differences among means of data were compared by Tukey's HSD *in vitro* experiment, *and* Duncan's Multiple Range Test *in vivo* trial. Differences at $p \leq 0.05$ were considered significant.

3. Results and discussion

3.1. Effect of some organic acids and plant oils on inhibiting mycelial growth of B. cinereain in vitro

The effect of salicylic and citric acids at different concentrations on inhibiting the growth of *B. cinerea* was represented in Table 1. The growth of *B. cinerea* was decreased on PDA medium containing salicylic and citric acids at different concentrations. But, the mycelial growth of *B. cinerea* was the most sensitive to salicylic acid compared to citric acid, where it completely inhibited the growth of tested fungus at concentrations 4 and 8 Mm.It was clear from the obtained results that increasing the concentration of citric acid from 7.5 to 30 mM increased gradually the effect of this acid in reducing the growth of tested fungus, These finding could be confirmed by Abd-El-Aziz and Salem (2011) and Abdel-Mageed et al. (2012) who recorded that salicylic acid was the best effective compound on reducing the growth of *B. cinerea* where it completely inhibited the grow of *B. cinerea*. Meanwhile, Saad et al. (2014) found that the linear growth of *Alternaria solani* and *Fusarium solani* decreased significantly as the concentrations of salicylic and

Table 1
Effect of different concentrations of salicylic and citric acids on inhibiting mycelial growth of *B. cinerea* on potato dextrose agar medium *in vitro* after 5 days at 22 ± 2 °C.

Treatment	Conc. mM	*B. cinerea*	
		Mycelial growth	
		mm	Efficiency (%)
Salicylic acid	2	31.3 c	65.2
	4	0.0 d	100.0
	8	0.0 d	100.0
Citric acid	7.5	76.7 ab	14.8
	15	73.7 b	18.1
	30	62.0 b	31.1
control	–	90.0 a	

Within each column, same letter/s indicates no significant difference among treatments at ($P < 0.05$).
mM: Male mol. mm: male mater. Control (un-treated).

citric acids increased, but SA was the most efficient caused complete reduction of the fungal growth at 150 ppm. Also, salicylic and citric acids reduced the growth of *Botrytis fabae* on PDA medium, but salicylic acid was the most effective inhibitor on the linear growth (Mbazia et al., 2016). Also, Vinai and Gurdeep (2018) who found that salicylic acid inhibit mycelial growth and conidial germination of *Fusarium mangiferae*.

3.2. Effect of clove and olive oils on mycelial growth of B. cinerea in vitro

Data in Table 2) indicate that both clove and olive oils at all concentrations were able to reduce the mycelial growth of *B. cinerea* compared with control. In this respect, clove and olive oils at rate 2.5 and 5 m/l were the best among all tested concentrations where their tested concentrations (2.5 and 5 m/L) reduced the growth of *B. cinerea* to 100%.While, the low concentration (1.25 m/L) of the two tested plant oils was less effective on growth of the tested fungus. The obtained results are in agreement with those obtained by Mostafa et al. (2013) who found that clove oils completely inhibited the mycelial growth of *B. cinerea* and *A. alternate*. In the other side, *Penicillium* spp. and *Aspergillus* spp. were also sensitive to clove essential (Jian et al., 2018). Meanwhile, the filter sterilized olive oil mill wastewater (OMW) inhibits the growth of *B. cinerea* mycelium *in vitro*. This effect probably due to the activity of the phenolic compounds contained on olive OMW (Vagelas et al., 2009). Also, many research workers reported that plant oils caused deformation and dysfunction of the membrane could lead to interference in the generation of ATP within the fungal cell, mainly inhibiting the enzymes and substrate usage of ATP production (Coutinho de et al., 2011; El-Mogy and Alsanius, 2012).

Table 2
: Effect of different concentrations of clove and olive oils on inhibiting mycelial growth of *B. cinerea* on potato dextrose agar medium *in vitro* after 5 days at 22 ± 2 °C.

Treatment	Conc. m/L	*B. cinerea*	
		Mycelial growth	
		mm	Efficiency (%)
Clove oil	1.25	20.7 b	77.0
	2.5	0.0 c	100.0
	5	0.0 c	100.0
Olive oil	1.25	21.3 b	76.3
	2.5	0.0 c	100.0
	5	0.0 c	100.0
control	–	90.0 a	

Within each column, same letter/s indicates no significant difference among treatments at ($P < 0.05$).
m/L: male / litter. mm: male mater. Control (un-treated).

3.3. Effect of salicylic and citric acid as pre-harvest treatment on controlling grey mold disease of pepper fruits under storage conditions

Spraying pepper plants under plastic houses condition with salicylic and citric acids at different concentration reduced development of grey mold on fruits stored at 12 ± 1 °C and 90–95% RH for 20 days comparing with control (un-treated, inoculated and un inoculated) during two seasons 2017 and 2018 (Table 3).Higher concentrations of tested acids were more effective on decreasing the gray mold on fruits and showed highly significance on the efficacy than lower concentrations for all naturally infected or artificially inoculated pepper fruits with *B. cinerea*. Also, positive correlation with high concentration of acids treatment and disease reduction was calculated. Data also revealed significant differences between salicylic and citric acid treatments to control pepper fruit rots caused by *B. cinerea* in the two seasons.

At 10 days from storage, all tested acids at high concentration completely prevented the disease severity percentages of *Botrytis cinerea* and natural infection on detached pepper fruits post-harvesting during seasons 2017 and 2018, except for citric acid under artificially inoculated with *B. cinerea* during season 2018. While, after 20 days storage, more suppressing of gray mold on stored pepper fruits was simultaneously obtained when pepper plants were pre-sprayed with acids comparing with control. Also, results cleared that salicylic acid treatment revealed significant control of *B. cinerea* infection than citric acid. Higher concentrations of salicylic acid (8 mM) was the most suppressive treatments to control grey mold incited by *B. cinerea* with efficacies reached 82.9%, in reducing the disease severity during season 2017 and 83.2%, during 2018. As naturally infected pepper fruits, only the higher concentrations of salicylic acid (8 Mm) totally suppressed fungal decay development of stored pepper fruits. Generally salicylic acid at 8 mM was the most suppressive treatment against grey mold incited by *B. cinerea* as well as the most preservative treatment for maintaining naturally infected pepper fruits. This approach of disease control using organic acids was also adopted by Mutphy et al. (2000) they found that application of SA to tobacco gave significant degree of protection against *Botrytis cinerea*. Anti-oxidant salicylic acid recorded the least percentage of infection for strawberry fruits naturally or artificially infected with *B. cirerea*. (Abd-El-Aziz and Salem, 2011). Also, Mohamed et al. (2015) reported that pre-harvest application with salicylic or citric acids on snap bean plants significantly reduced grey mold on bean pods caused by *B. cinerea* and completely suppressed the fungal decay on naturally infected bean pods. Meanwhile, Li and Zou (2017) found that SA application caused a remarkable increase in accumulation of hydrogen peroxide (H_2O_2) and superoxide anion (O_2-), phenylalanine ammonia-lyase (PAL) activity, expression level of PR gene(pathogenesis related protein) in tomato plants and resulted in increasing resistance against *B. cinerea*.

3.4. Effect of clove and olive oils as pre-harvest treatment on controlling grey mold disease of pepper fruits under storage conditions

Spraying pepper plants with clove and olive oils decreased the disease severity (DS) on pepper fruits incited by *B. cinerea* during storage at 12 ± 1^{O}C and 90–95 RH for 20 days. Data in (Table 4) indicated that there were significant differences among treatments in pepper fruits inoculated with spore suspension of *B. cinerea* and after treating with different concentrations of clove or olive oils during 20 days of storage in two seasons. The DS on pepper fruits increased with the passage of time and reached more than74% in two seasons after 20 days of storage in control fruits. The highest fungicidal effect was observed in those pepper fruits for all naturally and artificially inoculated with *B. cinerea* when spraying pepper plants with 5 m/l olive oil followed by clove oil with the same concentration after 10 and 20 days

Table 3
: Effect of pre-harvest spraying with salicylic and citric acids in controlling the post-harvest infection of grey mold of pepper fruits during cold storage at 12±1°C and 90–95% relative humidity, for 20 days, seasons 2017 and 2018.

Treatment	Conc. mM	Season 2017							
		Artificial inoculation				Natural infection			
		10 days from storage		20 days from storage		10 days from storage		20 days from storage	
		D.S%	EF%	D.S%	EF%	D.S%	EF%	D.S%	EF%
Salicylic acid	4	7.1 b	62.6	23.1 c	69.6	2.8 b	77.5	10.4 b	66.8
	8	0.0 c	100.0	13.0 d	82.9	0.0 c	100.0	0.0 d	100.0
Citric acid	15	5.1 b	73.1	30.3 b	60.0	2.0 bc	83.8	3.9 c	87.6
	30	0.0 c	100.0	24.7 c	67.5	0.0 c	100.0	1.8 cd	94.4
Control		19.0 a		75.9 a		12.3 a		31.4 a	
Season 2018									
Salicylic acid	4	7.6 b	59.0	21.8 c	70.7	2.9 b	75.2	9.8 b	68.1
	8	0.0 d	100.0	12.4 d	83.2	0.0 c	100.0	0.0 d	100.0
Citric acid	15	4.5 bc	75.9	28.9 b	61.1	1.8 b	84.8	3.6 c	88.4
	30	0.9 cd	95.2	24.0 bc	67.7	0.0 c	100.0	1.3 cd	95.7
Control		18.5 a		74.2 a		11.7 a		30.7 a	

Within each column, same letter/s indicates no significant difference among treatments at ($P < 0.05$).
mM: Male mol. EF% : efficiency as percentage D.S%: disease severity as percentage.
Control was infected with B. cinerea and without infection and un-treated.

from storage during two seasons.

Generally, the results showed that DS was decreased with increased olive and clove oils concentration. These results are in agreement with earlier findings of Vagelas et al. (2009) who found that olive oil mill wastewater significant decreased fungus mold formation on strawberry and pepper fruits infected with *B. cinerea*. The inhibition of yam tuber rot caused by *Rhizopus stolonifera* and *Fusarium solani* increased significantly when clove oil used at 200 ppm (Sameza et al., 2016). Soaking banana fruits in clove suspension (2%) completely inhibited (100%) crown rot, flower end rot and significantly reduced finger rot and neck rot diseases (Zoeir et al., 2017). Also, Vieira et al.(2018) reported that clove oil greatly reduced the blue mold lesions in apples caused by *Penicillium expansum*.

3.5. Effect of pre-harvest sprays with some organic acids and some plant oils on epidermis and cuticle thickness of pepper fruits

Data given in Table 5 and indicated in Fig. 1 indicate that application of clove oil as pre-harvest treatment on pepper plants was the most positive treatment to increase the cuticle thickness of the fruits causing thicknesses of 2.2 µm compared with the control (0.7 µm), followed by olive oils and citric acid treatments. This increase in cuticle thickness is coincided with the increase in less infection by, *B. cinerea*, compared with the disease development in the control treatment. This

Table 5
: Effect of pre-harvest spray of some organic acids and plant oils on thickness of pepper fruits cuticle and epidermis after 15 days from the last spray.

Treatment	Cuticle µm	Epidermis µm
Salicylic acid	0.5 c	18.8 a
Citric acid	0.7 c	18.3 a
Clove oils	2.2 a	17.7 a
Olive oils	1.4 b	17.5 a
Control	0.7 c	14.3 a

Within each column, same letter/s indicates no significant difference among treatments at ($P < 0.05$).
µm: Macro mater. Control (un-treated).

Table 4
: Effect of pre-hravest spraying with clove and olive oils in controlling the post-harvest infection of grey mold of pepper fruits during cold storage at 12±1°C and 90–95% relative humidity, for 20 days, seasons 2017 and 2018.

Treatment	Conc. m/L	Season 2017							
		Artificial inoculation				Natural infection			
		10 days from storage		20 days from storage		10 days from storage		20 days from storage	
		D.S%	EF%	D.S%	EF%	D.S%	EF%	D.S%	EF%
Clove oil	2.5	1.9 c	90.1	24.7 c	67.5	0.0 c	100.0	8.8 b	72.1
	5	0.0 c	100.0	17.7 d	76.7	0.0 c	100.0	1.8 c	94.3
Olive oil	2.5	5.7 b	70.2	30.3 b	60.0	1.8 b	85.6	9.9 b	68.6
	5	0.0 c	100.0	11.9 e	84.3	0.0 c	100.0	0.0 c	100.0
Control		19.0 a		75.9 a		12.3 a		31.4 a	
Season 2018									
Clove oil	2.5	1.3 c	92.8	24.0 c	67.7	0.0 b	100.0	8.5 b	72.5
	5	0.0 c	100.0	16.4 d	77.9	0.0 b	100.0	1.3 c	95.7
Olive oil	2.5	5.3 b	71.8	28.8 b	61.2	1.3 b	88.6	8.9 b	71.0
	5	0.0 c	100.0	10.7 e	85.6	0.0 b	100.0	0.0 c	100.0
Control		18.5 a		74.2 a		11.7 a		30.7 a	

Within each column, same letter/s indicates no significant difference among treatments at ($P < 0.05$).
m/L: male / litter EF% : efficiency as percentage. D.S%: disease severity as percentage.
Control was infected with B. cinerea and without infection and un-treated.

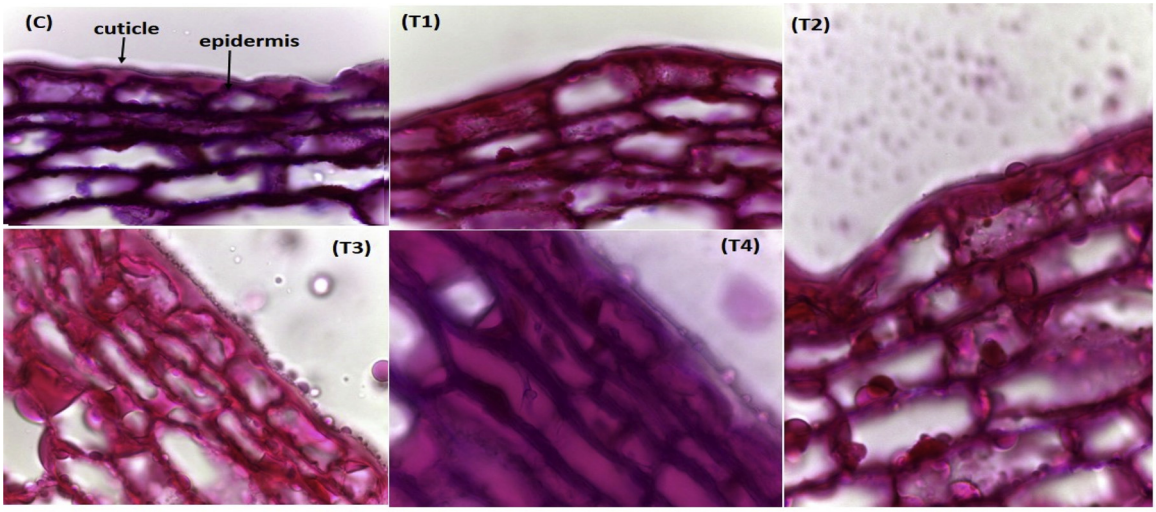

Fig. 1. Effect of pre-harvest sprays of some organic acids (Salicylic acids at 8 mM and citric acids at 30 mM) and plant oils (Olive and clove oils at 5 m/L) on the histological features of pepper fruits cell wall (cuticle and epidermis) of Khayrat variety after 15 days from the last spray.(x = 100).
C = Control, T1 = Salicylic acid, T2 = Citric acid, T3 = Clove oil, T4 = Olive oil.

finding refers to possible increase of pepper fruits resistance to fungal infection by spraying of certain plant oils (olive and clove oils) and citric acid on pepper plants through increasing the cuticle thickness as a mechanical barrier. While salicylic acid showed less thickness of the cuticle of pepper fruits than the control. On the other hand, the fruits of pepper collected from such sprayed plants with the tested organic acids and plant oils showed higher epidermis thickness than the control. salicylic acid followed by citric acid were the most effective treatments to increase the epidermis thickness of the fruits causing thicknesses of 18.8 and 18.3 µm, respectively, compared with the control (14.3 µm). Meanwhile, olive oil showed the least values of epidermis thickness compared with other treatments, but it showed higher epidermis thickness than the control. This finding referred to role of all tested organic acids and plant oils in increasing the epidermis thickness and decreasing simultaneously the disease infection incited by *Botrytis cinerea* on pepper fruits as obtained in Tables 3 and 4. Anatomical changes by acids were also obtained by Nour et al. (2012) who found that, spraying green bean plants with salicylic and citric acid increased thickness of leaflet blade, thickness of palisade and spongy, except salicylic acid at 100 ppm that had the opposite effect on these leaflet anatomical characters. Treating papaya plants with citric acid at 2 g/l increased thickness of each of epidermis, cortex, phloem zone and xylem zone in cross section of petiole flower (Ismaeil and Bakry, 2005). Spraying tomato plants with citric acid was positively correlated with increased thickness of each of layer of cuticle, epidermis, cortex and parenchymatous pith. In general, the stimulatory effects of citric acid upon the anatomy features of treated plants could be attributed to the increase of endogenous hormones level especially cytokinins and auxins (El-Desouky et al., 2011). Also Pre-harvest spraying of snap bean plants with salicylic or citric acids increased cuticle layer thickness of pods of Valentino variety and epidermis thickness of pods of Xara variety (Mohamed et al., 2015). In this respect, Exogenous application of SA on squash plants improved the leaf anatomical characteristics under deficit irrigation. The beneficial effect of SA on leaf structure may be due to the crucial role in cell division and cell expansion (Abd El-Mageed et al., 2016). Also, pre-harvest application with salicylic acid (100 ppm) on pea plants enhanced leaf anatomical characteristics, where salicylic acid led to a good translocation of the observed water and nutrients into cell to be used in different metabolic process which positively affected fresh weight of leaves and shoot and increased photosynthesis process activity as well as accumulation of photoassimilates (El-Saadony et al., 2017). On the other hand, field application with plant oils increased thickness of cuticle layer, epidermis thickness and Cortex Thickness as well as Xylem zone in cross sections of tomato stem (Mohamed, 2019).

3.6. Enzymatic studies

3.6.1. Polyphenoloxidase activity

Spraying pepper plants with different treatments affect the activities of polyphenoloxidase enzyme in pepper fruits compared with control

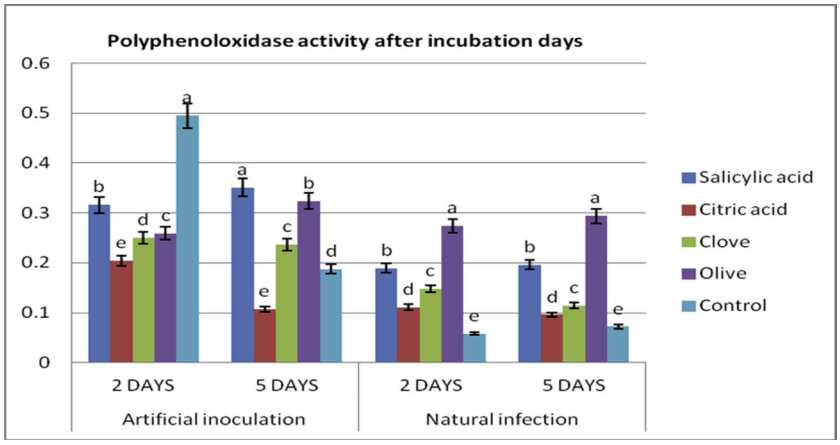

Fig. 2. Effect of spraying of salicylic acids (8 mM), citric acids (30 mM), olive and clove oils (5 m/L) under plastic house conditions on polyphenoloxidase activity (as optical density) in pepper fruits naturally infected and artificially inoculated with *B. cinerea* after 2 and 5 days incubation. Within each incubation period (after 2/ 5 days), same letter/s indicates no significant difference among treatments at ($P < 0.05$).

treatment (Fig. 2).In this respect, pre-harvest application with salicylic acid was the best effective in increasing the activity of polyphenoloxidase in pepper fruits at five DPI with *B. cinerea* compared with other treatments and control followed by olive oil and in naturally infected pepper fruits at two and five days DPI with olive oil and salicylic acid, respectively. This increase in enzymatic activity is consistent with reduced infection by *B. cinerea*, compared with the disease development in the control treatment. This finding refers to possible increase of pepper fruits resistance to *B. cinerea* infection by spraying pepper plants with salcylic acid and olive oil as obtained in Tables 3 and 4. But, the highest activity of polyphenoloxidase was recorded after two days in control treatment (un-treated and inoculated fruits with *B. cinerea*). Data also indicated that, spraying pepper plants with clove oil increased the activity of polyphenoloxidase in fruits at five DPI with *B. cinerea* and naturally infected fruits. Meanwhile, the activity of polyphenoloxidase was reduced in inoculated fruits with *B. cinerea* at two days after DPI spraying pepper plants with all tested treatments compared with control.

3.6.2. Peroxidase activity

As for, peroxidase activity, the maximum increase in peroxidase activity was recorded in pepper fruits at five DPI with *B. cinerea* of sprayed pepper plants with citric acid compared with other treatments and control followed by olive oil (Fig. 3). Also sprayed pepper plants with olive oil caused maximum increase in peroxidase activity in fruits at two and five DPI for all naturally infected and artificially inoculated with *B. cinerea*. Meanwhile, sprayed pepper plants with salicylic acid increased the activity of peroxidase in fruits during two DPI with *B. cinerea* and naturally infected fruits during two and five DPI. While, clove oil reduced the activity of peroxidase in naturally infected pepper fruits at two DPI. The obtained results could be interpreting in light findings of Mitlerass et al. (2006) who found that increasing of peroxidase activity was associated with increasing resistance against infection by many diseases, through the accumulation of phenolic compounds playing a role in disease resistance. Also similar results were obtained by Hassan et al. (2007) who revealed that, citric and benzoic acids were the most effective one, since they recorded the lowest percentages of disease severity of *B. cinerea* and/or *Botrytis fabae* on faba bean plants and the maximum levels of peroxidase activities. Moreover, pre-harvest treatment of faba bean plants showed some new isozymes and increasing in the density of original isozymes, especially in infected plants. Hegazi and El-Kot (2010) found that, the activities of peroxidase (POX) and polyphenoloxidase (PPO) were increased as a result of spraying clove oil on *Zinnia elegans* plants. On the other hand, Abdul Rashid et al. (2011) indicated that plants responded very quickly to SA at 1.5 mM and caused higher induction of POD and PPO activity, as well as higher accumulation of phenols, H_2O_2 and proteins. Sedghi et al. (2013) reported that spraying of sunflower with SA as pre-harvest treatment increased the activities of catalase, peroxidase, and super oxide dismutase. Also, Solanki et al. (2018) who found that exogenous application of SA increased the activities of peroxidase and polyphenoloxidase enzymes in black gram (Vigna mungo (L.) Hepper).

3.7. Effect of pre-harvest sprays with certain organic acids and certain plant oils on total phenolic content and antioxidant activity of pepper fruits

Basically, total phenolic content (TPC) is very important fruit constituents which can be used as an indicator of several functional properties like antioxidant or antibacterial capacities. TPC in pepper fruits are shown in Table 6.Phenolic content differs between species of the same family, even between cultivars (Veberic et al., 2005; Nopi et al., 2018).

Spraying pepper plants with olive oil at rate 2.5 m/l gave the highest content of total phenolic contents (TPC) (97.8 mg/100 g), which causes its stronger antioxidant ability as DPPH radical scavenging activity (IC_{50} 155.8 mg/ml). After infection with *B. cinerea* a significant increase in TPC was observed in clove 2.5 mg/ml (126.8 mg/100 g).Generally significant increase in phenolic compounds in pepper fruits was reported. According to Vermerris and Nicholson (2006) and Kulbat (2016) this increase may be due to inhibition of catalase activity which in turn induces phenylalanine lyase gene expression and synthesis of phenolic compounds. Phenolic compounds are considered as important defense-related compounds whose levels are naturally high in resistant varieties of many crops. On the other hand, phenolics are not the only resistance mechanism present in plants (Mikulic-Petkovsek et al., 2009). Additionally, the TPC quantity depends on the speed of the plant response (Veberic, 2016).

Data also, indicated that, total phenolic (TPC) and antioxidant ability as DPPH radical scavenging activity in naturally and artificially inoculated pepper fruits with *B cinerea* were increased, when spraying pepper plants with all tested treatments at different concentration compared with control. Generally, the addition of different amounts of antioxidant to the DPPH solution produced a rapid decrease in the optical density at 517 nm (Paixao et al., 2007). The results of investigated samples show that the higher the concentration of antioxidant is, the lower the amount of remaining DPPH and the higher the free radical scavenging activity. This finding referred to role of all tested treatments in increasing the TPC as well as antioxidant and decreasing simultaneously the disease infection incited by *B. cinerea* on pepper fruits as obtained in Tables 3 and 4. On the other hand, citric acid (30 mM) was the least effective in increasing the TPC and antioxidant ability as DPPH radical scavenging activity in inoculated pepper fruits with *B. cinerea* compared with other treatments.

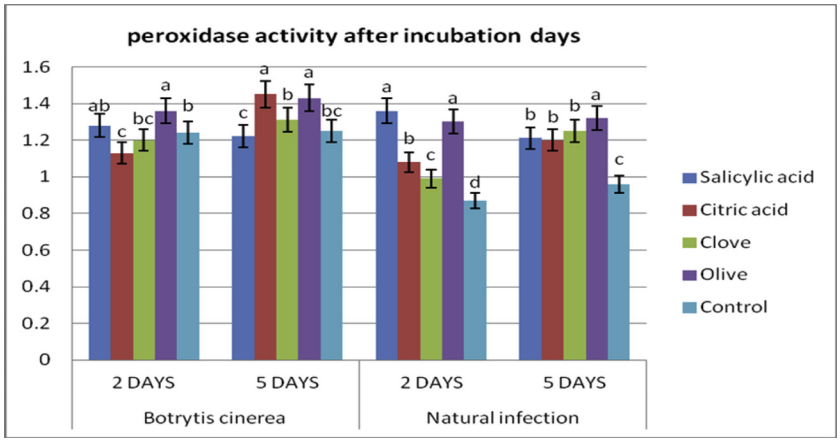

Fig. 3. Effect of spraying of salicylic acids (8 mM), citric acids (30 mM), olive and clove oils (5 m/L) under plastic house conditions on peroxidase activity (as optical density) in pepper fruits naturally infected and artificially inoculated with *B. cinerea* after 2 and 5 days incubation. Within each incubation period (after 2/ 5 days), same letter/s indicates no significant difference among treatments at ($P < 0.05$).

Table 6
: Total phenolic content and antioxidant activity of pepper fruits pre-treated with organic acids and plant oils.

Treatment	Conc.	Natural infection		*B. cinerea*	
		Phenolic compounds mg/ 100 g	DPPH radical scavenging activity (IC_{50} mg/mL).	Phenolic compounds mg/ 100 g	DPPH radical scavenging activity (IC_{50}mg/mL).
Salicylic acid	4 mM	90.5 c	266.8 b	107.4 e	74.3 i
	8 mM	97.5 a	163.4 g	99.0 g	134.7 d
Citric acid	15 mM	95.1 ab	184.1 e	103.4 f	180.1 c
	30 mM	87.9 d	280.6 a	92.6 i	234.4 a
Clove oils	2.5 m/L	94.5 b	200.2 d	126.8 a	85.9 h
	5 m/L	94.2 b	212.2 c	121.0 b	89.6 g
Olive oils	2.5 m/L	97.8 a	155.8 h	111.2 d	110.7 e
	5 m/L	95.9 ab	177.7 f	116.9 c	99.2 f
Control	–	87.8 d	277.5 a	95.2 h	197.2 b

Within each column, same letter/s indicates no significant difference among treatments at ($P < 0.05$).
Control was infected with B. cinerea and without infection and un-treated.

4. Conclusion

The results of this work demonstrated *in vitro*the high potential of olive, clove oils at 2.5 and 5 m/l and salicylic acid at 4 and 8 Mm, as a fungicide, where they completely inhibited the growth of B. cinerea. *in vivo*studies, high concentration of olive oil followed by salicylic acid significantly reduced development of grey mold on pepper fruits during cold storage. Clove oil was the most positive treatment to increase the cuticle thickness, while salicylic acid was the most effective treatments to increase the epidermis thickness of the fruits. This increase in cuticle and epidermis thickness is coincided with the increase in less infection by *B. cinerea.* The maximum increase in Polyphenoloxidase activity in pepper fruits was observed at 5 days post inoculation after sprays with salicylic acid. Meanwhile, the maximum increase in peroxidase activity was recorded in pepper fruits at five days post inoculation with *B. cinerea* after spraying pepper plants with citric acid. In addition, spraying pepper plants with clove oil at rate 2.5 m/l gave the highest content of total phenols, which causes its stronger antioxidant ability as DPPH radical scavenging activity.

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