Antagonistic Effect of Different Bioagents Against Two Different Soil-Borne Pathogens of Strawberry Plants

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Abstract :

Biological control agents *Trichoderma harzianum*, *Bacillus subtilis*, and *Streptomyces canescens*.are known as effective biocontrol agents for several soil-borne fungal plant pathogens including *Macrophomina phaseolina* and *Colletotricum fragariae*. The present work was carried out in the suburbs of Tripoli, Libya, during the spring of 2009 to illustrate the antagonistic effect and mode of actions of these three bioagents against *Macrophomina phaseolina* and *Colletotrichum fragariae* isolated from strawberry plants.The antagonistic effects of the different bioagents against pathogens as well as the interactions between them were evaluated in-vitro in dual culture and illustrated by scanning electron microscope (SEM) technology . *B. subtilis* (B1), *S. canescens* and *T.harzianum* showed antagonistic effect when used against *C. fragariae* and gave 73.7, 72.5, and 65.0 % of inhibition, respectively. SEM showed that *T. harzianum* act through several mechanisms such as hyperparasitism causing destruction the mycelium of the pathogens. Also a malformation and lysis of hyphae were observed in the mycelium of *M. phaseolina* and *C. fragariae* due to different antibiotics and enzymes produced by *B. subtilis* and *S. canescens*.

Keywords: Antagonistic effect ,*Trichoderma harzianum*, *Bacillus subtilis*, *Streptomyces canescens*, *Macrophomina phaseolina*, *Colletotrichum fragariae*, Scanning electron microscope.

Introduction:

Strawberry (*Fragaria* × *ananassa* Duch.) is one of the most popular fruits throughout the world and contains several important components, it is high in minerals; vitamins and macronutrients,and beneficial dietary compounds . Strawberry is an important crop worldwide because of its commercial value as well. Thus, increases in production and quality are required. Cultivated strawberries are now a commercially important fruit around the world and are also very popular in Libya. Strawberry production has increased over the resent years Nagamatsu *et al*., (2021); (Bianco *et al*., 2009).

Strawberry attacked by several soil-borne pathogens causing charcoal root-rot and crown rot diseasesas caused by *Macrophomina phaseolina*(Hutton et al.,2013)and *Colletotricum fragariae* (Smith, 1998) resnectvely. Crowns and roots of affected plants appeared dark, external and internal browning tissue with dark, oblong, subepidermal sclerotia.These pathogens causing high economic losses and high expensive control measures.Using chemical fungicides to control root rot pathogens leads to the toxic effect on animals and human health and causes environmental pollution problems and also the destruction of the biological communities (Karuppiah and Rajaram, 2011). Hence biological control methods are used widely as alternatives to chemical fungicides for controlling plant

pathogens (Juliatti *et al*.,2019). Biocontrol agents such as Trichoderma sp; Bacillus subtilis and Streptomyces sp. have been successfully and extensively used for the biocontrol of plant root rot diseases (Cawoy *et al*., 2011; Naher *et al*., 2014). The mechanism of actions of these antagonistic organisms may include mycoparasitism, production of secondary metabolites and antibiotics, competition for space and nutrients, and induction of defense responses including systemic resistance responses in the plant (Howell, 2003; Benítez *et al*., 2004; Compant *et al*., 2005). These microorganisms have been found to be highly effective biological control agents against root rot pathogens. (Heimpel and Mills, 2017).The present study aims to figure out the antagonistic effect and the mode of action of *Trichoderma harzianum*, *Bacillus subtilis*, and *Streptomyces* sp. towards *Macrophomina phaseolina* and *Colletotricum fragariae*

Materials And Methods

Isolation; purification and identification of root rots pathogens

Small pieces of the infected strawberry plant roots were cut longitidually with about of 3 cm long, washed, air-dried, surface-sterilized in 1% sodium hypochlorite solution for 3 minutes, twice times with sterilized distilled water, and dried between two sterilized filter papers. The sterilized root fragments were transferred to plates containing Potato Dextrose Agar(PDA) medium (Tuite,1969). Plates were incubated at 25ºC for 4-5 days. The developed mycelial growth was picked up and transferred onto a new PDA medium. Purification of each isolated fungus was carried out using the hyphal tip technique (Brown, 1924 and Hawker, 1956). Identification of the isolated fungi was carried out according to their cultural and morphological characteristics and was confirmed based on (Gilman, 1957; Barnett and Hunter 1987 ; and Singh 1982). The identified isolates were maintained on Gliotoxin Fermintaion medium (GFA) slants and kept in a refrigerator at 5 ºC for further studies.

Isolation, purification, and identification of biological microorganisms:

Isolation of Trichoderma spp:

Different Trichoderma strains were isolated from rhizosphere of healthy strawberry plants using serial dilution plate technique (Johnson and Curl 1972) on Rose Bengal Streptomycin medium(Johnson *et al*., 1960).Hyphal tips from developed colonies were transferred to (GFA) plates medium (Brian and Hemming 1945).Trichoderma species were identified according to their morphological and microscopic characteristics according to (Rifai, 1969) and stored in the refrigerator at 2ºC for further studies.

Isolation of *Bacillus* spp:

Soil samples (ten grams) from rhizosphere of healthy strawberry plants were heat-treated (80 °C) for 3 mins then transferred to 90 ml sterile distilled water and shaking the flask on a rotatory shaker for 5 min. Such suspension was subjected to serial dilutions of 10-2 to 10-6. One ml of each dilution was added to sterilized Petri dishes which were supplemented with about 10 ml of melted and cooled soil extract agar medium, and incubated at 30ºC (Abd-El-Moity, 1976). Three plates were used as replicates for each dilution. After 48 hours, the plates were examined. The separated, rough, and abundant colonies with waxy growth (1- 4 mm diameter) and irregular spreading edge were transferred to Nutrient Glucose Agar medium (NGA) (Dowson, 1957) for purification and identification. The identification was carried out according to cultural morphological and physiological characteristic, (Schleifer, 2009).

Isolation of actinomycetes:

Actinomycetes were isolated from rhizosphere of healthy strawberry plants using method of plate dilution technique (Abd El-Moity, 1976)on Starch Casein Agar (SCA) medium ((Kumar and Jadeja, (2016) and Njenga *et al*., (2017).

Antagonistic effect of different bioagents against *Macrophomina phaseolina* and *Colletotricum fragariae*:

Screening of the isolated *Bacillus subtilis*, *Streptomyces canescens* and *Trichoderma harzianum*. for their antagonistic capacities against *Macrophomina phaseolina* and *Colletotricum fragariae* were carried out using the dual culture plate method (Siddiqui *et al*., 2001). A loop full of 48 hrs old culture of B. subtilis or *S. canescens* grown on nutrient glucose broth was streaked on one side of a petri dish contained Nutrient Glucose Agar (NGA) medium. *T. harzianum*. isolate, A disc (0.5 cm in diameter) of 4 days old culture was inoculated at one side of a petriish contained GFA medium. The other side of each plate was inoculated with a disk (0.5 cm in diameter) of any of the tested pathogens taken from the periphery of 4 days-old colonygrown on Gliotoxin Fermentation Agar (GFM) agar medium. Plates inoculated only with one pathogen were used as a control treatment.Three replicates were used for each treatment. The plates were incubated at 25ºC and the inhibition zone (if any) was recorded when the pathogenic mycelium covered all the medium surface of the control plates. The percentage of reduction in the mycelial growth of the pathogenic fungi was calculated as follows:

%reduction of linear growth = 100 - [G2 / G1 x 100] suggested by(Abdel-Fattah *et al*., 2007).

Where;

G1= the growth diameter of the pathogenic fungus in the check plates (mm) and
G2= the growth diameter of the pathogenic fungus in treated plates (mm).

Dual culture slide technique for light and Scanning electron microscope to study the interaction between biological microorganisms and fungal pathogens:

For elucidating the modes of action of the biocontrol agents, a dual culture slide technique was carried out. This technique provided a clear view of any malformation in the mycelial growth of the pathogen (Bhat, 2017). A sterilized microscopic glass slide was covered by a thin film of diluted NGA medium (1: 10) under sterilized conditions. Diluted medium was used to decrease the fungal growth to facilitate its microscopic examination for any malformation in the *M. phaseolina* and *C. fragariae* mycelia. *B. subtilis* or S. *canescens* wasstreaked at one side of the slide whereas the pathogen was inoculated on the other side of the slide.*Trichoderma harzianum*. also was grown on a dual culture slides against *M. Phaseolina* and *C. fragariae*.

All inoculated slides were placed in sterilized Petri dishes contained two saturated filter papers with 10 ml of sterilized distilled water. All plates were incubated at 25 °C for 3 days. Discs from the contact area were taken from all slides to be examined under the scanning electron microscope (SEM) and to photograph any malformation or lysis at the National Research Center (NRC).

Statistical analysis:

Obtained data were statistically analyzed according to the standard procedures including one-way ANOVA according to Steel and Torrie (1981). COSTAT version 6.311 was used. Significant treatment differences were evaluated by using Duncan's multiple-range test (P=0.05).

Results and Discussion

Identification of the antagonistic bioagents:

The antagonistic microorganisms *Trichoderma harzianum,Bacillus subtilis* and *Streptomyces canescens* were identified according to their morphological and physiological characters (Rifai, 1969; Schleifer, 2009; Kumar and Jadeja, 2016 and Njenga *et al*., 2017).

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Antagonistic effect of different bioagents against *M. phaseolina* and *C. fragariae*:

Laboratory experiment was carried out to illustrate the ability of different antagonists to inhibit the pathogenic fungi.

All tested bioagents caused clear significant inhibition for mycelial growth of pathogenic fungi Table (1). Different antagonists showed different degrees of inhibition against different pathogens, *B. subtilis* (B1) and *S. canescens* showed the highest antagonistic effect when used against *C.fragariae* compared to *T.harzianum*. This can be explained in the light microscope of fact that *B.subtilis* produces different antibiotics such as subtilin, bacilysin, mycobacillisyn, and iturin which inhibit the pathogen growth (Kilian *et al*.,2000;Yoshida *et al*., 2001;Compant *et al*., 2005 and Živković, *et al*., 2010).The antagonistic activity of *S. canescens* is usually related to the production of extracellular hydrolytic enzymes and secondary antifungal metabolites (Priya *et al*.,2014). *T. harzianum* occupied third degree, after *B.subtilis* and *S.canescens*. when antagonistic effect against *C. fragariae* was considered.

T.harzianum gave the highest inhibition reduction (60.0%) compared with *B.subtilis* which gave (53.7%) when *M. phaseolina* was considered .The least effective antagonist was *S. canescens* as only (47.5%) inhibition were recorded against *M. phaseolina*. *T. harzianum* acts through different mode of actions as mycoparasitism, production of cell wall degrading enzymes, fast growing, prolific producers of spores and powerful mycotoxin producers and competition for nutrients. Similar observations were reported by (Aryantha and Guest, 2006; Woo *et al*., 2006; Živković, *et al*., 2010 and Gajera *et al*., 2012).

- Hyphal interaction between different bioagents against pathogenic fungi:

As observed by the light and SEM analysis, *T. harzianum* was found growing and coiling around the hyphal structure of *M. phaseolina* and *C. fragariae*. It is clear that *T. harzianum* antagonizes the pathogens by a process of hyperparasitism or mycoparasitism which involves the production of enzymes and secondary metabolites. Also, specific compounds such as chitinolytic enzymes can degrade the pathogen's cell walls and inhibit the production and release of active compounds by the pathogens (Fig. 1, 2). Other authors have referred this effect to the fact that *T. harzianum* produces different antibiotics and enzymes as chitinase, glucanase, protease and cellulose, where they degrade pathogen cell wall and play role in myoparasitism (Papavizas and Lumsden, 1980; Elad *et al*., 1982; Lorito *et al*., 1993; Lahsen *et al*., 2001; Howell, 2003;Khaledi and Taheri 2016).

The nature of antagonism involved in the interaction between *B.subtilis* or *S. canescens*. and *C. fragariae* or *M. phaseolina* was also studied by SEM. A clear distortion of fungal mycelium following lysis and destruction of the hyphae was observed at the interaction zonein dual cultures (Fig.3). The burst sites were surrounded bybacteria, some spores were found to be attached to the hyphae.Similar observation was noticed by Basha and Ulaganathan (2002) that mycelial deformations caused by

Bacillus sp. strain BC121.Some other studies reported that enzymatic dissolution of cell walls leads to loss of fungal protoplasm is one of the mainantagonistic mechanisms involved in the activity of biocontrol agents (Lim et al., 1991;Kilian et al., 2000; Kim and Chung, 2004). *B. subtilis* has been reported to produce several types of antibiotics,mycolytic enzymes, chitinase, glucanase that probably degrade the components of fungal cell wall such as chitin. (Chaiharn *et al*., 2008 and Kumar *et al*., 2012). SEM showed that *T. harzianum* act through several mechanisms such as hyperparasitism, inhibition and antibiosis. Also a malformation, lysis and destruction of hyphae was observed in the mycelium of *M. phaseolina* and *C. fragariae* due to different antibiotics and enzymes produced by *B. subtilis* and *S. canescens*.

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Table (1): Antagonistic effect of different bioagents against Macrophomina phaseolina and Colletotricum fragariae:

Bioagents	Reduction in linear mycelial growth (%)	
	Macrophomina phaseolina	*Colletotricum fragariae*
Trichoderma harzianum	60.0a*	65.0b
***Bacillus subtilis* (B1)**	53.7b	73.7a
Streptomyces canescens	47.5c	72.5a
Control	0.0d	0.0c

***Each value represents the mean of 3 replicates and means with the same letters, in each column, are not significantly different according to Duncan's multiple range test (P=0.05).**

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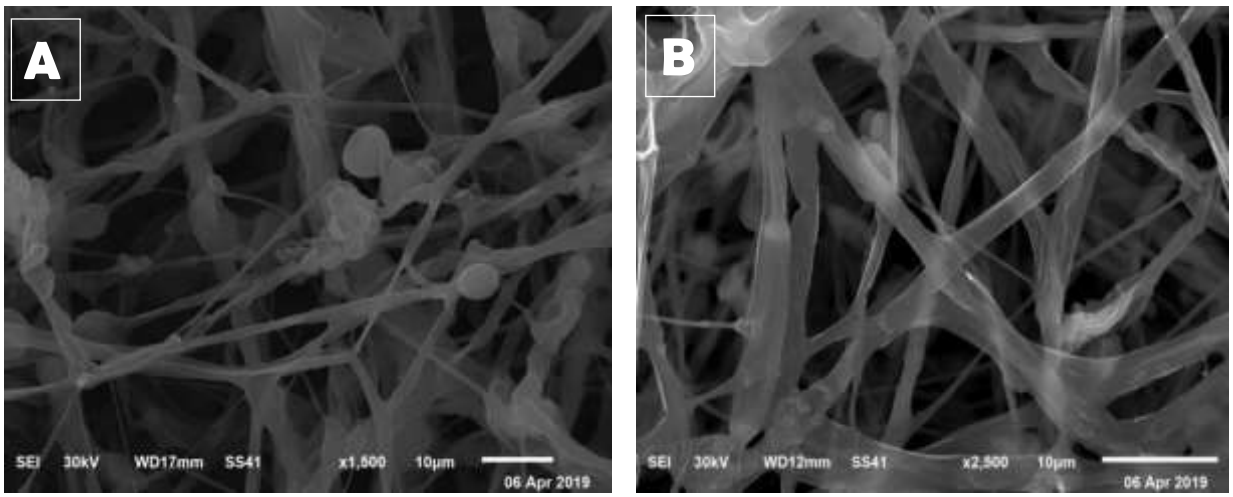

Fig.(1):(A)Mycoparasitism by massive envelopment of the hyphae. Disintegration of the pathogen's mycelium by sporulation of *T.harzianum* over the hyphae.

(B)Normal mycelium of *C. fragariae*.

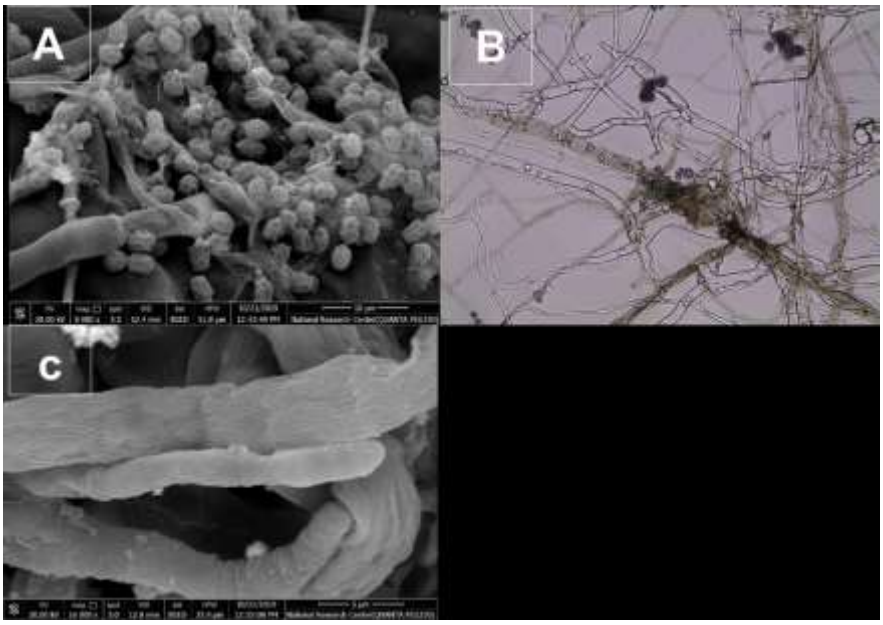

Fig. (2):(A)The presence of *T. harzianum* spores over the mycelium of the pathogen *M.phaseolina*. (B) interaction between *T. harzianum* and *M. phaseolina* under light microscope. (C) Normal mycelium of *M. phaseolina*.

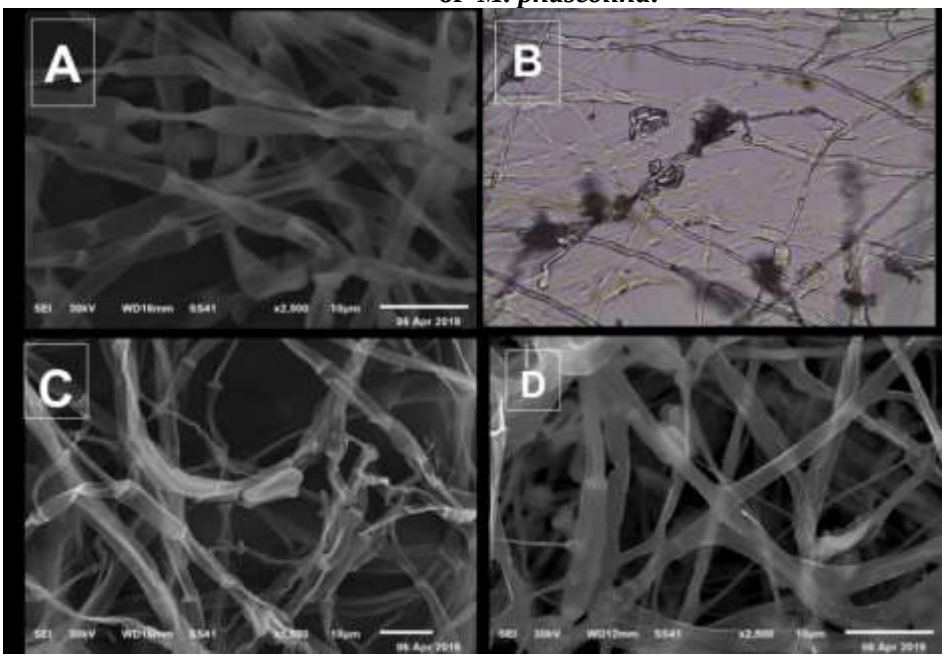

Fig. (3):(A)Scanning electron microscope (SEM) showed malformation and curling in themycelium ofthe *C.fragariae* due to the effect of *B. subtilis*. (B) lysis and malformation of *C. fragariae* under light microscope(C) Curling, deformation, and vacuolization, ending with lysis and destruction of the fungal mycelial due to effect of *S. canescens*.Normal mycelium of C. fragariae(D).

Conclusion:

Strawberry attacked by several soil-borne pathogens caused by *Macrophomina phaseolina* and *Colletotricum fragariae*. Using chemical fungicides to control root rot pathogens leads to variety of effects on organisms and ecosystem. Hence biological control methods are used widely as alternatives to chemical fungicides for controlling plant pathogens. The antagonistic effects of the different bioagents used in this experiment against pathogens as well as the interactions between them were evaluated in-vitro in dual culture and illustrated by scanning electron microscope (SEM) technology. *B. subtilis*; *S. canescens* and T.harzianum showed antagonistic effect when used against

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التأثير المضاد للعوامل الحيوية المختلفة ضد اثنين من مسببات الأمراض المختلفة التي تنتقل عن طريق التربة لنباتات الفراولة

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الملخص:

تعرف عوامل المكافحة الحيوية *Trichoderma harzianum, Bacillus subtilis, Streptomyces canescens* بأنها عوامل فعالة للمكافحة الحيوية للعديد من مسببات الأمراض الفطرية التي تنتقل عن طريق التربة بما في ذلك *Macrophomina phaseolina, Colletotrichum fragariae* تم تنفيذ العمل الحالي لتوضيح التأثير المضاد وطريقة عمل هذه العوامل الحيوية الثلاثة ضد مسببات الأمراض وكذلك التفاعلات فيما بينها ضمن أطباق بتري في المختبر في زراعة مزدوجة وباستخدامتقنية المجهر الالكتروني SEM . أظهرت البكتريا *B.* subtilis (B1) و*S. canescens* و *T. harzianum* تأثيرا مضادا عند استخدامها لمكافحة *C.fragariae* وأعطت تثبيط بنسب 73.7 و 72.5 و 65.0٪ على التوالي. أظهر SEM أن *T. harzhanum* يعمل من خلال عدة أليات مثل التطفل المفرط مما يتسبب في تدمير الفطريات المسببة للامراض، كما لوحظ تشوه وتحلل في الخيوط الفطرية لفطريات *M. phaseolina* و *C. fragariae*كنتيجة لمضادات حيوية وانزيمات مختلفة انتجت بواسطة *B. subtilis* , *S. canescens*.

الكلمات المفتاحية: التأثير المضاد, *Trichoderma harzianum, Bacillus subtilis, Streptomyces canescens, Macrophomina phaseolina, Colletotrichum fragariae*, المجهر اللالكتروني الماسح.