BIOLOGICAL CONTROL OF FABA BEAN CHOCOLATE SPOT DISEASE CAUSED BY *BOTRYTIS FABAE* L.

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ABSTRACT

Faba bean choclate spot disease causes a considerable losses under Egyptian conditions. Isolations of microorganisms from healthy faba bean leaf surface resulted in various microbial isolates comprising bacteria (60%), fungi (35%) and yeast (5%). *In vitro* antagonistic screening of theses isolates resulted in the isolation of 25 different bacterial isolates and eight fungal isolates exhibiting obvious antibiosis against the one or the two isolates of the *Botrytis fabae* pathogen. The bacterial isolates with the code B6, B7, B9 B13 and B15, as well as the fungal isolates with the code T6, T7 and T8 as the most antagonistic bacterial and fungal isolates, respectively were chosen for the greenhouse and field experiments. All selected bacterial and fungal bioagents were identified as *Bacillus subtilis* and *Trichoderma harizanum*, respectively. Under greenhouse experiments, results indicated that from all antagonistic selective isolates, the bacterial filtrates were more effective than fungal filtrates when it used directly for controlling the disease. However; the filtrate of B6 induced host resistance toward infection with the pathogen.

Plants treated with filtrate of B6 had a significant increment in the activity of peroxidase compared with the untreated plants. The activity of peroxidase has increased after the inoculation of the pathogen; however the activity of polyphenoloxidase did not change significantly in all treatments.

Natural chocolate spot infection was very high (65.0-70.0%) in both seasons of the study (2003-2004 & 2004-2005). Fungicide treatment proved its efficiency in inhibiting the disease severity to 13-15.9% in comparison with the control which was 65-70% for the both seasons respectively. While the yield was recorded 1650.3-1501.3 kg/fe in comparison with the control which was 1009.2-989.2 kg/fe for the both seasons respectively. Under the field conditions, the efficacy of the biocontrol agents (B6, B13, B15, T6) showed satisfactory control to *B. fabae* , as well as in increase the faba bean yield in comparison with the Benomyle fungicide treatment in both seasons.

Keywords: faba bean, *Botrytis fabae*, biological control, induced resistant, bioagents, *Bacillus subtilis*, *Trichoderma harizanum*, Benomyle .

INTRODUCTION

Faba bean (*Vicia faba* L.) is one of the important leguminous crops in Egypt. It is grown for human consumption due to its high protein content in seeds (about 25%) and total carbohydrates content (about 48%) (El-Noemani *et al.*, 1990). The acreage and production of faba bean in Egypt is high (303000 feddan, 426000 metric ton, respectively in 2005) as any other country in the mediterranean region, (FAO, 2006).

Several diseases attack faba bean, causing significant loss in the yield, however chocolate spot is considered a major one which caused by *Botrytis fabae* (Sardina) and *B. cinerea* (pers. ex. sr.). The severe infection might cause 40-50% loss in the total yield (Mansour and Amer, 1976)

The problem of adequately protecting plants against the fungi by using fungicides became more and more controversial by the development of fungicidal resistance and/or potential undesirable environmental effects on human, host plant and accompanied microflora. Therefore. biological control lend itself as a suitable alternative of chemical control.

Mechanisms of biological control against plant pathogens fall into four broad categories, none of which is mutually exclusive: pre-emptive competitive exclusion (competition); antagonism by antibiosis; hyphal interference (mycoparasitism); and induction of host resistance. (Whipps, 1986; Deacon & Berry, 1993; and Kohl & Fokkema, 1998).

Induced resistance is defined as an enhancement of the plant's defensive capacity against a broad spectrum of pathogens, which is acquired after appropriate stimulation (Hammerschmidt & Kuc, 1995). In 1991, an alternative approach to inducing systemic resistance was reported by several investigators (Alström, 1991; Van Peer *et al.*, 1998; and Wei *et al.*, 1991). These authors independently showed that selected strains of nonpathogenic microrganisms were able to elevate plant resistance. Induced systemic resistance (ISR) mediated by nonpathogenic or biocontrol agents, especially *Trichoderma* spp., *Pseudomonas* spp and *Bacillus* spp., has been demonstrated in some plant species (Kloepper *et al.*, 1992; Pieterse *et al.*, 1996; Van Loon *et al.*, 1998; Benítez *et al.*, 2004; and Kloepper *et al.*, 2004).

Therefore, the present work was designed to investigate the potential of bioagents to reduce chocolate spot disease caused by *Botrytis fabae*, their possibility to induce resistance and estimate their effect on yield of faba bean.

MATERIALS AND METHODS

Isolation of *Botrytis fabae* and the preparation of the inocula

Faba bean leaves with typical symptoms of chocolate spot were cut to small portions (0.5-1.0 cm^2) of tissue from the advancing margin of a lesion. Portions were surface sterilized by immersing in 0.25 % sodium hypocoloride solution for 2 minutes, then washed several times with sterilized water and plotted between two sterilized filter paper. The portions were put in faba bean dextrose agar (FDA) on 20 ±3 °C. Purification of the isolates were carried out by single spore technique. The causal pathogen were identified using the key of imperfect fungi according to Barnett and Barry (1972). For the antagonistic test, the most two virulent isolates were used coding with the numbers 5 and 9 from the different collected isolates (un published data).

In vivo (Under greenhouse conditions), isolate number 9 was used. For spore production, we exposed the pathogen culture in the Petri plates to 12 h. light (using 40W fluorescent tube) and 12 h. darkness for 3 days, then plates were incubated in completely darkness up to 11 days old.

In vitro,

Isolation of phylloplane microorganisms and selection of the antagonistic agents.

Leaves of Broad bean (*Vicia faba* L.) collected from different localities of Sakha, Kafr El-Sheikh governorate were used. 10g of leaves were cut and putt in conical flask containing 90ml of sterilized tap water plus one drop of Tween 20, under septic condition. Aliquots (0.1 ml) from serial of dilutions (10^{-1} up to 10^{-6}) were spreaded on the surface of Potato dextrose agar medium (PDA) and Nutrient agar medium (NA) by using Driglasky glass triangle. Plates were incubated at 20 ±3°C for four days until arising a maximum numbers of bacterial and fungal colonies. The used purification techniques were based on isolation of a single spore or hyphal-tip for fungi and single colony for the other microorganisms on agar media.

To determine the relative power of antibiosis (RPA) for bacterial biocontrol agents, pure isolates from healthy faba bean leaves surface were subjected to standard test against virulent isolates (No. 5 and 9) of *B. fabae* as follows: PDA medium was poured into 9 cm-dim Petri-dishes with 15ml/dish. After solidification each plate was inoculated in the center with a disk (6 mm in diameter) from three days old cultures of *B. fabae* and plates were simultaneously inoculated at the periphery by standard amounts of the bacterial growth (4 isolates/dish). Plates not inoculated with antagonists were used as control. Experiments were made in three replicates. Plates were incubated at 20 ±3 °C until full growth of control treatments. Diameter of inhibition zone surrounding each antagonistic isolate was recorded, and (RPA) of each isolate was estimated as described by Ibrahim *et al.*, (1987). While for fungal biocontrol agents, a disk bearing mycelium of *B. fabae* was placed in the center of plate containing PDA medium and at periphery, similar disks (2-3/dish) of potential antagonists were placed. Antagonistic efficiency was carried out as described by Bell *et al.*, (1982).

The efficient antagonist was identified according to Rifai, (1969), Parry *et al.*, (1983) and Bergey's Manual of Systematic Bacteriology (1984). The best eight antagonistic isolates (five bacteria and three fungi) were individually re-cultured in nutrient broth for bacteria and potato dextrose broth for fungi up to 10 days in shaking incubator. Cultures were adjusted to 10^8 cfu/ml for bacteria as well as 10^7 condia/ml individually. Cultures were divided into 2 groups, the first group was unfiltered and used for the open field, while the second group was filtrated by bacterial sterilized filter (0.22 µm) to avoid the cells from their filtrates which used only in a greenhouse.

Evaluation of antagonism against *B. fabae* under a greenhouse

Seeds of faba bean cv. Misr 1, which is susceptible to chocolate spot disease were sown in 15 cm pots. Four replicates per each treatment were carried out. Plants at the third leaf stage, were treated carefully by brushing the filtrates of the bioagents on the first leaf. One, four, and eight days later, plants were inoculated by spraying suspension of *B. fabae* (10^5 condia/ml) on whole plants. Control treatment was sprayed with water instead of the filtrates. Plants were incubated after inoculation for 12 h in complete darkness at 20 ±5°C and 100% relative humidity by ultrasonic-humidification unit, then transferred to a greenhouse at 20 ± 5°C and with 80-85 % relative

humidity in the first 3 days. This experiments were repeated three times.

Same experiment steps were carried out with only filtrate of the B6 bioagent isolate. However, plants were only inoculated after 8 days. Both untreated and treated plants by the filtrate of B6 were divided into two groups, the first was inoculated and the second was not inoculated. Second and third leaf were used in analysis of peroxidase and polyphenoloxidase enzyme activities at 7 days after inoculation.

Evaluation of the selected antagonistic bioagents under field conditions

Two field experiments were conducted at the experimental farm, Faculty of Agriculture, Kafr El-Sheikh, Tanta University, Egypt during the two successive seasons of 2003/2004 and 2004/2005. Seeds of faba bean cultivar Misr 1 were sown in plots in four rows, 50 cm apart by 1 m long. Seeds were planted at 25cm distance, 2 seeds per hill. Cultural irrigation and fertilization were carried out as recommended in program of production improvement of faba bean crop by the ministry of agriculture and land reclamation of Egypt.

Treatments were: the best selective bioagents, benomyle fungicide [methyl 1-(butylcarbomyl) benzimidazol-2-ylcarbamate] at 400g/fe., and untreated plot as a control treatment. All treatments were arranged in five randomized blocks. Treatments were applied in four times, 15 days intervals starting from med February in the two seasons by hand

Diseases assessments

For the greenhouse experiments, chocolate spot disease was assessed by the determination of the diameter of spots in mm 7 days after inoculation (Mansfield & Deveall, 1974 and Mohamed *et al.*, 1994).

For the field experiments, chocolate spot disease was assessed as percentage of leaf lamina affected. Twenty five plants from each replicate were determined. The assessment was carried out at 7 April in the two seasons.

Enzymes extraction and assay

One gram leaves was frozen in liquid nitrogen, ground in precooled mortar and pestle, suspended in 10.0 ml of precooled (4°C) 0.1 **M** potassium phosphate buffer (pH 7.1). The homogenates were centrifuged at 10 000 xg for 20 min at 4°C. The clear supernatant was collected and considered as crude enzyme extract. Peroxidase activity was determined according to Allam & Hollis (1972). by measuring the oxidation of pyrogallol to pyrogalline in the presence of hydrogen peroxide. Peroxidase activity was measured following the change in absorbance at 425 nm every 15s up to 90s. Polyphenoloxidase (PPO) was determined according to Maxwell & Batman (1976). The change in absorbance was following spectrophotometrically measured at 495 nm, and recorded every 30s up to 120s. All measurements were assayed by using Beckman Spectrophotometer Du® 7400.

Statistical analysis:

Data were subjected to analysis of variance using SPSS Computer Program. Means were compared using Duncan's range test (Duncan, 1954).

RESULTS

Isolation trials carried out on faba bean leaves with chocolate spots which were collected from many locations in Kafr El-Sheikh governorate resulted in isolation of different isolates of *B. fabae* and *B. cinerea* fungi. The virulence of the fungal isolates were tested in a pathogencity test (un published data). The most virulent isolates (number 5 and 9 from *B. fabae*) were selected to be in this study.

Isolations of microorganisms from healthy faba bean leaf surfaces resulted in various microbial isolates comprising bacteria (60%), fungi (35%) and yeasts (5%). The *in vitro* screening of theses isolates resulted in the isolation of 25 different bacterial isolates and eight fungal isolates exhibited obvious antibiosis against either one or both isolates of *B. fabae*. All selective bacterial and fungal bioagents were identified as *Bacillus subtilis* and *Trichoderma harzianum*, respectively.

Data in Table (1) show the efficiency of each of the selected antagonistic bacterial isolates against two isolates of *B. fabae*. Some of these antagonistic isolates had a limited inhibitory spectrum to one

pathogen isolate (i.e B11 and B14 which inhibited the isolate number 5 of the pathogen and not the isolate number 9 and the reverse for B2 which inhibited the isolate number 9 of the pathogen and not the isolate number 5). The most antagonistic bacteria to the pathogen isolate number 5 were B3 ,B6 ,B7 ,B9, B13 and B15. However; for the other isolate of the pathogen the most antagonistic bacteria were B1, B6, B7, B9 ,B13 ,B15 and B18. For the greenhouse and field experiments B6, B7, B9 B13 and B15 were selected as the most antagonistic isolates for both pathogen isolates.

Table (1)|: Relative power of antibiosis (RPA) of different isolates of *Bacillus subtilis* against two virulent isolates of *Botrytis fabae* which cause chocolate spot disease

Code No. of *Bacillus subtilis* isolates	Relative power of antibiosis (RPA*).	
	Isolate No. 5 of *B. fabae*	Isolate No. 9 of *B. fabae*
B1	1.10 ij	2.51 b
B2	0.00 l	1.22 efg
B3	2.30 cd	1.14 fgh
B4	1.45 f	1.14 fgh
B5	1.05 k	1.20 efgh
B6	2.62 a	2.71 a
B7	2.10 e	2.40 b
B8	1.37 fg	1.04 h
B9	2.52 b	2.10 c
B10	1.06 k	1.20 efgh
B11	1.05 k	0.00 i
B12	1.11 ij	1.21 efgh
B13	2.23 d	2.40 b
B14	1.21 hi	0.00 i
B15	2.36 c	2.56 ab
B16	1.01 k	1.19 efgh
B17	1.04 k	1.25 def
B18	1.01 k	2.43 b
B19	1.40 gh	1.40 d
B20	1.10 ij	1.05 gh
B21	1.03 k	1.17 fgh
B22	1.01 k	1.35 de
B23	1.20 hi	1.40 d
B24	1.21 hi	1.29 def
B25	1.15 ij	1.21 efgh

*Data presented are the ratio between the diameter of inhibition zone / diameter of spotted antagonistic isolate. In the same column. Means followed by the same letter are not significantly different according to Duncan multiple range test P≤0.05.

Data in table (2) show the efficiency of each of the selected antagonistic fungal isolates against the two isolates of *B. fabae*. The most antagonistic fungi to the pathogen were T6, T7 and T8. that used in a greenhouse and field experiments.

Table (2) Efficiency of *Trichoderma harzianum* isolates against the causal pathogen of *B. fabae* which causes chocolate spot disease

Code No. of *Trichoderma harzianum* isolates	Antagonistic efficiency*	
	Isolate No. 5 of *B. fabae*	Isolate No. 5 of *B. fabae*
T1	3	4
T2	4	4
T3	4	5
T4	4	5
T5	4	4
T6	1	1
T7	2	1
T8	2	2

*Antagonistic efficiency was carried out as the scale of Bell *et. al.*, (1982), where: 1= Antagonist completely over grow the pathogen and covered the entire medium surface. 2= Antagonist over grow at least two thirds of the medium surface, 3= antagonist and the pathogen each colonized one-half of the medium, 4= The pathogen colonized at least two-thirds of the medium surface, and 5= The pathogen completely over grew the antagonist.

F ficacy of the selected bioagents on leaf spots diameter

Data in table (3) indicate that the application of any filtrates led to a significant reduction on the leaf spot diameter of the first leaf. Such results occurred when the applications were done one or four days before the inoculations by the pathogens. The most effective treatment was the filtrate of B9, which the leaf spot reduced to 20,3 and 19.9 mm on the first leaf compared to untreated plants (40.0 and 39.0 mm) at 1 and 4 days, respectively. In general, the bacterial filtrates were more effective than fungal filtrates in all treatments. Only bacterial filtrates were effective when the inoculation was after 8 days which indicated that the bacterial filtrates were more preciste than the fungal filtrates.

Data also show, from the third leaves (not treated with the filtrates), that all treatments were not effective on the leaf spots diameter except the filtrate for the isolate B6. Which was not effective if it is applied 1 day before the inoculations, however its effect started (30.9 mm) compared with the control (38.6 mm) when it is applied 4

days before the inoculation. It's effect became more clear (25.9 mm) compared with the control (40.8 mm) when it is applied 8 days before inoculations.

Table (3) Efficiency of filtrates for *Bacillus subtilis* and *Trichoderma harzianum* isolates against *Botrytis fabae*, the causal agent of chocolate spot disease under greenhouse conditions.

Code No. of bacterial and fungal isolates	Leaf spots diameter (mm) after filtrate treatments.					
	First leaf			Third leaf		
	Treatments of filtrates at different times before inoculation					
	1 days	4 days	8 days	1 days	4 days	8 days
B. subtilis						
B 6	20.7 f	21.4 g	29.3 c	40.2 ab	30.9 b	25.9 b
B 7	20.5 f	28.9 d	30.5 c	39.5 bc	38.5 a	38.9 a
B 9	20.3 h	19.9 i	30.3 c	42.2 a	40.3 a	41.0 a
B 13	22.0 e	21.2 h	30.3 c	38.9 bc	39.7 a	38.8 a
B 15	21.9 e	27.9 e	30.1 c	37.3 c	37.8 a	40.0 a
T. harzianum						
T 6	30.9 b	29.8 c	37.2 b	39.2 bc	41.1 a	38.9 a
T 7	25.8 d	26.9 f	38.4 ab	40.6 ab	39.9 a	39.1 a
T 8	29.0 c	30.0 b	38.9 ab	39.6 bc	43 0 a	40.0 a
Control	40.0 a	39.3 a	41.0 a	39.5 bc	38.6 a	40.8 a

In the same column. Means followed by the same letter are not significantly different according to Duncan multiple range test $P \leq 0.05$

The activity of peroxidase (POX) and polyphenoloxidase (PPO) were determined after 7 days of the inoculation. Data in table (4) show that inoculated plants (untreated and treated with B6) had a significant increment in the activity of POX compared to non-pathogen inoculated plants (untreated and treated with B6 filtrate) respectively. Treated plants with B6 filtrate increased the activity of POX especially in treated and inoculated plants (0.489) compared with untreated and non-inoculated plants (0.221). The activity of polyphenoloxidase did not show any significant different among treatments.

Table (4) The effect of *Bacillus subtilis* filtrate of isolate B6 on the activity of peroxidase (POX) and polyphenoloxidase (PPO) of faba bean plants whether inoculated or not with *Botrytis fabae*

Treatments		POX activity*	PPO activity**
Untreated	Non-inoculated	0.221 c	0.086 b
	Inoculated	0.315 b	0.084 b
Treated with B6 filtrate	Non-inoculated	0.344 b	0.089 b
	Inoculated	0.489 a	0.091 b

*Data presented are enzymes activity as Δ_{abs}/1.0 min/0.01 g fresh wt.
** Data presented are enzymes activity as Δ_{abs}/1.0 min/0.1 g fresh wt.
In the same column. Means followed by the same letter are not significantly different according to Duncan multiple range test $P \leq 0.05$

Biological control of chocolate spot disease under field conditions

The effect of the selected bioagents (B6, B7, B9, B13, B15, T6, T7 and T8) on the disease severity and yield were conducted under field conditions in two seasons (2003-2004 and 2005-2006).

Data in table (5) show that fungicide treatment proved its efficiency in inhibiting the disease severity to 13-15.9% in comparison with the control which was 65-70% for the both season respectively. While the yield was recorded 1650.3-1501.3 kg/fe in comparison with the control which was 1009.2-989.2 kg/fe for the both seasons respectively.

Data in table (5) also show that the biocontrol agents (B6, B13, B15, T6) demonstrated their efficacy in controlling *B. fabae* and in increasing the yield as compared with the fungicide treatment in the two seasons. The biocontrol agent B6 was the best effective one and the T6 was the less one. However, B7, B9, T7 and T8 had no effect on disease severity under the field conditions.

Table (5) Effect of *Bacillus subtilis* and *Trichoderma harzianum* isolates on faba bean yield and chocolate spot disease severity.

Code No. of bacterial and fungal isolates	Season 2003/2004		Season 2004/2005	
	Yield Kg/Fe	% leaf area infected	Yield Kg/Fe	% leaf area infected
B. subtilis				
B6	1509.4 ab	9.3 d	1450.3 ab	10.1 e
B7	1096.2 c	64.7 b	979.6 d	70.2 a
B9	1009.3 c	69.1 ab	997.0 d	65.8 b
B13	1500.0 ab	14.1 c	1123.1 c	20.7 c
B15	1559.2 ab	13.4 cd	1456.9 ab	21.4 c
T. harzianum				
T6	1436.4 b	14.0 c	1369.3 b	23.0 c
T7	1100.6 c	70.0 a	900.4 d	68.8 ab
T8	959.8 c	66.9 ab	990.5 d	72.0 a
Fungicide	1650.3 a	13.0 b	1501.3 a	15.9 d
Control	1009.2 c	65.0 cd	989.2 d	70.0 a

In the same column. Means followed by the same letter are not significantly different according to Duncan multiple range test $P \leq 0.05$

DISCUSSION

The primary effect of a foliar disease is to cause loss of photosynthetic area for a particular time. Whether or not yield is affected depends on the size of this loss and on whether or not the period in which it was sustained was essential for the fulfillment of the yield potential set by other limitations (McEwen & Yeoman, 1990).

Several investigators had worked on controlling faba bean chocolate spot disease with fungicides (Eliott, 1980; Doto, 1980 and Giltrap, 1991). However their effect on the environment should be considered.

It has been established that modified cultural practices, the use of low seeding rates and the choice of the planting date to avoid extended periods of suitable conditions, rotating faba bean with non-host crops such as cereals, significantly reduce sclerotial amounts and chances of primary infections which can play an important role in reducing chocolate spot disease. (Harrison, 1979 and Hanounik & Hawtin, 1982). Breeding for resistance is the most adequate practices to

control strategy, but only moderate level of resistance are available in commercial cultivars, which make necessary the integration of several control strategies including biological control. (Dobson & Giltrap, 1991 and Jackson *et al.*, 1991)

Our results show that some isolates from *B. subtilis* and *T. harzianum* could be used, as the effect of fungicide Benomyle, in biological control of chocolate spot disease depending on their antibiosis. The best one from these biocontrol agents was B6 isolate of *B. subtilis*.

The effect of B6 filtrate was very interesting in this study which it had dual mechanisms. The first was depending on its antibiosis, however the second was depending on its effect as induced resistance. It was appear from the results in the greenhouse that B6 filtrate induce the resistance to the disease spread in the above leaves which were not treated directly by it. If this filtrate have a systemic biofungicide then the effect might be decreases by the time but the surprise results, revealed the contrast. Which the effects of the B6 filtrate on declining leaf spot by the time was increased. So, we think that the productions of related pathogenesis proteins (PRs) like oxidative enzymes might be responsible for such effect. Peroxidase activity results of this work support this idea.

The ability of *B. subtilis* and *T. harzianum* strains to protect plants against root pathogens has long been attributed to an antagonistic effect against the invasive pathogen. However, these microorganisms may also stimulate plant defensive mechanisms. At a molecular level, resistance results in an increase in the concentration of metabolites and enzymes related to defensive mechanisms, such as the enzymes phenyl-alanine ammonio-lyase (PAL) and chalcone synthase (CHS), involved in the biosynthesis of phytoalexins (HR response), chitinases, glucanases, peroxidase (POX) and polyphenoloxidase (PPO). These comprise pathogenesis-related proteins (PRs) (SAR response) and enzymes involved in the response to oxidative stress (Chet, *et al.*, 1997; Dana, *et al.*, 2001 and Howell 2003).

It could be concluded that *B. subtilis* isolate B6 proved to be the most effective antagonism. Such results are in agreement with Kloepper *et al.*, 2004. Therefore, this biocontrol agent could be a candidate for the commercial production to control chocolate spot disease on faba bean. Further study is necessary to find a suitable

formulation to optimize fermentation conditions to yield larg amounts of the bacteria as well as with its the best filtrate.

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الملخص العربي

المكافحة البيولوجية لمرض التبقع البنى فى الفول المتسبب عن (*Botrytis fabae* L.)
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يسبب مرض التبقع البني في الفول خسارة معنوية في المحصول .ولذلك فقد تمت هذه الدارسة بعزل العديد من الكائنات الحية الدقيقة من على اسطح اوراق الفول البلدى. وقد تم التعرف على العزلات المتحصل عليها وقسمت الى حوالي ٦٠% عزلات بكتيرية ، ٣٥% فطريات و ٥% خمائر .

وقد تم إجراء تجارب لدراسة مقدرة هذه العزلات على التضاد مع الفطر *Botrytis fabae* L. المسبب لمرض التبقع البني في الفول داخل المعمل. وأوضحت النتائج أن هناك ٢٥ عزلة بكتيرية بالإضافة إلى ٩ عزلات فطرية ذات قدرة على التضاد مع المسبب المرضي المذكور . ولقد كانت افضل العزلات من حيث التضاد تلك التى تحمل الكود B6 , B7 , B9 , B13 , B15 من البكتريا و T6, T7, T8 من الفطريات. وبالتالى تم اختيارهم لتكملة الدراسة سواء فى الصوبة أو فى الحقل وقد عرفت جميع عزلات البكتريا على أنها (*Bacillus subtilis*) وجميع عزلات الفطريات على أنها (*Trichoderma harizanum*).

وقد أوضحت نتائج الدراسة فى الصوبة أن راشح مزارع العزلات البكتيرية افضل من راشح العزلات الفطرية. ولقد تفردت العزلة البكتيرية ذات الكود B6 بأن راشح مزرعتها استحث مقاومة نبات الفول تجاه المسبب المرضى ، كما كان واضحا زيادة معنوية فى نشاط إنزيم البيروكسيد يز فى النباتات المعاملة براشح هذه العزلة بالمقارنة بالكنترول. كما أن نشاط إنزيم البيروكسيديز كان عاليا فى النباتات الملقحة بالمسبب المرضى *B. fabae* L . ولم يكن هناك فارق بين جميع المعاملات بالنسبة لإنزيم البولى فينول أوكسيديز .

أما تجارب الحقل فقد أوضحت أنه خلال موسمين زراعيين ناجحين (٢٠٠٣-٢٠٠٤/٢٠٠٤-٢٠٠٥م) بسبب الإصابة الطبيعية الشديدة لنباتات الفول لمرض التبقع البنى فى الفول .ولقد كان ذلك واضحا من خلال معاملة المبيد الفطرى (بينو ميل) التى خفضت نسبة الإصابة بالمرض من ٦٥,٠ – ٧٠,٠ % إلى ١٣,٠ – ١٥,٩ % وبالتالى زاد الانتاج من ١٠٠٩,٢ – ٩٨٩,٢ كجم/فدان إلى ١٦٥٠,٣ – ١٥٠١,٣ كجم/فدان فى الموسمين على التوالى . وتحت هذه الظروف فأن الكائنات المضادة ذات الكود (B9,B13,B15,T6) أوضحت كفاءتها فى مقاومة *B. fabae* وفى زيادة المحصول بالمقارنة بمعاملة المبيد خلال الموسمين .