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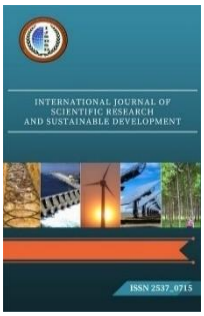

Controlling strawberry fruit rots with the alternative of fungicides

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Abstract

Strawberry fruits are subjected to many pathogens, especially the necrotrophic fungus *Botrytis cinerea*. Many salt compounds, plant extracts, and bio-agents were used to control gray mold in strawberry fruits caused by the fungus *Botrytis cinerea*. All the tested salt compounds reduced the mycelial growth of *B. cinerea*. The salt KH_2PO_4 was the most effective followed by the salt $NaHCO_3$ and $CaCl_2$ respectively. Fore disease severity, the highest concentration of any salt was the most effective in reducing disease severity. Spray strawberry fruits with three plant extract types, i.e., Garlic, Marjoram, and Thyme at two concentrations (10 and 20%) were effective in reducing mycelial growth. The highest effective concentration of Garlic extract in reducing disease severity was 20% compared with the other plant extracts and the control treatment. The results of the bio-agent experiment indicated that *Trichoderma harzianum* was the most effective in reducing the growth of ***B. cinerea*** in Petri dishes. In contrast, *Trichoderma viride* was the most effective one in reducing disease severity.

Keywords: Strawberry, *B. cinerea*, Salts, Plant extract, Bio-agent.

Introduction

Strawberry (*Fragaria* sp.) is among the most favorite and delicious on which the demand has been increased all over the world during the last decades. It is grown in some governorates in Egypt. Such as El-Qalyubia, Tahrir, Ismaelia and Garbia. Fruits are consumed not only fresh but may be canned and processed in different forms. Also, fresh, as well as canned strawberry fruit are exported in considerable quantities to Arab and Europe countries.

Strawberry fruit rots cause many losses to fruits both in quantity and quality in the field and market. Numerous pathogenic fungi cause rots of fruits such as leather rot caused by *Phytophthora cactorum*, gray mold caused by *Botrytis cinerea*, *Rhizopus* leak caused by *Rhizopus nigricans*, and hard brown rot caused by *Rhizoctonia (corticium*) *solani.*

All the above-mentioned causals of fruit rots of strawberries were isolated and recorded a long time ago. (**Stefan Petrash *et al.*, 2019)**

Botrytis cinerea is an airborne plant pathogen and necrotrophic fungus that infects over 200 plant species worldwide.

This pathogen infects fruit in the field, storage, transport, and market-leading to significant economic losses. *B. cinerea* mainly enters the host via wounds or natural openings.

For controlling gray mold caused by *B. cinerea* many workers used many plant extracts **(Awad and Al-Shennay, 2015; Antonov *et al.*, 1997; Williamson *et al.*, 2007).** Many salt compounds are more used in inducing resistance by many workers (**El-Mougy and Abdel-Kader, 2009; Smilanick *et al.*, 1999; Horst *et al.*, 1992 Droby *et al.*, 2003).**

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Bio-control agents were used for controlling gray molds such as *Trichoderma* spp. and *Bacillus* sp. (**Stanley Freeman *et al.*, 2004; Tronsmo and Dennis 1983; Trongsmo and Dennis, 2000).**

The presence of gray mold is the most common reason for fruit rejection by growers, shippers, and consumers, leading to significant economic losses.

The aim of this study is to find many alternatives to conventional fungicides that are characterized by a low impact on the environment and on human health. These include plant extracts, bio-agents, and some salt compounds that produce induced resistance.

Material and Methods

3.2. Isolation and identification of the causal organism:

Diseased strawberry (*Fragaria sp*.) fruits showing rots disease symptoms were collected from two governorates, i.e., El-Qalubia and El-Behaira.

Collected samples were subjected to isolation trials. The infected tissues were small pieces and were surface sterilized with sodium hypochlorite (0.5%) for 3 minutes. Then washed several times with sterilized distilled water and dried between sterilized filter papers and transferred directly to the PDA medium in Petri dishes 9 cm. The plates were incubated for 7 days at 22 ± 2°C. The fungi growing from the lesion pieces were transferred to potato dextrose agar (PDA) slants. The fungus was purified by the single spore technique and kept on the slant of PDA in test tubes at 5°C. Pure isolates were identified according to the morphological characteristics of mycelium, spores.

3.3. Pathogenicity test:

Pathogenicity tests for isolates of *B. cinerea* were carried out in the lab. The following technique was adopted in the pathogenicity study.

Healthy strawberry fruits of California cv. (El-Qalyubia) were surface sterilized by immersing in 2.0% sodium hypochlorite solution for two minutes, then washed several times by sterilized tap water and immediately at the rate of three fruits per group. Three replicates for each treatment were used.

The number of fruits having specific rots disease symptoms was counted after 7 days of incubating and the percentage of infection was calculated according to **Balogum *et al.*, 2005.**

The purified isolated fungi were identified according to their morphological features using the description of **Barnett and Hunter (1998)**. The isolated fungi were maintained on a PDS slant, kept in the refrigerator at 5-8°C, and sub-cultured till used.

The identification was confirmed at the Disease Survey and Mycology Department, Plant Pathology Institute, Agriculture Research Center, Egypt. The identified are *Botrytis cinerea*.

Disease assessments:

Disease readings were determined for fruits according to disease index rating which was made to include the average diameter of the infected areas. The following numerical rates were suggested to facilitate visual determination and to give a satisfactory comparison:

- 0 = No rot
- 1 = Scattered small rots
- 2 = Rots coalescing and including about 0.25 to 0.50 of fruit area.
- 3 - More than 0.50 of the fruit area was infected.
- Readings were converted to disease index according to the equation by **Townsend** and **Heuberger** (**1943**) as follows:

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$$\textbf{Disease index \% = Sum } \frac{(n\times r_1)+(n\times r_2)+(n\times r_3)}{3N}\times 100$$

Where:

- n: is the number of fruits in each numbered
- rates: r_1, r_2, and r_3 are the ratings
- N: is the total number of inoculated fruits multiplied by the maximum numerical rates 3

The percentage of infection was determined according to the following formula.

$$\textbf{\% Infection = } \frac{\text{Number of rotted fruits}}{\text{Total number of tested fruits}}\times 100$$

Effect of some salt compounds on *B. cinerea*

Used salts:

Potassium dihydrogen phosphate, sodium bicarbonate, and calcium chloride were purchased from Merck chemicals (Merck, Germany) Egypt as are presented in Table (1).

Table 1: Chemical products, Formula, and Molecule weight:

Chemical products	Formula	MW g/mol
1- Potassium dihydrogen phosphate	KH_2PO_4	136.08
2- Sodium bicarbonate	$NaHCO_3$	84.00
3- Calcium chloride	$CaCl_2$ (monohydrate)	128.99

a- Effect of some salt's compounds on the mycelial growth of *Botrytis cinerea*.

The tested fungus was grown on PDA amended with the tested salts with different concentrations (250, 500, and 1000) at 25°C using PDA agar disks (diameter = 5 mm) of actively growing mycelium of *B. cinerea*, a fungus was used to inoculate the plates. Colony diameter was determined one - week post-incubation. Colony diameter was measured as the average of the longest diameter (cm) and the shortest diameter.

Inhibition of mycelial growth (IMG) was calculated using the following equation:

IMG = control radial growth - salt amended radial growth × 100.

Three replicates were used for each treatment.

b- Effect of some salt's compounds on disease severity of *Botrytis cinerea*:

Different concentrations of salt (250, 500, and 1000 mM) were used to determine their effect on disease severity (Dis. %) of strawberry fruit rot inoculated with *B. cinerea* fungus.

Matured fruits were inoculated with a spore suspension of the young culture of *B. cinerea* fungus and then sprayed with different concentrations of used salt and disease assessments were calculated according to **Townsend** and **Heuberger** (**1943**) as mentioned before.

c- Plant extracts experiments:

Plant extraction: (Garlic, Marjoram, and Thyme) were collected locally or brought to local markets in Zagazig city. Samples were thoroughly masked using tap water, air-dried at room temperature for 3-4h, and finally dried in a hot-air oven at 45-50°C for 1-2 days. Dried samples were ground using a small grinder, then placed in polyethylene bags, and stored at 4°C until required, for each sample, 50g of sample powder were added to 150 ml L of 80% ethanol.

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The mixtures were agitated for 72h on a rotary shaker (130 rpm). The obtained extracts were centrifuged at 8,000 rpm for 10 minutes, filtered through Whatman filter paper No. 1, and transferred to 250mLround - bottom flasks. Finally, the extracts were evaporated using a rotary evaporator at 45°C. Concentrated extracts were allowed to dry in a hot-air oven, weigh again, and kept at 45°C until required for the antifungal assay.

d- Effect of some plant extracts on mycelial growth of *B. cinerea*

Crude plant extracts were in vitro tested for their efficacy against fungi mycelial growth using the poisoned food technique. The crude plant extracts were reconstituted to have concentrations 10 and 20%. Then 1mL of each extract was used for mixing with 19mLof worm PDA and poured into a 9cm sterile Petri dish. After solidification, the plates were inoculated with the 6mm agar piece containing week-old mycelia. For each crude plant extract, the experiments were performed in three replicates and sterile distilled water as a control. Inhibition of mycelial growth was calculated using the following formula:

%inhibition = 100 × (x – yx).

X : diameter of a fungal colony grown on a negative control plate.

Y : diameter of a fungal colony grown on plates containing crude plant extracts and sterile distilled water served as control. Plates were inoculated at 25 ± 18 and monitored for 7 days.

e- Effect of some plant extracts on disease severity of *B. cinerea*

%Disease incidence = [Number of rotted fruits / Total number of tested fruits] × 100

Disease severity was recorded according to an empirical scale with six degrees: 0 = healthy strawberry, 1=1% to 20% fruit surface infected, 2=21% to 40% fruit surface infected, 3=41% to 60% fruit surface infected, 4= 61% to 80% fruit surface infected, 5= more than 81% of fruit surface infected **(Romanzzi *et al.*, 2000).**

Bioagent experiments:

The isolates of *Trichoderma harzianum*, *T.viride* and *Bacillus subtilis* were obtained from department of nigtable disease, plant disease institute, ARC.

f- Effect of some bioagents on mycelial of *B. cinerea in vitro*:

Two discs (5mm) of 7 days old plain agar culture of both antagonistic fungi (*Trichoderma harzianum* and *T. viride*) and *B. cinerea* isolate from El-Qalubya were inoculated simultaneously each opposite the other 1 cm apart from the plate edge in individual plates (09 cm) contained 10 mL PDA medium. In the control treatment, the plate was inoculated each with 1 disc of mycelial growth of a given isolate of ***B. cinerea.*** Three plates were used for each treatment. All dishes were incubated at 22+2°C for 10 days. The percentage of the fungal growth reduction (X) was calculated by using the following formula suggested by **Abd-El-Moity, 1985.**

$$X = G_1\text{-}G_2 / G_1 \times 100$$

Where:

- X = fungal growth reduction
- G_1 = linear growth of the pathogen inoculated alone.
- G_2 = linear growth of the pathogen inoculated against the antagonistic fungus.

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g- Effect of Bioagent *Bacillus subtilis* on mycelial growth of *B. cinerea:*

Studying the effect of antagonistic bacteria isolate of *Bacillus subtilis* on the growth of *B. cinerea* isolated from El-Qalubyia was conducted as follows, individual plates (09cm) contained PDA medium were streaked at one side 1 cm apart from the plate edge with a loop full of the antagonistic bacteria (48 hrs. - old) grown on nutrient broth medium (NB) and for 24 hrs. at 22°C. Thereafter the same plate was inoculated at the opposite side 1 cm apart from the plate edge with a 5 mm disc of 7-days-old plain agar culture of *B. cinerea.* All plates were incubated at 22±2°C for 5 days.

Results and Discussion

1) Collected sample and number of isolates:

The collected samples from El-Qalubia governorate were 10 while the collected sample from El-Behiar were 25. The Fungal isolates were 14 from El-Qalubia while 33 from EL-Behiara (Table 2).

Table (2): Isolates Table:

Governorate	Sample Number	Isolate Number
El-Qalyubia	10	14
El-Behiara	25	33
Total	**35**	**47**

Isolation of the causal organisms:

Botrytis cinerea, *Rhizopus nigricans* and *Rhizoctonia* solani were isolated from the diseased strawberry fruits.

Pathogenicity test of the isolated fungi:

Pathogenicity tests (% infection and % disease severity) were carried out for the isolated fungi i.e *Botrytis cinerea*, *Rhizopus nigricans* and *Rhizotonia solani*. Data in Table (3) indicate that *B. cinerea* was the most effective effect on inducing rot strawberry fruits.

Table (3): Pathogenicity test of the isolated fungi:

Isolated Fungi	% Infection	% Disease severity
Botrytis cinerea	75	80
Rhizopus nigricans	50	60
Rhiztonia soluni	35	40

Salt experiments:

a. Effect of some salt compounds on mycelial growth of *B. cinerea*

Data presented in Table 2 indicate that all the tested salts significantly reduced the mycelial growth of *Botrytis cinerea* compared with the control treatment. The salt of KH_2PO_4, was the most effective in reducing radial growth of *Botrytis cinerea*, followed by the salt of $NaHCO_3$ and $CaCl_2$, respectively, with increasing the concentration of any salt from 250 up to 500 mm caused an inhibition of the hyphal growth of the fungus of *Botrytis cinerea.*

To achieve successful control, it is necessary to use the effective active salt at the appropriate concentration and applied at the right time. In addition, the use of different chemical salts in a rotational and/or repetitive program will prevent fungi from developing resistance to a given active ingredient (**Doster and Michailides, 1999**).

Similar results were obtained by **Arslan *et al.*, 2009, Erper *et al.*, 2011** and **Latifa *et al.*, 2011** who concluded that chemical salts significantly differed in their action against the fungi.

b. Effect of different compounds salts on disease severity % of *Botrytis cinerea:*

Results in Table 3 indicate that the isolated *Botrytis cinerea* from strawberry fruits reduced the disease severity % by application of the tested chemical salts. The highest concentration of any salt significantly reduced on disease severity percentage of *Botrytis cinerea* compared to the control treatment. The salt of KH_2PO_4 is the most effective on disease severity percentage of gray mold disease compared with the other salts and control treatments.

The obtained results agree with those reported by **Fallik *et al.*, 1996, Sugimoto *et al.*, 2009 and Turkkan *et al.*, 2017** who illustrated that the stimulative effect of chemical salts differed responses to inhibition of the development and disease severity percentage of gray mold disease.

Plant extracts experiments:

a) Effect of plant extracts on mycelial growth of *B. cinerea:*

Plant extract types, i.e., Garlic (*Allium cepa* L.), Marjoram (*Majorana hortensis* L), and Thyme (*Thymus vulgaris* L.) at both concentrations (10 and 20%) were the most effective in used to study their effect on reducing mycelial growth of *B. cinerea* (Table 4).

The garlic plant extract was the most effective in reducing the mycelial growth of *B. cinerea* isolated from strawberry fruits cv. Florida, followed by marjoram and thyme extracts, respectively. Increasing the concentration of plant extract from 10% to 20% significantly decreased the mycelial growth of *B. cinerea* on strawberry fruits. There is a gradually decreased in mycelial growth due to thyme, marjoram, and garlic extracts, respectively in both concentrations compared to the control treatment (**Erper *et al.*, 2011).**

Garlic extract was the most effective in growth inhibition of *B. cinerea* due to its content of the Sulphur compounds, i.e., *S. allylcysteine*, diallyldisuifide, allylpropylddisulfide and allylmercaptan (**Sulaiman and Abdulhafcdh, 2013**).

As well as, the active compounds of marjoram extract are camphor, ocimene, cadinene, and tannic acid, and the active compounds of thyme extract are thymol and carvacrol, which inhibited the mycelial growth of *B. cinerea* (**El-Cali and Hypa, 2018 and Tamim and Akrama, 2019).**

Obtained data have coincided with those of **Awad and Al-Shennawy (2015), Bozik *et al.*, (2017), Singh *et al.*, (2019) and Sernaite *et al.*, (2020).**

b) Effect of plant extracts on disease severity of *B. cinerea*:

Data in Table 5 show that all plant extracts with different concentrations decreased disease severity % of *B. cinerea* when compared with the control treatment. In this respect, increasing the concentration of plant extracts decreased gradually the determined disease severity %. The tested concentrations of garlic, marjoram, and thyme were more effective than the control treatment, in controlling strawberry fruit rot infection. Also, the Florida cultivar exhibited clear resistance to fruit rot infection with all plant extracts at both concentrations (10 and 20%).

The sprayed strawberry fruits with garlic extracts were more effective in reducing the strawberry fruit rot infection at both concentrations than the other plant extracts. The highest effective concentration of garlic extracts in reducing the fruit rot infection was 20% compared with the other plant extracts and control treatment.

It can be assumed that garlic extract contained the highest amounts of antifungal compounds, i.e., diallyldisulfide and allylpropyldisulfide which reduced the disease severity % of *B. cinerea* in strawberry fruits infection (**Sernaite *et al.*, 2020; Choudhury *et al*., 2018).**

The results agree with those illustrated by **Bozik *et al.*, (2017), El-Naggar *et al.*, (2017), and Choudhary *et al.*, (2018).**

Bioagent experiments:

a- Effect of bio-agents (Biocontrol) on mycelial growth of *B. cinerea*:

Mycelial growth:

Results in Table 6 illustrate that the inhibition of mycelial growth of strawberry pathogen *B. cinerea* was determined by using bio-agents, i.e., *Trichoderma harzianum, Trichoderma viride,* and *Bacillus subtilis.*

The results indicated that all the tested bio-agents were effective in inhibiting the fungal growth of *B. cinerea* in all experimental trials in Petri dishes. In this respect, the *Trichoderma harzianum* was more effective in inhibiting the growth of *B. cinerea* than the other bio-agents.

The impact of the treatment was followed by the treatments of *Trichoderma viride and Bacillus subtilis, respectively.*

These bio-agents produce a wide range of antibiotic compounds that are inhibiting fungi growth.

Trichoderma strains have highly effective antagonistic mechanisms to survive and colonize the competitive environment of the rhizosphere, phyllosphere, and spermosphere. One of its main mechanisms, mycoparasitism, relies on the recognition, binding, and enzymatic disruption of the host-fungus cell wall. The important role of *Trichoderma* strains for an astonishing variety of secreted lytic enzymes, including endochitinases, N- acetyl - β - glucosaminidase, chitin 1,4 - β - chitobiosidaes, proteases, endo and exo glucan β -1,3 - glucosidases, endoglucan β -1,6 - glucosidases, lipases xylanases, mannanases, pectinases, pectin lyases, phospholipases, R, Nases and D. Nases **(Lorito, 1998).**

In addition, *Trichoderma* spp**.** is now the most common fungal biological control agent that has been comprehensively researched and deployed. Concluded that several fungal cell wall degrading enzymes, amongst them chitinase and glucanase, seem to play an important role in the antagonistic action of *Trichoderma* against a wide range of fungal pathogens.

Moreover, ***Bacillus subtilis*** are known to produce a wide range of antibiotic compounds that are inhibitory to fungi, it is capable to use chitin and β - glucan as substrates, which inhibit the growth of fungus (**Bargabus *et al.*, 2004**). The obtained results are supported by the findings of **Ayduki *et al.*, 2016.**

b. Effect of Bioagent on disease severity of *B. cinerea*:

Disease severity (D.S. %):

Data presented in table 7 show that the bio-agents, i.e., *Trichoderma harzianum, Trichoderma viride,* and *Bacillus subtilis* significantly decreased the disease severity (D.S.) percentage of *Botrytis cinerea.*

The isolate of *Trichoderma viride,* being the most effective one, inhibited the disease severity percentage. This treatment was followed by the treatments of *Bacillus subtilis* and *Trichoderma harzianum,* respectively in disease severity.

The bio-agents of *Trichoderma viride* and *B. subtilis* may inhibit pathogens by producing antibiotic and fluorescent siderophores.

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Moreover, *Bacillus subtilis* produce other metabolites including biosurfactants, chitinase, and other fungal cell-wall degrading enzymes, volatiles, and compounds that elicit plant resistance mechanisms, and are involved in several mechanisms of biological control not only an antibiosis but also competition **(Harman *et al.*, 2004).**

These results are confirmed by those recorded by **Awad and Al-Shennay, (2015)** who reported that *Trichoderma* isolates were the most effective bioagent in reducing the mycelial growth of *B. cinerea*.

Table (4): Effect of different concentrations of some salts on mycelial growth of *B. cinerea:*

Salt Compound	Concentration		
	(mm)	(mm)	Mycelial Growth
Concentration	250	500	1000
KH_2PO_4	18	4	1
$NaHCO_3$	11	6	2
$CaCl_2$	33	25	15
Control	90	90	90
LSD (0.05)			

Table (5): Effect of different concentrations of some salts on disease severity of *B. cinerea*:

Salt Compound	Concentration		
	Disease Severity %		
Concentration	250	500	1000
KH_2PO_4	3.0	2.0	0.5
$NaHCO_3$	3.5	2.8	0.8
$CaCl_2$	3.1	3.5	3.5
Control	74.3	74.3	74.3

Plant Extracts:

Table (6): Effect of different concentrations of some plant extracts on mycelial growth of *B. cinerea*:

Plant extract	Concentration	
	10%	20%
Garlic	10	5
Marjoram	19	13
Thyme	33	26
Control	90	90
LSD (0.05)		
Extracts	**2.7**	
Concentrations	**1.9**	
EXC	**4.8 (N.S)**	

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Table (7): Effect of different concentrations of some plant extracts on disease severity of *B. cinerea*:

Plant extract	Concentration	
	10%	20%
Garlic	1.3	0.3
Marjoram	3.2	2.7
Thyme	10.8	8.5
Control	75.3	75.3
LSD (0.05)		
Extracts 2.0		
Concentrations 1.7		
EXC 4.8 (N.S)		

Bio-agent

Table (8): Effect of some bio-agents on mycelial growth of *B. cinerea*:

Bioagent	Mycelial Growth (mm)
Trichoderma harzianum	11.6
T. viride	17.3
Bacillus subtilis	24.00
Control	90
LSD (0.05)	4.6

Table (9): Effect of some bioagents on disease severity of *B. cinerea:*

Bioagent	D.S% (Disease severity)
T. harzianum	16.32
T. viride	9.00
B. subtilis	12.55
Control	76.66
LSD (0.05)	3.02

Conclusion:

For controlling *B. cinerea* using plant extracts *in vitro*, all tested plant extracts concentrations were effective in inhibiting fungal growth in all experimental trials.

A significant effect for most used salts solutions in inhibiting the growth of the fungus was noticed. On the other hand, biological agents, *Trichoderma* species, and *Bacillus subtilis* against *B. cinerea* were very effective in the disease incidence of *B. cinerea.*

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