Annals of Agric. Sci., Moshtohor **ISSN 1110-0419**
Vol. 60(1) (2022), 169 – 180 **https://assjm.journals.ekb.eg**

Integrated Treatments between Some Essential Oils and Chitosan for Controlling Gray Mold Disease of Bell Pepper Fruits during Storage

Amira M. Ewide[1]; Eman O. Hassan[1]; F Abd-El-kareem[2]; M. A Mady[3] and Abdou M.M. Mahdy[1]
[1]Plant Pathology Dept., Fac. Agric, Moshtohor, Benha Univ., Egypt
[2]Plant Pathology Dept., National Research Centre, Giza, Egypt
[3]Agric. Botany Dept., Fac. Agric, Moshtohor, Benha Univ., Egypt

Abstract

Gray mold disease caused by *Botrytis cinerea* is the most sever diseases attacking pepper fruits. Four purified isolates of *Botrytis cinerea* were tested for their pathogenic ability to pepper fruits. The most sever isolate is *Botrytis cinerea* no. 3 which caused disease incidence by 90.0, 35.0 & 40.0 % and disease severity by 74.0, 30.0 and 15.0 % for green, red and yellow fruits respectively. The highly pathogenic isolate of *Botrytis cinerea* no.3 causing gray mold of pepper fruits, was identified using molecular biology and used in the following experiments. *In vitro* experiments, five essential oils, *i.e*. thyme, citral, lemongrass, methyl anthranilate and camphor at concentrations of .0, 0.25, 0.5 and 1.0 % were evaluated for their capability to suppress fungal growth and spore germination of *Botrytis cinerea*. Results demonstrated that complete suppression of mycelial growth and spore germination was achieved with concentration of 0.5 and 1.0 % of all tested essential oils except that Camphor. Also chitosan at concentration of 6.0 g/L caused complete suppression of mycelial growth and spore germination of *Botrytis cinerea.* In vivo experiments, essential oils and chitosan were tested alone or in combination with chitosan for controlling gray mold of bell pepper (cvs. Khayrat (green), Antonio (red) and Cleopatra (yellow). The most effective treatments are combination between thyme or citral with chitosan which significantly reduced disease incidence and disease severity. Meanwhile, single treatments showed moderate effect.

Key word: Grey mold - *Botrytis cinerea* - Pepper fruit- Essential oils- Chitosan – Storage

Introduction

Bell peppers are a popular vegetable crop in Egypt and around the world. (Abd-**Elgawad, 2020)**.*Capsicum annuum* L., which includes all sweet and most hot pepper types, is a member of the Solanaceae family. Nonetheless, many kinds and groups of *C. annuum* exist, each with a distinct flavor profile, such as glabriusculum, bola, Bell, Cayenne, Jalapeno, and New Mexico Chile **(Zayed *et al*. 2017).**

Grey mold disease of bell pepper fruit caused by *Botrytis cinerea* Persoon, resulting in economic losses during storage and probable negative effects on fruit quality , water-soaked lesions appear on the fruit, which turn brown before being covered with powdery, grey spore masses **(Naz *et al*. (2018)**.

Essential oils, which are natural, volatile, complex molecules with antibacterial, antioxidant, and therapeutic characteristics, are used (**Guha, 2018).**
Plant essential oils have been shown to have antifungal properties against fruit diseases in numerous investigations. (**Ding and Lee, 2019).**
Several researchers have reported on the possibilities of employing essential oils to treat postharvest infections by spraying or dipping fruits **(Abdel-Mageed *et al.,* 2014** and **Abd-El-Kareem, *et al.,* 2021).**
In four apple cultivars, treatments with emulsions of 1 % essential oil from oregano, savory, and thyme demonstrated substantial efficacy in lowering the diameters of *B. cinerea* lesions (**Lopez- Reyes *et al.,* 2010**). Chitosan is a flexible biopolymer that shows antimicrobial activity against a variety of foodborne pathogens, attracting interest as a possible preservative (**Ganan *et al*., 2009**).

The chitosan coating forms a semi-permeable barrier that reduces water loss and alters the natural exchange of gases between the fruit and the external atmosphere, lowering respiration, slowing senescence, and limiting microbial deterioration (**Gao *et al*., 2013**).

The aims of this study research are the evaluation of some essential oils and chitosan alone or in combinations for controlling postharvest diseases of pepper fruits during storage.

Materials and Methods

Identification of *Botrytis cinerea*

Isolation fungi from infected bell pepper fruits. Hyphal tips or single spore cultures of grown fungi were maintained on PDA medium. All fungi were purified using single spore technique described by **Fang *et al.* (1983).**Isolate No.3 of *Botrytis* spp. were DNA extraction, PCR amplification and sequenced for species identification using the internal transcribed spacer region of rRNA (ITS) Trimmed sequences (ITS 573 bp) (**Staats *et al*., 2004)** .

DNA extraction:

DNeasy® Plant Mini Kit used to extraction DNA from fungal growth was carried out according to (**Fan *et al.* 2015).**

PCR amplification:

Botrytis cultures were identified molecularly using the conserved ribosomal internal transcribed spacer (ITS) region **(White, *et al*., 1990).**

Sequencing

Using the Basic Local Alignment Search Tool for Nucleotide Sequences, the ITS nucleotide sequences of each isolate were compared to those in the public domain databases NCBI (National Center for Biotechnology Information; www.ncbi.nih.gov) (BLASTN). The Clustal W tool was used to align ITS DNA sequences. CLC Sequence Viewer Version 6.3 was used to generate a phylogenetic tree based on UPGMA (unweighted pair group method for arithmetic analysis). Bootstrap analysis was used to determine the branching's confidence (**Fan *et al.* 2015).**

Pathogenicity test of different isolates of Botrytis *cinerea* under wounded or unwounded technique of different cultivars of bell pepper fruits

Four purified isolates of *Botrytis cinerea* were tested for their pathogenic ability to bell peeper fruits (green, Red and Yellow). Fresh harvested apparently healthy pepper fruits
were cleaned with sterilized distilled water and then surface sterilized by dipping in 70% ethanol for one minute and drying in a sterile environment.

Inoculum preparation:

To make standard inoculum, pure *Botrytis cinerea* isolates were grown separately on PDA plates for 10 days at 20±2°C. Spore suspension was obtained from tested fungus by brushing the culture surface in the presence of 10 mL sterilized distilled water for each plate, then filtering the spore suspension through muslin. A haemocytometer slide was used to regulate the spore suspension concentration to roughly 10^6 spores/mL.

Inoculation of pepper fruits.

Apparently healthy pepper fruits were surface sterilized by dipping for one minute in 70% ethyl alcohol, then washing several times with sterilized distilled water and drying at room temperature. Fruits were divided in two groups, the first group of sterilized pepper fruits was artificially wounded by a sterilized needle, while the second group was not. For each fungus, sprayed two groups using an atomizer at a rate of 200 mL spore suspension (10^6spores/mL) per 100 fruits. As a control, some of the fruits were sprayed with the same amount of sterilized distilled water. To improve the relative humidity, all inoculated and un-inoculated fruits were maintained in a foam tray (25) and put in polyethylene bags. Each treatment had four duplicates and five fruits per replicate. Fruits that had been inoculated and those that had not been inoculated were kept at 20°C for 15 days. The number of infected fruits as a percentage of the total number of fruits was used to calculate disease incidence **(Mercier, *et al.*, 2001).** The severity of the disease was determined by calculating the weight percentage (g) of the infected part of the fruit relative to the total weight of the fruit according to **Spalding and Reeder (1974)** as follows:

Disease assessment

$$\text{Disease incidence \%} = \frac{\text{Number of infected fruits}}{\text{Total number of fruits}} \times 100$$

$$\text{Disease severity \%} = \frac{\text{Rotted part weight}}{\text{Fruit weight}} \times 100$$

***In vitro* experiment.**

The highly pathogenic isolate of *Botrytis cinerea* No.3 causing gray mold of pepper fruits, was used in the following experiments.

Essential oils

Source of essential oils:

Different essential oils *i.e*. thyme, citral , lemongrass, methyl anthranilate and camphor were obtained from Oils Extract Unite, National Research Center, Giza, Egypt.

Evaluation of different concentrations of some essential oils on linear growth and spore germination of *Botrytis cinerea*

a- on linear growth

Five essential oils, *i.e*. thyme, citral, lemongrass, methyl anthranilate and camphor were evaluated for their capability to suppress fungal growth of *Botrytis cinerea*. PDA medium was infused with varying amounts of each oil to obtain the proposed concentrations of 0.0, 0.25, 0.5 and 1.0 % with 0.1% Tween-80. Treated or not treated (control) medium with oils were poured into 5 Petri dishes per each concentration After medium solidification, Petri dishes were inoculated with 5 mm discs of 7 days old culture of *Botrytis cinerea* then incubated at 20±2ºC for 7 days. Five plates of each treatment were used as replicates. Linear growth of ***Botrytis cinerea*** was measured daily to control plats are completely filled. The percentage of inhibition was calculated as previously mentioned. Plates were examined. The reduction percent on mycelial growth of *Botrytis cinerea* was calculated. **Fokemma (1973)** as follows:

$$\text{Reduction \%} = \left[\frac{C - T}{C} \; X100\right]$$

Where:

C = Linear growth of *Botrytis cinerea* in control.

T = Linear growth of *Botrytis cinerea* in treatment.

b- On spore germination

However, the effect of essential oils against spores germination of *Botrytis cinerea* was tested as method described by **Chien, *et al*., (2007).** Broth potato dextrose broth (PDB) (5mL) was added to 10 mL test tube and sterilized for 20 min. at 121°C . Essential oils were added to PDB to obtain finial concentrations of 0.0, 0.25, 0.5 and 1.0 % then mixed gently with 0.1% Tween 80 (Sigma). Test tubes were inoculated with one mL of spore suspension (10^6 spores /mL) and incubated at 25 ± 2°C for 24 h on rotary shaker. Examination of percent germinated spores were carried out microscopically. Experiment was represented by five replicates per treatment.

Chitosan:

Source of chitosan.

Chitosan was purchased from El-Gomhoria chemical Co, Cairo, Egypt.

Evaluation of different concentration of chitosan on linear growth, and spore germination of ***Botrytis cinerea***

Certain weights of chitosan were dissolved into PDA medium before it's solidifying to obtain final concentrations of 1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 g/L, and then transferred into Petri-plates. Plates were inoculated with 5 mm mycelial discs cut from the periphery of a seven-day-old culture after solidification, and incubated at 20±2°C. Five repetitions for each treatment were used. When fungal mycelium covered the control plates, the linear growth of the tested pathogen was measured.

***In vivo* experiments**

Testing of essential oils and chitosan alone or in combination on gray mold disease of bell pepper fruits

Four essential oils, *i.e*. thyme, citral, methyl anthranilate and lemongrass were evaluated at concentrations of 1.0 % alone or in combination with chitosan at 0.6 % for their inhibitory effect against gray mold disease of pepper fruits *in vivo*.

Apparently healthy pepper fruits cvs. Khayrat (green), Antonio (red) and Cleopatra (yellow fruits) were surface sterilized by dipping for one minute in 70% ethyl alcohol, then washing several times with sterilized distilled water and drying at room temperature.

Sterilized pepper fruits were artificially wounded by sterilized scalpel. Fruits were divided in two groups, first, dipped individually in previous concentrations of salts for one minute then air dried. The Second dipped individually in previous concentrations of salts for one minute then air dried followed by dipped in chitosan solution for one minute, then air dried.

Inoculum preparation

To make standard inoculum, a pure *Botrytis cinerea* fungal isolate was grown on PDA plates for 10 days at 20±2°C. Spore suspension was collected from fungus by brushing the surface of the culture in the presence of 10 ml sterilized water for each plate, followed by muslin filtering. A hemocytometer slide was used to regulate the spore suspension concentration to 10^6 spores/mL.

Inoculation of pepper fruits

Pepper fruits that appeared to be healthy were surface sterilized by dipping them in 70% ethyl alcohol for one minute, then washing them several times with sterilized water and drying them at room temperature. Sterilized pepper fruits were wounded by sterilized needle and inoculated with prepared *Botrytis cinerea* inoculum at a rate of 200 mL spore (10^6 spores/mL.) per 100 fruits using an atomizer for fungus. As a control, some of the injured fruits were sprayed with the same amount of sterilized water. All pepper fruits, inoculated and uninoculated, were maintained in a foam tray (25cm) and placed in polyethylene bags to improve relative humidity. Each treatment had four duplicates and five fruits per replicate. Fruits that had been inoculated and those that had not been inoculated were kept at 20±2°C for 14 days. As previously stated, the incidence and severity of disease were assessed.

Statistical analysis

Tukey test for multiple comparisons among means was utilized (**Neler *et al*., 1985**).

Experimental Results

Pathogenicity test of different isolates of *Botrytis cinerea* under wounded or unwounded technique of different pepper fruits

Four purified isolates of *Botrytis cinerea* were tested for their pathogenic ability to peeper fruits pepper cvs. Khayrat (green), Antonio (red) and Cleopatra (yellow fruits) *in vivo* experiments.

Results in Table 1 showed that all tried isolates of *Botrytis cinerea* caused gray mold disease on pepper green, red and yellow fruits under wounded fruits. The most sever isolate is *Botrytis cinerea* no 3 which caused disease incidence by 90.0, 35.0 & 40.0 % and disease severity by 74.0, 30.0 and 15.0 % for green, red and yellow fruits respectively. While, other isolates had moderate impact.

Table 1. Pathogenicity test of different isolates of *Botrytis cinerea* under wounded or unwounded technique of different cultivars of pepper fruits

Isolate	Disease incidence %					
	Unwounded fruits			Wounded fruits		
	Khayrat (green)	Antonio (red)	Cleopatra (yellow)	Khayrat (green)	Antonio (red)	Cleopatra (yellow)
Botrytis cinerea (1)	0.0 c	0.0 b	0.0 b	70.0 b	20.0 c	15.0 b
Botrytis cinerea (2)	5.0 b	0.0 b	0.0 b	75.0 b	25.0 b	15.0 b
Botrytis cinerea (3)	10.0 a	5.0a	5.0 s	90.0 a	35.0 a	40.0 a
Botrytis cinerea (4)	5.0b	0.0 b	0.0 b	70.0 b	25.0 b	15.0 b
Control	0.0 c	0.0 b	0.0 b	0.0 c	0.0 d	0.0 c

Isolate	Disease severity %					
	Unwounded fruits			Wounded fruits		
	Khayrat (green)	Antonio (red)	Cleopatra (yellow)	Khayrat (green)	Antonio (red)	Cleopatra (yellow)
Botrytis cinerea (1)	0.0 b	0.0 b	0.0 b	65.0 bc	18.0 c	13.0 b
Botrytis cinerea (2)	8.0 a	0.0 b	0.0 b	74.0 b	30.0 bc	15.0 b
Botrytis cinerea (3)	7.0 a	5.0 a	5.0 a	86.0 a	32.0 a	37.0 a
Botrytis cinerea (4)	7.0 a	0.0 b	0.0 b	70.0 c	25.0 c	12.0 b
Control	0.0 b	0.0 b	0.0 b	0.0 d	0.0 d	0.0 c

Values followed by the same letter are not significantly different (P= 0.0 5).

Identification of *Botrytis cinerea*

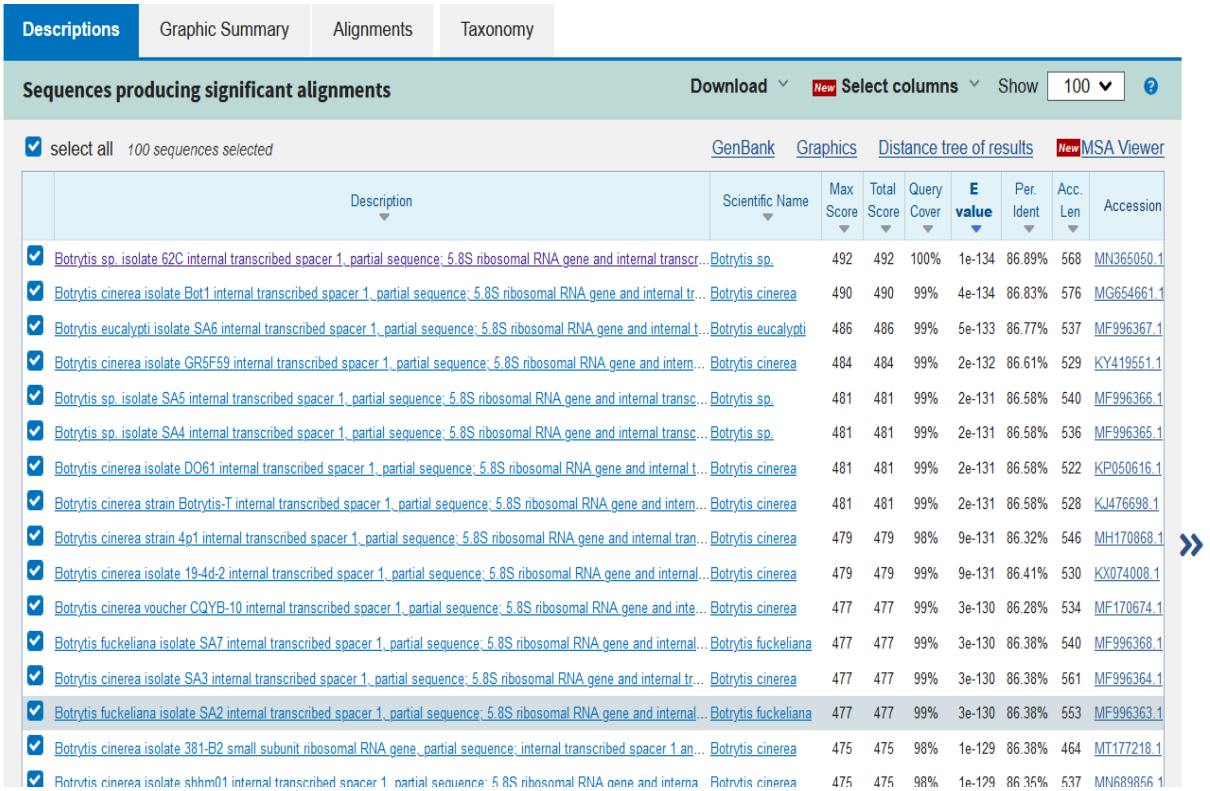
The highly pathogenic isolate of *Botrytis cinerea* no.3causing gray mold of pepper fruits, was identified using molecular biology Results in Fig. 1 and 2 indicate that The NCBI (National Center for Biotechnology information) alignment showed the percentage of identity (100%) of *Botrytis cinerea* between our isolated no.3 and Gene bank isolate. *Botrytis cinerea* no.3 used in the following experiments.

Identification of Botrytis cinerea using molecular biology

Isolate 3. *Botrytis cinerea*

NCBI relationship

>GCGTACTGCGGAGACATTACAGAGTTCATG
CCCGAAAGGGTAGACCTCCCACCCTTGTGTA
TTATTACTTTGTTGCTTTGGCGAGCTGCCTCC
GGGCCTTGTATGCTCGCCAGAGAATACCAAA
ACTCTTTTTATTAATGTCGTCTGAGTACTATA
TAATAGTTAAAACTTTCAACAACGGATCTCT
TGGTTCTGGCATCGATGAAAAACGCAACGAA
ATGCGATAAGTAATGTGAATTTCAAAATTCC
ATTAATCATCAAATTCTTTAACGCATTTTGCT
CCCCTTGGTATTCATGGGGGAACCAAGAAAT
TCGAGCGTCAATGCACCCCCCAATCTAACTT
GGGTATAGAGTCTTTGTAAAAAAGGGTTTGG
TCTTAAATCGGGGGCGGCCCCCGGTGCGGCC
CGGAACTTAGCAAAATCACCCAGTTACAGGT
TCCTCGGGGTG



Descriptions | Graphic Summary | Alignments | Taxonomy

Sequences producing significant alignments Download | New Select columns | Show 100

select all *100 sequences selected* GenBank | Graphics | Distance tree of results | New MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Botrytis sp. isolate 62C internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcr...	Botrytis sp.	492	492	100%	1e-134	86.89%	568	MN365050.1
Botrytis cinerea isolate Bot1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal tr...	Botrytis cinerea	490	490	99%	4e-134	86.83%	576	MG654661.1
Botrytis eucalypti isolate SA6 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal t...	Botrytis eucalypti	486	486	99%	5e-133	86.77%	537	MF996367.1
Botrytis cinerea isolate GR5F59 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and intern...	Botrytis cinerea	484	484	99%	2e-132	86.61%	529	KY419551.1
Botrytis sp. isolate SA5 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transc...	Botrytis sp.	481	481	99%	2e-131	86.58%	540	MF996366.1
Botrytis sp. isolate SA4 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transc...	Botrytis sp.	481	481	99%	2e-131	86.58%	536	MF996365.1
Botrytis cinerea isolate DO61 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal t...	Botrytis cinerea	481	481	99%	2e-131	86.58%	522	KP050616.1
Botrytis cinerea strain Botrytis-T internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and intern...	Botrytis cinerea	481	481	99%	2e-131	86.58%	528	KJ476698.1
Botrytis cinerea strain 4p1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal tran...	Botrytis cinerea	479	479	98%	9e-131	86.32%	546	MH170868.1
Botrytis cinerea isolate 19-4d-2 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal...	Botrytis cinerea	479	479	99%	9e-131	86.41%	530	KX074008.1
Botrytis cinerea voucher CQYB-10 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and inte...	Botrytis cinerea	477	477	99%	3e-130	86.28%	534	MF170674.1
Botrytis fuckeliana isolate SA7 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal...	Botrytis fuckeliana	477	477	99%	3e-130	86.38%	540	MF996368.1
Botrytis cinerea isolate SA3 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal tr...	Botrytis cinerea	477	477	99%	3e-130	86.38%	561	MF996364.1
Botrytis fuckeliana isolate SA2 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal...	Botrytis fuckeliana	477	477	99%	3e-130	86.38%	553	MF996363.1
Botrytis cinerea isolate 381-B2 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1 an...	Botrytis cinerea	475	475	98%	1e-129	86.38%	464	MT177218.1
Botrytis cinerea isolate shbm01 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and interna...	Botrytis cinerea	475	475	98%	1e-129	86.35%	537	MN689856.1

Fig.1: The NCBI alignment showed the percentage of identity (100%) of *Botrytis cinerea* between our isolated and Gene bank isolate.

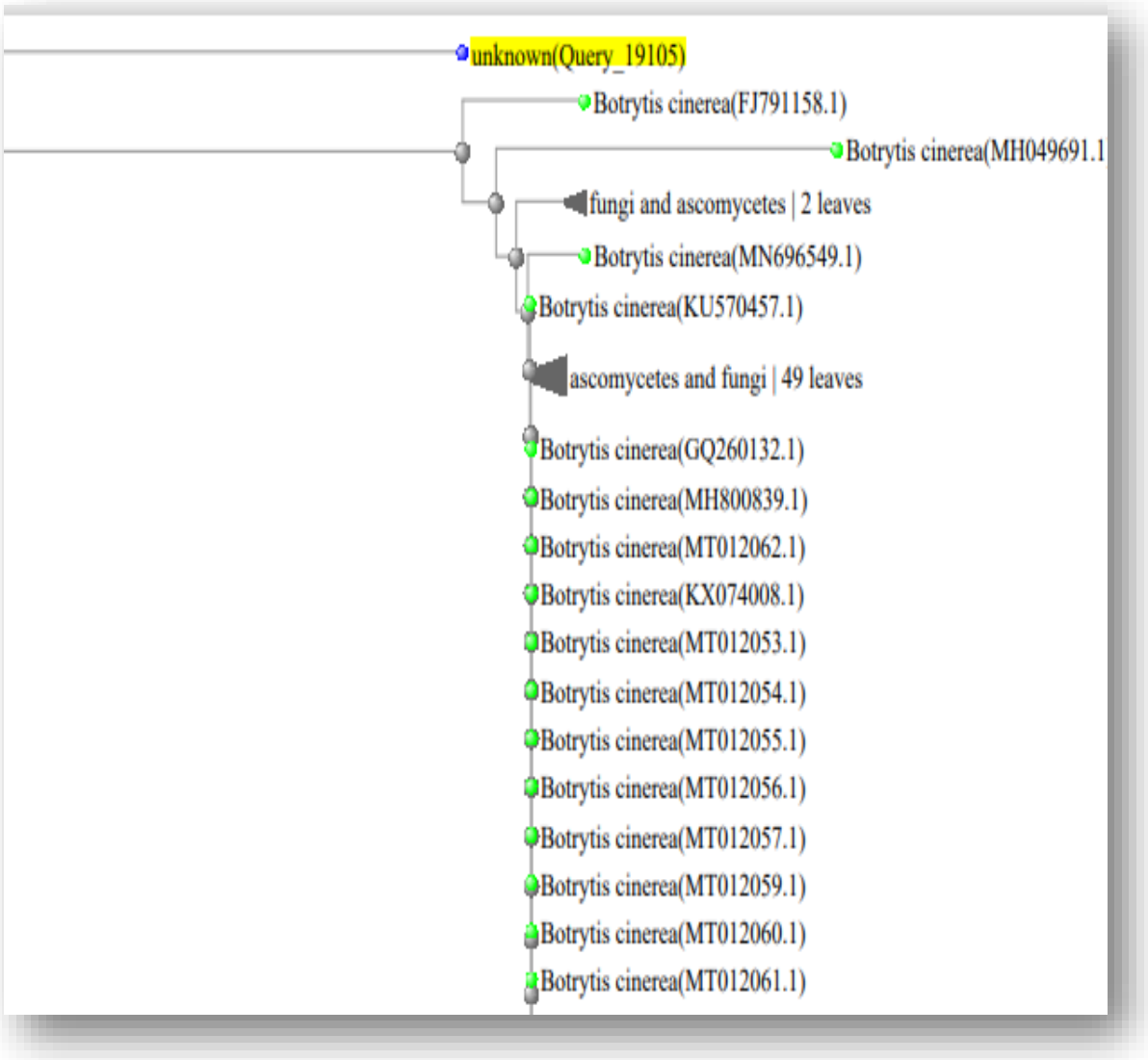


Fig.2: The phylogenic tree showed Convergence between our isolated (Yellow color) and Gene bank isolate. Our isolate showed in separated cluster which mean it's had diversity (0.050).

***In vitro* experiments**

Evaluation of different concentrations of essential oils on linear growth and spore germination of *Botrytis cinerea*

Five essential oils, *i.e*. thyme, citral, lemongrass, methyl anthranilate and camphor at concentrations of .0, 0.25, 0.5 and 1.0 % were tested for their capability to suppress fungal growth and spore germination of *Botrytis cinerea*. **Results in table 2** reveal that all tested concentrations of all tested essential oils reduced the linear growth and spore germination of *Botrytis cinerea*. Complete suppression of growth and spore germination was obtained with concentration of 0.5 and 1.0 % of all tested essential oils except that Camphor.

Table 2. Effect of different concentrations of essential oils on linear growth and Spore germination of *Botrytis cinerea*

Essential oil	Conc. g/L	Linear growth (mm)	Reduction %	Spore germination	Reduction %
Thyme	0.25	21.0 c	76.7	15.0 c	
	0.5	0.0e	100.0	0.0 d	100.0
	1.0	0.0e	100.0	0.0 d	100.0
Citral	0.25	22.0 c	75.6	14.0 c	
	0.5	0.0e	100.0	0.0 d	100.0
	1.0	0.0e	100.0	0.0 d	100.0
Lemon grass	0.25	18.0	80.0	12.0 c	86.7
	0.5	0.0e	100.0	0.0 d	100.0
	1.0	0.0e	100.0	0.0 d	100.0
Camphor	0.25	54.0 b	40.0	44.0 b	51.1
	0.5	23.0 c	74.4	16.0 c	82.2
	1.0	0.0e	100.0	0.0 d	100.0
Methyl anthranilate	0.25	14.0 d	84.4	0.0 d	100.0
	0.5	0.0e	100.0	0.0 d	100.0
	1.0	0.0e	100.0	0.0 d	100.0
Control	0.0	90.0 a	0.0	94.0 a	0.0

Values followed by the same letter are not significantly different (P= 0.0 5).

Evaluation of different concentrations of chitosan on linear growth, and spore germination of *Botrytis cinerea*

Chitosan solutions of various concentrations 1.0, 2.0, 3.0, 4.0, 5.0, and 6.0 g/L were studied to see how they affected *Botrytis cinerea* linear growth and spore germination. **Results in Table 3** shows that all of the chitosan doses tested greatly inhibited *Botrytis cinerea* linear growth and spore germination. With a dosage of 6.0 g/L, linear growth and spore germination were completely inhibited.

Table 3. Effect of different concentrations of chitosan on linear growth and spore germination of *Botrytis cinerea*

Chitosan g/L	Linear growth (mm)	Reduction %	Spore germination	Reduction %
1.0	58.0 b	35.6	47.0 b	47.8
2.0	41.0 c	54.4	33.0 c	63.3
3.0	28.0 d	68.9	19.0 d	78.9
4.0	18.0 e	80.0	8.0 e	91.1
5.0	8.0f	91.1	0.0 f	100.0
6.0	0.0 g	100.0	0.0 f	100.0
Control	90.0 a	0.0	94.0 a	0.0

Values followed by the same letter are not significantly different (P= 0.0 5).

***In vivo* experiments**

Effect of essential oils and chitosan alone or in combination on gray mold disease of bell pepper fruits

Four essential oils, *i.e*. thyme, citral, methyl anthranilate and lemongrass were evaluated at concentrations of 1.0 % alone or in combination with chitosan at 0.6 % for their inhibitory effect against gray mold disease of pepper fruits *in vivo*.

Gray mold disease of pepper cv. Khayrat (green fruits)

Results in Table 4 show that all of the treatments studied, whether used alone or in combination with chitosan, lowered disease incidence and severity. The combination of thyme or citral with chitosan was the most effective treatment, reducing disease incidence by 87.5 % and severity by 90.5 %.

Gray mold disease of pepper cv. Antonio (red fruits)

Results in Table 5 all of the treatments studied, whether used alone or in combination with chitosan, showed a significant reduction in illness incidence and severity. The combination of thyme or citral with chitosan was the most effective treatment, reducing disease incidence by 83.3 % and severity by 87.0 %.

Gray mold disease of pepper cv. Cleopatra (yellow fruits)

Results in Table 6 illustrate that all of the treatments studied, whether used alone or in combination with chitosan, showed a significant reduction in illness incidence and severity. The most effective treatment was a combination of thyme or citral with chitosan, which lowered disease incidence by 84.6 percent and severity by 86.9%.

Table 4. Effect of essential oils and chitosan alone or in combination on gray mold disease of bell pepper cv. Khayrat (green fruits).

Treatment	Conc. %	Gray mold disease Day after storage					
		7		14			
		Disease incidence	Disease severity	Disease incidence	Efficacy %	Disease severity	Efficacy %
		Single treatments					
Thyme	1.0	25.0	21.0bc	20.0d	75.0	17.0d	77.0
Citral	1.0	20.0 c	13.0d	25.0cd	68.8	22.0cd	70.3
Methyl anthranilate	1.0	20.0 c	19.0c	30.0bc	62.5	28.0b	62.2
Lemon grass	1.0	25.0b	23.0b	35.0b	56.3	33.0b	55.4
Chitosan	0.6	25.0b	23.0 b	25.0 cd	68.8	23.0cd	68.9
		Combined treatments					
Thyme + chitosan		5.0 f	4.0f	10.0e	87.5	7.0e	90.5
Citral + chitosan		5.0 f	4.0 f	10.0e	87.5	7.0e	90.5
Methyl anthranilate + chitosan		10.0 e	8.0ef	15.0 e	81.3	16.0 d	78.4
Lemon grass + chitosan		15.0 d	12.0de	25.0cd	68.8	22.0cd	70.3
Control		60.0 a	52.0 a	80.0 a	0.0	74.0a	0.0

Values followed by the same letter are not significantly different (P= 0.0 5).

Table 5. Effect of essential oils and chitosan alone or in combination on gray mold disease of bell pepper cv. Antonio (red fruits).

Treatment	Conc. %	Gray mold disease Day after storage					
		7		14			
		Disease incidence	Disease severity	Disease incidence	Efficacy %	Disease severity	Efficacy %
		Single treatments					
Thyme	1.0	15.0d	17.0	20.0	66.7	18.0	66.7
Citral	1.0	15.0d	14.0	20.0	66.7	17.0	68.5
Methyl anthranilate	1.0	20.0c	18.0	25.0	58.3	21.0	61.1
Lemon grass	1.0	25.0b	23.0	30.0	50.0	28.0	48.1
Chitosan	0.6	25.0b	23.0	25.0	58.3	23.0	57.4
		Combined treatments					
Thyme + chitosan		5.0f	4.0	10.0	83.3	7.0	87.0
Citral + chitosan		5.0f	4.0	10.0	83.3	7.0	87.0
Methyl anthranilate + chitosan		10.0e	7.0	15.0	75.0	12.0	77.8
Lemon grass + chitosan		20.0c	14.0	25.0	58.3	21.0	61.1
Control		40.0 a	34.0	60.0	0.0	54.0	0.0

Values followed by the same letter are not significantly different (P= 0.0 5).

Table 6. Effect of essential oils and chitosan alone or in combination on gray mold disease bell pepper cv. Cleopatra (yellow fruits)

Treatment	Conc. %	Gray mold disease					
		Day after storage					
		7		14			
		Disease incidence	Disease severity	Disease incidence	Efficacy %	Disease severity	Efficacy %
		Single treatments					
Thyme	1.0	15.0d	16.0c	20.0b	69.2	21.0b	65.6
Citral	1.0	15.0d	12.0d	20.0b	69.2	17.0b	72.1
Methyl anthranilate	1.0	20.0c	17.0c	25.0b	61.5	21.0b	65.6
Lemon grass	1.0	25.0b	17.0c	25.0b	61.5	22.0b	63.9
Chitosan	0.6	25.0b	23.0b	25.0b	61.5	23.0b	62.3
		Combined treatments					
Thyme + chitosan		5.0e	4.0e	10.0c	84.6	8.0d	86.9
Citral + chitosan		5.0e	6.0e	10.0c	84.6	8.0d	86.9
Methyl anthranilate + chitosan		15.0d	12.0d	20.0b	69.2	14.0cd	77.0
Lemon grass + chitosan		20.0c	17.0c	25.0b	61.5	22.0b	63.9
Control		40.0ab	37.0a	65.0a	0.0	61.0a	0.0

Values followed by the same letter are not significantly different (P= 0.0 5).

Discussion

Botrytis cinerea, the cause of gray mold, is considered one of the most important postharvest decays of fresh fruit and vegetables (**Elad *et al.*, 2015**). In the present study results, demonstrated that the most severe isolate is *Botrytis cinerea* no 3 which caused disease incidence by 90.0, 35.0 & 40.0 % and disease severity by 74.0, 30.0 and 15.0 % for green, red and yellow fruits respectively. Meanwhile, other isolates showed a moderate effect. Isolate no.3 of *Botrytis* cinerea was identified using DNA extraction, PCR amplification and sequenced for species identification using the internal transcribed spacer region of rRNA (ITS) Trimmed sequences (ITS 573 bp) (**Staats *et al.*, 2004)**. In this respect, **Naz *et al.* (2018)** reported that gray mold of bell pepper fruit, causing economic losses during storage and possible adverse effects on fruit quality, more specifically, grey mold is a major pre- and post-harvest disease caused by *Botrytis cinerea* (**El- Hifny and El-Sayed, 2011**).

Essential oils are natural, volatile, complex compounds known for their antimicrobial, antioxidant, and medicinal properties (**Ding and Lee, 2019).** Results in the present study indicated that all tested concentrations of all tested essential oils significantly reduced the linear growth and spore germination of *Botrytis cinerea*. Complete inhibition of linear growth and spore germination was obtained with the concentration of 0.5 and 1.0 % of all tested essential oils except that Camphor. Meanwhile, concentration of 0.25 % showed the moderate effect. Generally, the efficacy of EOs is investigated through direct contact with fruit, by application through spraying or dipping **(Ding and Lee, 2019).**

The chitosan coating fruits can reduce water loss and alter the natural exchange of gases between the fruit and the external atmosphere, thereby reducing respiration, slowing senescence in fruit and vegetables, and inhibiting microbial decay (**Gao *et al.*, 2013**). Results in the present study indicated that all tested concentrations of chitosan significantly reduced the linear growth and spore germination of *Botrytis cinerea*. Complete inhibition of linear growth and spore germination was obtained with a concentration of 6.0 g/L.

In the present study, results indicated that all tested treatments applied as single or in combination with chitosan significantly reduced the disease incidence and severity. The most effective treatments were the combination between Thyme or Citral with chitosan which reduced disease incidence and disease severity with all tested pepper fruits. Meanwhile, single treatments showed moderate effect. In this respect, **Li *et al.*, (2015)** reported that chitosan was effective for controlling blue mold decay of apple fruit caused by *P. expansum*. Both lesions' sizes of blue mold decay were significantly inhibited by chitosan treatment. The combination of chitosan and aloe vera fractions as edible coating materials have great potential in expanding the shelf-life of blueberries **(Vieiraa *et al.*, 2016).** Chitosan treatment has been reported to prolong life shell and control decay of cucumber, carrot, apple, citrus, kiwifruit, peach, pear, strawberry, and sweet cherry (**Ben *et al.* 2003**). This chitosan coating reduces water loss, decreases nutrient loss, and prevents the growth of pathogens that causes fruit decay. The importance of chitosan in plant hormone production and systemic acquired resistance has been widely demonstrated (**Colman *et al.*,**

2019; Fooladi vanda *et al*., 2019; Iglesias *et al.*, 2019 and Ma *et al.*, 2019). This correlation between chitosan and phenolics has been previously studied (**Park *et al*., 2019; Jaisi and Panichayupakaranant, 2020 and Samari *et al.*, 2020)**. Chitosan enhances metabolic pathways (e.g., phenylpropanoid) involved in the biosynthesis of phenolic compounds (**Fooladi vanda *et al*., 2019 and Singh *et al*., 2020).**

The chitosan also activates chitinases, glucanases, and lipoxygenases, stimulating the generation of reactive oxygen species.

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تكامل المعاملات بين بعض الزيوت الطياره والكيتوزان لمكافحة مرض العفن الرمادي في ثمار الفلفل الحلو خلال التخزين

أميرة محمد عويضة[1] و ايمان عثمان حسن[1] و فريد عبدالكريم[2]و محمد احمد ماضي[3] و عبده مهدي محمد مهدي[1]

1- قسم أمراض النبات – كلية الزراعة – جامعة بنها – مصر

2- قسم أمراض النبات- المركز القومي للبحوث – مصر

3- قسم النبات الزراعي – كلية الزراعة – جامعة بنها – مصر

يعتبر مرض العفن الرمادي من أخطر الامراض التي تصيب ثمار الفلفل . تم اخيبار القدرة الامراضية لاربع عزلات من الفطر بوتريتيس سينيريا لدراسة قدرتها الامراضية علي ثمار الفلفل وسجلت العزلة رقم (3) أعلي قدرة امراضية حيث سجلت 90 و 35.0 و 40.0 % كنسبة حدوث المرض و 74.0و 30.0 و 15.0% شدة حدوث المرض علي ثمار الفلفل الاخضر والاحمر والاصفر علي الترتيب. بينما باقي العزلات سجلت قدرة امراضية متوسطة . وتم تعريف العزلة رقم (3) بواسطة الطرق الميكروسكوبية وطرق البيولوجيا الجزئية . تم اختبار تأثير 5 زيوت نباتية طيارة وهي الزعتر و السترال و حشيشة الليمون وميثيل أنثرانيليت والكافور بتركيزات 0.25 و 0.5 و1.0 % لدراسة تأثيرها علي النمو الطولي وانبات الجراثيم للفطر بوتريتيس سينيريا تحت ظروف المعمل . تم التثبيط الكامل للنمو الطولي وانبات الجراثيم بواسطة التركيز 0.5 و 1.0% مع كل الزيوت الطيارة المختبرة باستثناء زيت الكافور . وادي استخدام الكيتوزان بتركيز 6 جم / لتر الي تم التثبيط الكامل للنمو الطولي وانبات الجراثيم للفطر بوتريتيس سينيريا. نم اختبار معاملة الثمار بالزيوت الطيارة والكيتوزان بصورة منفردة أو مجتمعة ثم العدوي بالفطر اللمرض لمكافحة مرض العفن الرمادي في ثمار الفلفل الاخضر (صنف خيرات) والفلفل الاحمر (صنف انطونيو) والفلفل الاصفر (صنف كليوباترا) ثم التخزين وأوضحت النتائج ان تكامل المعاملات بين زيت الزعتر أو السترال ثم الكيتوزان أدي الي مكافحة معنوية لمرض العفن الرمادي (نسبة حدوث وشدة المرض) بينما أدت المعاملات الفردية الي مكافحة متوسط للمرض .

الكلمات الدالة : مرض العفن الرمادي- ثمار الفلفل- الزيوت الطيارة – الكيتوزان – النخزين