Effectiveness of Acetic Acid, Essential Oils and *Trichoderma* spp. in Controlling Gray Mold Disease on Cucumber

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ABSTRACT

Gray mold disease caused by *Botrytis cinerea* is an important postharvest disease of cucumber and many other horticultural products. Effectiveness of acetic acid, two essential oils (thyme and clove) and two species of *Trichoderma* (*T. harzianum* and *T. viride*) against gray mold fungus, *Botrytis cinerea* infected cucumber fruits, were evaluated under laboratory conditions. Five isolates of *B. cinerea* were isolated from 85 cucumber samples collected from local markets of Jazan governorate. Only *B. cinerea* 4th isolate was the most virulent than the other isolates. Treatments with 7.5 % of acetic acid, 500 µl/ml of either clove and thyme essential oils and 10^8 spores/ml of *T. harzianum* showed the most significant inhibitions (65.6- 83.3%) on *B. cinerea* 4th isolate growth. Also, same treatments under artificial infection resulted in the most significant reductions in gray mold disease severity of tested cucumber fruits comparing with other treatments as well as check treatment. All treatments resulted in conspicuous decrease in the activity of peroxidase enzyme and affected negatively on activity of polyphenoloxidase enzyme. Obtained results, lead to suggest that application of acetic acid, *T. harzianum* or essential oils are considered an applicable, safe and cost-effective method for controlling grey mould disease on cucumber fruits.

Key words: *Botrytis cinerea*, *Trichoderma* spp., essential oils, control, acetic acid, thyme and clove oils, cucumber fruits, postharvest disease.

INTRODUCTION

Cucumber (*Cucumis sativus* L.) is an important vegetable and one of the most popular members of the family Cucurbitaceae (Lower & Edwards, 1986). Cucumber also, considers basic part of almost any human diets. *Botrytis cinerea* Pers. ex. Fr., which also known as "gray mold fungus" causes serious pre- and post-harvest diseases in at least 200 plant species including agronomically important crops and harvested commodities, such as grapevine, tomato, strawberry, cucumber, bulb flowers, cut flowers and ornamental plants (Jarvis, 1977; Morsy *et al.,* 1999). Grey mould symptoms could be noticed on all parts of the infected plant since the pathogen could attack all old and fresh plant parts. Symptoms of *Botrytis* infection of cucumber fruits likely show as aggressive grey rot of the fruit (Ten Have *et al.,* 2002). The major factors limiting the epidemic are the requirements of water and nutrients for germination of conidia (Thomas *et al*., 1988).

The biocontrol agent *Trichoderma* spp. was intensively used against gray mould fungus (Harman, 2006). Several investigators reported that *Trichoderma* isolates were effective in controlling grey mould under laboratory and greenhouse conditions (Elad, 2000; Card *et al.,* 2009; Robinson-Boyer *et al.,* 2009).

Recently, essential oils are also considered a promising alternative with many having antifungal properties. However, very high concentration is needed when applied to real food systems (Hammer *et al*., 2003; Ahmet *et al*., 2005; Perricone *et al.,* 2015; Ghaderi *et al.,* 2017). Application of essential oils is a very attractive method for controlling different diseases. Essential oils and their components are gaining increasing interest because of their relatively safe status, their wide acceptance by consumers, and their exploitation for potential multi-purpose functional use (Ormancey *et al*., 2001).

Treated vegetable crops with safety chemicals such as dipping in acetic acid has shown promise for the control of gray mold disease (Morsy *et al.,* 1999). Acetic acid is a universal metabolic intermediate occurring in plants and animals. It is commonly used by food manufacturers as antimicrobial preservative or acidulate in a variety of food products (Davidson & Juneja, 1990).

This study aims to evaluate the effect of *Trichoderma harzianum* and *T. viride*; two essential oils, thyme and clove oils and acetic acid as safety chemicals on gray mold disease incidence and on activities of peroxidase and polyphenoloxidase in cucumber fruits.

MATERIALS AND METHODS

Source of *Botrytis cinerea* isolates:

Eighty five collected samples of naturally infected cucumber fruits were collected from local markets located in Jazan Governorate. Surface sterilized small pieces of these fruits were planted on potato dextrose agar (PDA) plates and incubated at 25 °C for 7 days. The growing fungi were purified using the hyphal tip technique (Hawker, 1950). The purified isolates were identified according to their morphological features using the descriptions of

Barnett and Hunter (1972) and Jarvis (1977). Five isolates of *B. cinerea* were isolated from collected samples. Stock cultures of *B. cinerea* isolates were maintained onto PDA slants and stored in a refrigerator.

Preparation of spore suspension of *B. cinerea*:

Spores suspension of 4th isolate of *B. cinerea*, which isolated from collected cucumber fruits were prepared at Biology Department, Jazan University, Saudi Arabia by culturing *B. cinerea* on PDA medium for seven days at 28±2°C. *B. cinerea* conidia of two weeks old cultures were removed by sterile distilled water supplemented with 0.8% Tween solution. The collected conidia were counted in a Burker chamber and the concentration was adjusted to be 10^5 spores/ml using Haemocytometer slide.

Pathogenicity test:

Healthy cucumber fruits were thoroughly washed with tap water then surface sterilized with ethanol 70 %, two superficial pores (4 mm in diameter) were made on the surface of the fruits (using cork borer 4 mm), then inoculated with 10^5 spores/ml of *B. cinerea* spores of each isolate. Five isolates of *B. cinerea* were used for pathogenicity test. Five replicates were used for each isolate. All replicates were incubated in a plastic moist chamber 70-80% RH and 20-25 °C. Five cucumber fruits treated only with sterilized water served as check treatment. A week later, decayed area on cucumber fruits were measured (El-Habaa, 1997).

Biological control agents:

Two species of biocontrol agent *Trichoderma* spp., *T. harzianum* Rifai and *T. viride* Pers., which used through the current study were obtained from the collection of Biology Department, Jazan University, KSA. *T. harzianum* and *T. viridi* spores suspensions were adjusted to be 10^8 spores/ml.

Source of essential oils:

Pure essential oils of thyme (*Thymus vulgaris*) and clove (*Syzygium aromaticum*) were obtained from Masarat El-Safay Chemicals Company, Jazan.

Effect of acetic acid, essential oils and *Trichoderma* spp. on *Botrytis cinerea* growth inhibition *in vitro*:

Ninety Petri plates (9 cm), containing 10 ml of PDA medium/each were used as a culture media to determine the activity of two dilutions; 5 and 7.5% of acetic acid, 250 and 500 µl/ml of each of thyme and clove essential oils and 10^8 spores/ml spores suspension of *T. harzianum* and *T. viride* against *B. cinerea* 4th isolate. Plates were incubated at 28±2°C and observations of *B. cinerea* inhibition growth was determined and recorded 7 days after incubation. Each treatment replicated ten times. Ten plates inoculated only with *B. cinerea* were served as check treatment.

Effect of acetic acid, essential oils and *Trichoderma* spp. on disease severity on cucumber fruits inoculated with *Botrytis cinerea*:

Under laboratory conditions healthy cucumber fruits were sterilized, each fruit were wounded (as pathogenicity test) and inoculated with 10^5 spores/ml of 4th isolate of *B. cinerea*. Inoculated fruits were treated with the following treatments; two dilutions 5% and 7.5% of acetic acid, 250 and 500 µl/ml of thyme and clove essential oils and 10^8 spores/ml spores suspension of *T. harzianum* and *T. viride*. Each treatment was replicated five times. All treatments were incubated in a plastic moist chamber and 20-25 °C. Five cucumber fruits inoculated only with 4th isolate of *B. cinerea* were served as check treatment. Disease severity was recorded a week after treatments.

Effect of acetic acid, essential oils and *Trichoderma* spp. on activities of-peroxidase and polyphenoloxidase in cucumber fruits inoculated with *B. cinerea*:

Treated and non-treated cucumber fruits with tow dilution of acetic acid, essential oils and 10^8 spores/ml of *Trichoderma* spp. suspension were used for enzymatic assay according to Tuzun *et al.* (1989) as follows: 0.5 g of fruit tissues was ground with 1.5 ml of 0.2M Tris (tris hydroxymethyl aminomethane)-HCL buffer (pH 7.8) containing 14 mM (-mercaptoethanol). The extracts were centrifuged at 10000x gravity for 20 min at 4°C.

The supernatant was collected and stored at 20°C until use. It was used as a crude enzyme extract for the polyphenoloxidase and peroxidase enzymes. Peroxidase, and polyphenoloxidase detection was carried out using a Spectrophotometer (SPECTRONIC 20-D) at 27±2°C.

Readings of the Spectrophotometer were recorded every 30 sec, for 5 min of the two tested enzymes. The reference cuvette for the spectrophotometer always contained the same concentrations of components as the sample cuvette, except that the substrate solution was replaced by extraction buffer. The activity of peroxidase enzyme was measured as described by Chance and Maehly (1955). Polyphenoloxidase was assayed following the method of Taneja and Sachar (1974).

Statistical analysis

Data obtained were statistically analyzed according to SAS software program (SAS, 1997). Comparison among means was made via the least significant difference test (LSD) at ≤ 5% level of probability.

RESULTS

Data in Table (1) showed that, there are five isolates of *B. cinerea* all of them were capable of causing gray mold infection onto cucumber fruits. *B. cinerea* 4th isolate was the most virulent, followed by the other isolates. Also, the decayed area in mm

which caused by *B. cinerea* 4th isolate was more broad on cucumber fruits than the other isolates (Table 1).

Table 1: Pathogenicity test of *B. cinerea* isolates on wounded cucumber fruits.

Treatment	Decayed area on cucumber fruits (in mm)
Check*	0.1 a
B. cinerea isolates	
1st isolate	10.2 b
2nd isolate	13.2 bc
3rd isolate	15.6 c
4th isolate	51.9 d
5th isolate	9.4 b

*Check = Uninoculated fruits. Data are averages of 5 replicates. Values, within each column, followed by the same letter (s) are not significantly different at ($P \leq 0.05$).

Data in Table (2) reported that treatments with 10^8 spores/ml of *T. harzianum* and 7.5 % of acetic acid resulted in great inhibitions of 83.3 and 81.1% on growth of *B. cinerea* 4th isolate, respectively followed by treatment with 500 µl/ml of clove and thyme essential oils with 65.6 and71.1% inhibition, respectively. In addition, treatments with 250 µl/ml of the tested oils caused 46.7-52.2% inhibition on *B. cinerea* growth. Meanwhile, treatments with 5% of acetic acid and 10^8 spores/ml of *T. viride* showed 28.9 and 43.3 % inhibition on growth of *B. cinerea*, respectively as compared with the check treatment.

Data in Table (3) indicated that, treated cucumber fruits with acetic acid, essential oils and *Trichoderma* spp. decreased severity of the grey mould infection of *B. cinerea* 4th isolate comparing with the check treatment.

Table 2: Effect of acetic acid, essential oils and *Trichoderma* spp. on *B. cinerea* growth inhibition under *in vitro* conditions

Treatment	Radial growth (cm)	Growth inhibition %**
Check*	9.0 a	0.0
Acetic acid		
5.0 %	6.4 b	28.9
7.5 %	1.7 e	81.1
Essential oils		
Clove		
250 µl/ml	4.8 bc	46.7
500 µl/ml	3.1 cd	65.6
Thyme		
250 µl/ml	4.3 c	52.2
500 µl/ml	2.6 d	71.1
T. harzianum (10^8 spores/ml)	1.5 e	83.3
T. viride (10^8 pores/ml)	5.1 bc	43.3

* = Check treatment = *B. cinerea* only. **Growt inhibition %= (growth zone in check plate-growth zone in test plate)/growth zone in check plate × 100. Data are averages of 10 replicates. Values, within each column, followed by the same letter (s) are not significantly different at ($P \leq 0.05$).

Table 3: Effect of acetic acid, essential oils and *Trichoderma* spp. on gray mould disease severity on cucumber fruits infected with *B. cinerea*

Treatment	Gray mold disease incidence	Severity %
Check*	100.0 a	0.0
Acetic acid		
5.0 %	52.6 b	47.4
7.5 %	29.1 cd	70.9
Essential oils		
Clove		
250 µl/ml	50.6 b	49.4
500 µl/ml	20.9 cd	79.1
Thyme		
250 µl/ml	49.8 bc	50.2
500 µl/ml	19.3 d	80.7
T. harzianum (10^8 spores/ml)	9.1 e	90.9
T. viride (10^8 spores/ml)	38.1 c	61.9

*Check = cucumber fruits treated with *B. cinerea* only. Data are averages of 5 replicates. Values, within each column, followed by the same letter (s) are not significantly different at ($P \leq 0.05$).

In this respect, using 10^8 spores/ml of *T. harzianum* showed greatest prevention 90.9 % of the grey mould infection on cucumber fruits, followed by treatments with 500 µl/ml of clove and thyme essential oils and 7.5% of acetic acid which showed prevention ranged from 70.9-80.7%. Meanwhile, treatments with 5% of acetic acid, 250 µl/ml of used essential oils and 10^8 spores/ml of *T. viride* showed lower efficiency in prevented gray mold disease, ranged from 47.4-61.9 %, comparing with check treatment.

Data in Table (4) indicated that treatments with 500 µl/ml of both clove and thyme essential oils, 7.5% of acetic acid and 10^8 spores/ml of *T. harzianum* resulted in conspicuous decrease in the activity of peroxidase enzyme in the treated cucumber fruits comparing with check treatment. Also, the same treatments showed negative effect on the activity of polyphenoloxidase enzyme in the treated fruits.

DISCUSSION

The present efforts indicated that all *B. cinerea* isolates have the ability of causing gray mold infection onto cucumber fruits. Isolate No. 4 was the most virulent than the other isolates and decayed area was more broad on cucumber fruits than the other isolates. These results are in harmony with those finding of (Kim *et al.,* 1997; Ten Have *et al.,* 2002). El-Habaa (1997) verified the ability of two *B. cinerea* isolates for decaying cucumber and pepper fruits after harvesting and found that both isolates were able to infect pepper and cucumber fruits, however, cucumber isolate was more virulent on cucumber than pepper isolate, which was more virulent on pepper than cucumber.

The present data indicated that the application of *T. harzianum* showed the greatest reduction on grey mould disease on cucumber fruits. This data was thoroughly discussed by several researchers (Navaneetha *et al.,* 2015; Menzel *et al.,* 2016). Robinson-Boyer *et al.* (2009) and Xiangming *et al.* (2010) reported that the combinations of commercially available biological control agents containing *T. harzianum* and *T. atroviride* were more effectively in controlling *B. cinerea* on strawberry. The present efforts also indicated that treatment with acetic acid was significantly reduced the growth of *B. cinerea*. These results are in harmony with those of Hassenberg *et al.* (2010); Abd-Alla *et al.* (2011) and Hesami *et al,* (2013). Archbold *et al.,* (1997) mentioned that the efficacy of acetic acid might be due to its volatile compounds which showed promising results as a post-harvest fumigants for controlling *Botrytis* on strawberry fruits. Tohamy *et al*. (2003) also stated that, acetic acid was effective in controlling gray mold on tomatoes.

Current data revealed that treatment with essential oils decreased grey mold disease on cucumber. This data was in agreement with those of other workers (Azizi *et al*., 2008; Phillips *et al.,* 2012). Meepagala *et al.* (2002) reported that application of essential oils were effective for management of fungal rotting of fruit and vegetables, and prolonging shelf life. Liu *et al*. (2009) and Lopez-Reyes *et al*. (2010) reported that essential oils from oregano, savory and thyme showed significant efficacy against *B. cinerea, Penicillium expansum* and *Geotrichum citri-aurantii*.

Our treatments resulted in conspicuous decrease in the activity of peroxidase enzyme, but negatively affected the activity of polyphenoloxidase enzyme.

Table 4: Effect of acetic acid, essential oils and *Trichoderma* spp. on activities of peroxidase and polyphenoloxidase in cucumber fruits inoculated with *B. cinerea*

Treatment	Peroxidase	Polyphenoloxidase
Check*	1.285 a	0.159 a
Acetic acid		
5.0 %	0.784 b	0.075 bc
7.5 %	0.275 e	0.053 cd
Essential oils		
Clove		
250 µl/ml	0.430 d	0.095 b
500 µl/ml	0.160 g	0.040 d
Thyme		
250 µl/ml	0.415 de	0.074 bc
500 µl/ml	0.204 f	0.041 d
T. harzianum (10^8 spores/ml)	0.374 e	0.065 c
T. viride (10^8 spores/ml)	0.480 c	0.070 bc

*Check = cucumber fruits treated with *B. cinerea* only. Data are averages of 5 replicates. Values, within each column, followed by the same letter (s) are not significantly different at ($P \leq 0.05$).

These results were not harmony with those of Chile (1984), who found that fruits of apple, mango, guava and lemon, infected with *B. theobromae,* showed higher polyphenoloxidase activity than healthy ones. In contrast, peroxidase activity increased in rotten apple and lemon but remained suppressed in guava and mango. Shalaby *et al*. (2000) revealed that higher levels of peroxidase and phenoloxidase activity, phenols and non-reducing and total sugars were recorded in inoculated leaves of resistant tomato cultivar than those susceptible to early blight disease.

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الملخص العربي

كفاءة استخدام حمض الخليك، والزيوت النباتية وفطر التريكودرما فى مقاومة العفن الرماد على نبات الخيار

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يعتبر مرض العفن الرمادى المتسبب عن الفطر بوتريتس سنيريا (*Botrytis cinerea*) من الأمراض الهامة التى تصيب الخيار والكثير من النباتات والمنتجات البستانية بعد الحصاد. تمت دراسة معملية لتقييم كفاءة استخدام حمض الخليك، وزيت الزعتر، وزيت القرنفل، ونوعين من الفطر التضادى تريكودرما هرزيانم(*Trichoderma harzianum*)، وتريكودرما فيريدى (*T. viridi*) وذلك لمقاومة مرض العفن الرمادى على ثمار الخيار. واستخدم فى ذلك خمسة عزلات من فطر البوتريتس سنيريا، عزلت من ٨٥ عينة من ثمار الخيار تم جمعها من الأسواق المحلية بمحافظة جازان. وأوضحت نتائج دراسة القدرة الإمراضية لها أن العزلة رقم (٤) هى الأكثر شراسة وقدرة امراضية مقارنة بباقى العزلات المختبرة. وبينت النتائج أن استخدام حمض الخليك بتركيز ٧,٥%، وتركيز ٥٠٠ ميكروليتر/مل من كل من زيت الزعتر، وزيت القرنفل، وتركيز ١٠ ^{٨} جرثومة فطرية من أى من الفطرين تريكودرما هارزيانم أو فيريدى كانت الأكثر كفاءة فى تثبيط الفطر بواتريتس سنيريا بنسبة تراوحت بين ٦٥,٦ – ٨٣,٣ % وذلك باستخدام العزلة رقم ٤ من الفطر. كذلك أعطت نفس المعاملات نفس النتيجة عند عمل عدوى صناعية لثمار الخيار مقارنة بباقى المعاملات أو معاملة الكنترول. وتسببت كل المعاملات المختبرة فى خفض واضح لنشاط إنزيم البيروكسيديز وكذلك إنزيم البولى فينول أكسيديز. وبذلك تشير النتائج المتحصل عليها من امكانية استخدام حمض الخليك وفطر التريكودرما هرزيانم أو الزيوت الاساسية فى مقاومة مرض العفن الرمادى على ثمار الخيار بطريقة آمنة وغير مكلفة اقتصادياً.