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EFFICACY OF BIOCOTROL AGENTS AGAINST BOTRYTIS *CINERA* A CAUSING FRUIT A GRAY MOLD OF STRAWBERRY AND ITS EFFECT ON SOME QUALITY PARAMETERS.

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ABSTRACT

In vitro and in vivo studies were conducted to evaluate the antagonistic activities off our biological control agents (*Trichoderma harzianum*, *Pseudomonas fluorescens, Bacillus subtilis* and *Streptomyces spp.*) against *Botrytis cinerea* that cause gray mold of strawberry fruits. *In vitro* antagonistic study showed that highest reduction% in *B. cinerea* linear growth was induced by *Trichoderma harzianum* by 51.3% followed by *Pseudomonas fluorescens*, *Bacillus subtilis* and *Streptomyces* spp. (46.8, 33.2 and 24.6 % respectively). Also, *In vivo* antagonistic study on strawberry fruits showed that the lowest disease incidence and highest reduction percentage in disease incidence was obtained by *Trichoderma harzianum* (14 and 76 % respectively)while the highest disease incidence and lowest reduction percentage in disease incidence was obtained by *Streptomyce sspp*. (22 and 67.3% respectively). On the other hand treatment of fruits with *Bacillus subtilis* resulted in the highest TSS% (total soluble solids %), titratable acidity and protein content followed by *Streptomyces spp*., *Pseudomonas fluorescens* and *Trichoderma harzianum.* The longer shelf life of fruits was obtained by treatment with *Trichoderma harzianum* (5.9 days) followed by *Pseudomonas fluorescens, Bacillus subtilis* and *Streptomyces* spp. (5.7, 4.3, 4.1 days respectively). Also the lowest fruit weight loses was obtained by *Trichoderma harzianum* treatment (6.3%) and while the highest was obtained by *Streptomyces spp*. (9.1%). *Trichoderma harzianum*, *Pseudomonas fluorescens, Bacillus subtilis* and *Streptomyces* spp. showed potent antagonistic activity against *B. cinerea* that cause strawberry gray mold and reduce the disease severity as well as increase fruits quality characteristics.

Keywords: Gray mold, Strawberry, *Botrytis cinerea*, Antagonistic, *Trichoderma harzianum*, *Pseudomonas fluorescens*, *Bacillus subtilis*, *Streptomyces spp.*

INTRODUCTION

Strawberries (Fragaria x ananassa Duch) are a highly important vegetable crop for consumption and export, but they have a short postharvest lifespan mainly because of fungal decay. Every year 10 to 20% of world food production lost due to plant fungal and bacterial diseases which lead to loss of billions of dollars. *Botrytis cinerea* is necrotrophic fungal plant pathogens that affect hundreds of plant species producing gray molds **(Williamson et al., 2007)**. It infects a wide range of plants via mycelia or dispersed spores **(Clarkson et al., 2003)**.The effect of such pathogenic mold varies greatly from year to year, depending on temperature, host plant and inoculum conditions. Excessive chemical fungicides have been used for the effective control of pathogens for many decades, resulting in increased costs for growers, destruction of natural biological processes, growth of pathogen resistance and adverse effects on other species, the environment and human health **(Abdel Wahab et al., 2020)**. **(Laura et al., 2020)** They evaluate the use of *Bacillus velezensis* XT1 as plant growth – promoting rhizo bacterium (PGPR) and biocontrol agent against *B .cinerea* in Tomato and Strawberry plant. They found that, foliar and radicular application of strain XT1 increased plant total biomass as compared to the control and *B. cinerea* infected plant. Application of the bacterium was found to reduce infection and severity by 50% and 60% respectively. The study also highlights the potential of preventive application of strain XT1 to activate defense mechanisms in Strawberry and Tomato plants through hormone regulation. It was believed that biocontrol agents were safe environmental solutions that enhance food production while decreasing the use of chemical fertilizers, pesticides, and other artificial supplements. (**Esitken et al., 2005; Bhattacharyya and Jha, 2012**). Biological control of many fungal plant diseases has been achieved by using *Trichoderma harzianum*, an efficient biocontrol agent from the *Trichoderma spp*. Group **(Elad, et al., 1998; Abdel-Fattah, et al., 2007)**. *Pseudomonas fluorescence, Bacillus subtilis*, exhibited a propensity toward E. carotovora subsp. Carotovora **(Vanneste and Yu, 1996; Salem and Abd El-Shafea, 2018).** Streptomyces is a widely recognized genus in the Actinomycetales order. Its main function is to inhabit soil and enhance soil fertility. These prokaryotes possess properties that enable them to act as biocontrol agents against bacterial plant pathogens **(Keiser et al. 2000).**

Vasantharaj David (2008) asserted that Biological control is a promising method for managing diseases in crop fields due to the increasing awareness of the possible harmful effects of fungicides on the ecosystem and the increasing interest in pesticide-free agriculture products. Biological control is effective in increasing crop yield and suppressing disease, while also avoiding environmental pollution.

The aim of this research was to assess the protective effect of four biocontrol agents (*Trichoderma harzianum*, *Pseudomonas fluorescens*, *Bacillus subtilis*, and *Streptomyces spp.*) against strawberry gray mold caused by

B. cinerea on both *in vitro* and *in vivo*, with a focus on reducing the severity of disease and improving fruit quality characteristics.

MATERIALS AND METHODS

The strawberries collected from local markets in Cairo governorate were grouped into two categories, healthy and decayed, and then stored at 13 °C for three days until the developing fungal colonies were picked up and examined.

Isolation, purification and identification of causal organisms:

Rotted strawberries were rinsed by sterilized water for several times then disinfected by 70% ethanol, dried and cut into small pieces. Sterilized Petri dishes containing Potato Dextrose Agar (PDA) were employed to culture these parts and incubate them at 20 °C for 3 days. PDA was used to isolate and purify the growing fungi, which were then identified according to **Raper and Thom (1968)**.

Antagonistic organisms

The antagonistic organism *Trichoderma harzianum, Pseudomonas fluorescens, Bacillus subtilis* and *Streptomyces* spp. were provided by the Bacteriological Lab at Faculty of Science, Zagazig University, Egypt.

Preparation of inoculums

The isolation of *Botrytis cinerea* from infected Strawberries and its maintenance on PDA has been confirmed. The mycelial suspension of a 2 week old culture was filtered through 3 layers of sterile cheese cloth to recover conidia of *B. cinerea*. The conidial suspension's concentration was modified to 2 x 10^5 conidia per ml. Fungal infection of healthy strawberries were done by immersing in a conidial suspension of *B. cinerea* containing 0 .1% tween 80 and allowed to air dry at room temperature for 2 hrs.

Following treatment of both healthy and infected strawberries using an antagonistic organism, the fruits of the strawberries were checked for (diseases Severity, %)

In Vitro antagonistic effect between bioagents and *B. cinerea*

Antagonistic effect between *Trichoderma harzianum, Pseudomonas fluorescens, Bacillus subtilis and Streptomyces spp* and *B. cinerea* were studied. Using a sterilized L. glass shape rod spreader, the *B. cinerea* suspension was applied to the plate surface. Next, an agar disk with a diameter of 7 mm was cut from a plate on which the biocontrol strain had been grown for 48 hours at 28 °C for bacteria and for 7 days for actinomyces. This disk was then placed in the center of the plates. After *B. cinerea* was incubated for 48 hours at 28 °C, the diameter of its inhibition zones was measured, and the mean value of three replicates was computed.

In vivo antagonistic impact between bioagent and *B. cinerea*

Uniform strawberry fruits (cultivar Fragaria xananassa Duch) were selected by shape, size and color free of physical damage and diseases. This fruits were sterilized by sodium hypochlorite (100 µl. L^{-1}). The fruits were divided into two lots; one uninoculated (natural infection) and the other artificially inoculated with *B. cinerea* by immersing fruits in prepared suspension (10^6 spores/ml)

containing 0.1% Tween 80 for 1 min and left at room temperature (20±2°C) for 2 hours in order to fix fungal infection. Each lot was divided into four parts and each part was subjected to one of the following four bio agents, then carboxymethyl cellulose (as sticker agent) was added to the actinomycetes, or antagonistic bacteria (2.8&10 cfuml) with 1% (W/V) for one hour **(Anuratha and Gnaanamanickam, 1990**). Strawberry fruits were soaked in the above mixture, stored and the disease incidence (%) and % reduction in disease incidence were recorded.

Severity of infection (%)

Based on a visual examination of every fruit infection, **Chastanger and Ogawa (1979)** determined the infection's severity. Five categories were used to classify contaminated fruits: 0 = Superficial flack (no rot)
1 = 1-24% of the surface spoiled
3= 50-74% of the surface spoiled
2 = 25-49% of the surface spoiled
4= 75% or more of the surface spoiled

The decay index (DI) was calculated as follows:

$$DI = \frac{\text{Sum (number of fruits per category x category number)}}{\text{Total number of fruits infected}}$$

% severity of infection = (DI/4) x 100

The percent inhibition of growth of the pathogen: was calculated by using formula suggested by **Vincent (1947)**.

I= C-T/C *100
Where I= percent inhibition
C= growth in control
T= growth in treatment

Total soluble solids (TSS %)

Hand refractometer was employed to estimation of TSS, according to **(A.O.A.C, 1990).**

Titratable acidity (TA %)

Determination of titratable acidity was expressed as percentage of citric acid (g citric acid / 100 ml juice), according to **(A.O.A.C. 1990).**

Protein (mg/gm)

The dried fruits were ground in a mortar and then extracted using tris borate (PH=7.9) as an extraction buffer in a ratio of 1:2.5 (W/V).After extracting the samples, they were centrifuged for 15 minutes at 4000 rpm at room temperature. The supernatants with the soluble proteins were utilized for further chemical analyses. The soluble protein was measured in accordance with **Bradford (1976)**.

Fruit weight loss (WL %)

Determination of WL was done according to **(Kabel, 1990)** as follow:-

$$WL\ \% = \frac{\text{Fruit initial weight – fruit weight at each sampling date}}{\text{Fruit initial weight}} \times 100$$

Fruit shelf- life (in days)

Shelf -life was estimated by removing fruits sample from cold storage and 3 replicates from each treatment were left at room temperature (25 °C ± 2), when 50 % of fruits were scalded and the number of days was recorded.

Statistical analysis

All treatments were randomized. The obtained results (%) were presented as mean of replicates ± SE and statistically analyzed according to procedure of **SAS (1985),** and Duncan's multiple range test **(Duncan 1955)**.

RESULTS AND DISCUSSION

Enhancing acquired resistance of strawberry by using biological control seems to be one of alternatives to substitute or at least to decrease the use of fungicides in plant disease control. The current study was planned mainly to investigate the possibility of minimizing gray mold of strawberry fruits caused by *B. cinerea* by antagonistic activities of *T. harzianum*, *P. fluorescens*, *Bacillus subtilis* and *Streptomyces spp*.

The results in table (1) indicated that all antagonistic isolates significantly reduce linear growth of *B. cinerea*, the most effective antagonistic isolate is *T. harzianum* which reduced linear growth of *B. cinerea* by 51.3% followed by *P. fluorescens, B. subtilis and Streptomyces spp*. which give reduction % in linear growth of *B. cinerea* by 46.8 , 33.2 and 24.6 % respectively.

Table (1): *In vitro* antagonistic activities of *T. harzianum, P. fluorescens, Bacillus subtilis and Streptomyces spp* on gray mold of strawberry caused by *B. cinerea*

Treatment	**% Reduction in Linear growth of *B. cinerea***
T. harzianum	51.3 ± 0.3^{a}
P. fluorescens	46.8 ± 0.15^{b}
B. subtilis	33.2 ± 0.18^{c}
Streptomyces spp	24.9 ± 0.31^{d}
LSD	0.68

Superscript letters in each column indicate significant differences (Duncan, $p<0.05$). (n=3)

In vivo experiment in strawberry fruits was carried out to study antagonistic effect of *T. harzianum, P. fluorescens , B. subtilis and Streptomyces spp* against *B. cinerea* the causal agent of gray mold on disease incidence and % reduction in disease incidence. The results obtained in table 2 were aligned with that *in vitro T. harzianum* showed the strongest

antagonistic effect with lowest disease incidence (14) and the highest % reduction in disease incidence by 76.0 followed by *P. fluorescens , B. subtilis and Streptomyces spp* which give disease incidence 16,19 and 22 respectively and % reduction in disease incidence by 72.9, 70.8 and 67.3 respectively.

Table (2): *In vivo* Antagonistic activities of *T. harzianum, P. fluorescens, B. subtilis* and *Streptomyces spp* on gray mold of strawberry caused by *B. cinerea*

Treatment	Gray mold caused by *B. cinerea*	
	Disease incidence	**% Reduction in disease incidence**
T. harzianum	14	76.0±0.71[a]
P. fluorescens	16	72.9±0.30[b]
B. subtilis	19	70.8±0.32[c]
Streptomyces spp	22	67.3±0.32[d]
LSD		1.13

Superscript letters in each column indicate significant differences (Duncan, $p<0.05$). (n=10)

Increase shelf life is considered as a very important target in postharvest studies especially for strawberry fruits which have short shelf life. The current experiment is designed to study the effect of selected bio agents in increasing shelf life of strawberry fruits. Data in table (3) demonstrated that fruits treated with *T. harzianum* exhibits longest shelf life 5.9 (days) followed by *P. fluorescens, B. subtilis and Streptomyces spp.* (5.7, 4.3 and 4.1 (days) respectively). Moreover, fruits that treated with *T. harzianum* recorded the lowest fruit weight losses (6.3 %), while, *Streptomyces spp.* resulted in highest fruit weight losses (9.1%) as compared with control. Regarding to strawberry fruits chemical components (total soluble solids , titratable acidity and protein content) *T. harzianum* give the lowest chemical component when compared with *Streptomyces spp.* give highest chemical component (total soluble solids , titratable acidity and protein content) Also all antagonistic isolates give chemical component higher than control.

Table (3): *In vivo* effect of different treatments to control gray mold of strawberry fruits on some quality parameter of fruits

Treatment	TSS %	Titratable acidity %	Protein (mg/gm)	Fruit weight losses (%)	Shelf life (days)
T. harzianum	8.2±0.15^{b}	0.62±0.02^{b}	2.97±0.09^{b}	6.3±0.12^{d}	5.9
P.Fluorescens	8.2±0.15^{b}	0.64±0.01^{b}	3.75±0.05^{c}	6.7±0.21^{d}	5.7
B. subtilis	8.8±0.23^{a}	0.70±0.02^{a}	4.82±0.05^{a}	8±0.12^{c}	4.3
Streptomyces spp.	8.7±0.10^{ab}	0.70±0.02^{a}	4.80±0.05^{a}	9.1±0.15^{b}	4.1
Control	6.4±0.15^{c}	0.60±0.02^{b}	2.53±0.04^{d}	20.0±0.21^{a}	2.0
LSD	0.51	0.045	0.19	0.52	

Superscript letters in each column indicate significant differences (Duncan, p<0.05). (n=3)

Chemical control is not always completely effective in controlling plant disease since pathogen may develop resistance to some fungicides **(Gullino** and **Wardlow**, **1990)**, in addition to the harmful effects of these fungicides to human and environment. The use of microorganisms that can grow in the rhizosphere and control fungi biologically is an ideal way to use them as biocontrol agents. Antibiosis is a type of interaction between microorganisms and plant hosts that lead to biocontrol, since the rhizosphere acts as the front line defense for roots against pathogen attack via production of volatile toxic metabolites, mycolytic enzymes, the development of host resistance, and the competition for spaces and nutrients **(Sobiczewski, 2002)**. *In vitro* and *in vivo* obtained data demonstrated that *T. harzianum* was the strongest antagonistic against *B. cinerea* followed by *P. fluorescens*, *B. subtilis* and *Streptomyces spp.* respectively, which are in agreement with **YigalElad (2000)** who explained the beneficial effect of *T. harzianum* isolates T39 against many foliar pathogens like *B. cinerea*, Powdery mildew and *Sclerotinia sclerotioum* through enhancing local resistance and/or systemically through protection uninfected leaf tissue by increasing the formation of phytohormones and fighting the reactive oxygen species (ROS) production. Also three mechanisms explained the inhibitory effect of *Trichoderma spp.* on fungal growth: 1- compete pathogen for space and nutrients, 2- parasitism and 3- antibiosis (through produced inhibitory metabolites that acting as antibiotic) **(Harman, 2006)**. **Mercier and Lindow (2000)** reported that *T. atroviride* inhibits

B. cinerea on strawberry leaves through competition for glucose. Moreover, **Fakhouri, et al., (2001)** recorded that, *P. fluorescens* and *Streptomyces spp*. produce wide spectrum of biocotrol factors such as: siderophores, antibiotic and extracellular enzymes. Also **Singh et al, (2008)** attributed the antagonistic effect of *B. subtilis* isolates against pathogenic fungi to production of many of antifungal substances. Meanwhile, *P. fluorescens* induce antagonistic activity against pathogenic fungi through chelating iron from medium which used in its metabolism to promote its immobilization and prevent other microorganisms to utilize this element **(Naureen et al., 2009)**. Also it is found that *P. fluorescens* regulates competition among nutrient through facilitate the absorption of Mn, N, Zn, and P and regulate the uptake of Cu, Na, Ca, and K by plants, which may lead to increases fruit size, weight, yield and quality parallel to decreasing the use of chemical fertilizers and pesticides (**Martínez et al. 2019)**, in addition to production of some pigments and antibiotic **(Jayaraj et al., 2007).** The same mechanism was found by using *Streptomyces spp.* as antagonistic, since *Streptomyces spp*. produce antimicrobial metabolites or antibiotic that active against several plant pathogens **(Said, 2002)**.

CONCLUSION

In conclusion, *Trichoderma harzianum*, *Pseudomonas fluorescens*, *Bacillus subtilis* and *Streptomyces* spp. were exceptional biocontrol agents that showing wide antagonistic and inhibitory effect against *B. cinerea* that infects strawberry fruits in the postharvest stage through producing antimicrobial metabolites or antibiotic, as well as increase fruits quality characteristics.

Abbreviations

PDA: Potato Dextrose Agar; ML2: Mycological lab.2; CFU: Colony-forming unit; TSS: Total soluble solids; A.O.A.C.: Association of Official Analytical Chemists;

TA: Titratable acidity**;** SAS: Science Analysis System; ROS: Reactive oxygen species

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الملخص العربي

المسبب للعفن الرمادي لثمار الفراولة *Botrytis cinera* فعالية عوامل المكافحة الحيوية ضد وتأثيرها على بعض معايير الجودة.

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أجريت دراسات في المختبر وفي الثمار لتقييم التأثير المثبط لأربعة عوامل مكافحة بيولوجية (*Trichoderma harzianum* ،*Pseudomonas fluorescens* ،*Bacillus subtilis* و *Streptomyces spp.*) ضد *Botrytis cinerea* المسبب للعفن الرمادي لثمار الفراولة. أظهرت الدراسة المضادة في المختبر أن أعلى نسبة انخفاض في النمو الخطي *B. cinerea* كانت مستحثة بواسطة *Trichoderma harzianum* بنسبة 51.3٪ تليها *Pseudomonas fluorescens* و *Bacillus subtilis* و *Streptomyces* spp.(46.8و 33.2 و 24.6٪ على التوالي). و أيضاً أظهرت نتائج الدراسات علي الثمار أن أقل نسبة حدوث للمرض وأعلى نسبة انخفاض في الإصابة تم الحصول عليها بواسطة *Trichoderma harzianum* (14و 76%) بينما أعلى معدل للإصابة وأقل نسبة انخفاض في حدوث المرض تم الحصول عليها بواسطة *Streptomyces* spp. (22 و 67.3 %). من ناحية أخرى المعاملة ب *Bacillus subtilis*أعطت أعلى نسبة من المواد الصلبة الذائبة والحموضة والمحتوى البروتيني يليه *Streptomyces spp* ثم *Pseudomonas* fluorescens و *Trichoderma harzianum*. أعلى فترة صلاحية للثمار تم الحصول عليها من المعاملة ب *Trichoderma harzianum* (5.9 أيام) تليها *Pseudomonas fluorescens* و *Bacillus subtilis* و *Streptomyces spp.* ب(5.7, 4.3 و4.1 أيام على التوالي). كما تم الحصول على أقل خسارة في وزن الفاكهة عن طريق المعامله ب Trichoderma harzianum (6.3%) بينما تم الحصول على أعلى خسارة في وزن الفاكهة بواسطة Streptomyces spp. (9.1%). أظهرت عوامل المكافحة الحيوية المستخدمة تأثيراً قوياً في السيطرة على العفن الرمادي للفراولة الذي تسببه *Botrytis cinerea* و كذلك تقليل شدة المرض وتحسين خصائص جودة الثمار.

الكلمات الدالة: العفن الرمادى، الفراولة، المكافحة الحيوية.