



## Role of certain bioagents against Guava decline disease and in enhancement of the growth of guava trees

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### Abstract

Biocontrol agents, *Bacillus subtilis*, *Pseudomonas fluorescens* and *Trichoderma harzianum* were evaluated against fungi causing guava (*Psidium guajava*) decline disease caused by *Botryodiplodia theobromae*, *Fusarium oxysporum*, and *Rhizoctonia solani*. Generally, our results showed high antagonistic effects of tested biocontrol agents against previous pathogens. *T. harzianum* isolates showed an average of 58% inhibition against all pathogens. *T. harzianum* T4 was the most prominent isolate in inhibiting the growth of guava pathogenic fungi. Based on the secretion of volatile substances, T4 had the most significant inhibition ability as compared to other *Trichoderma* isolates. By using *B. subtilis*, the radial growth of *R. solani* and *F. oxysporum* was significantly decreased as compared to *B. theobromae*. In case of *P. fluorescens*, the radial growth of *R. solani* was decreased more than *B. theobromae* followed by *F. oxysporum*. Different densities of T4 significantly decreased the disease severity and increased plant height, dry weight of shoots and roots and total pigments (chlorophyll a and b, and carotenoids) in guava trees in comparison with infected trees only. We concluded that the application of biocontrol agents decreased guava decline disease and improved the growth of guava trees.

**Key words:** bioagents, Guava, decline disease, volatile compounds, *Trichoderma*, *Bacillus*, *Pseudomonas*.

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## Introduction

The reduction in the use of chemicals against plant pathogens became an inevitable task due to their severe risks on humans, animals and plants, in addition to polluting the environment. The promising strategies of managing plant pathogens include the use of microbial antagonists such as yeasts, fungi, and bacteria. The use of these antagonists against plant pathogens is a very important component in improving crop yields (Didwania et al., 2013; Droby, 2006). Disease suppression, as mediated by biocontrol agents, is the consequence of the interactions between the plant, pathogens and the microbial community (Sivan & Chet, 1986). Soil-borne plant pathogens, e.g. *Botryodiplodia theobromae*, *Fusarium oxysporum*, and *Rhizoctonia solani*, affecting agricultural plants can be controlled by using *Trichoderma* spp., *Bacillus subtilis* and *Pseudomonas fluorescence* (Anitha & Dass, 2011). *Trichoderma* spp. contains a wide range of soil-borne species (Bouregghda et al., 2008). It shows several antagonistic mechanisms towards pathogens which include: 1) competition for nutrients and space, 2) release and/or secretion of antibiotic compounds such as trichodermin, trichothecenes, trichorzianins, or gliotoxins; 3) exerting indirect toxic effects due to volatile compounds, and 4) having mycoparasitic capabilities (Chaube et al., 2003; Howell, 2002). The mycoparasitism is based on the relationship in which one fungus obtains directly or indirectly its nutrients by invading another fungus (Gao et al., 2005). Isolates of *T. harzianum* can produce lytic enzymes (Haran et al.,

1996), antifungal antibiotics (Almassi et al., 1991), they can also be competitors of fungal pathogens (Whipps, 1987), and promote plant growth (Inbar et al., 1994). *Trichoderma* spp. directly or using culture filtrate inhibit many fungal pathogens (Nawar, 2005; Abdel-Kader et al., 2002; Marchetti et al., 1992). Four species of *Trichoderma* (*T. harzianum*, *T. hamatum*, *T. viride* and *T. virens*), *Aspergillus niger*, and *Gliocladium virens* were used for biocontrol of guava wilt disease. Among these bioagents, *T. harzianum* worked out to be the best (Srivastava et al., 2009). *T. harzianum* showed best antagonistic effect for the control of guava decline disease complex which caused by *F. oxysporum* f. sp. *psidii*, *R. solani* and *B. theobromae* (Bokhari et al., 2008). Dwivedi et al. (2000) studied the activity of *T. harzianum* and *A. flavus* singly or in combination, against the soft rot disease of guava caused by *Rhizopus stolonifer*. The mycelial growth was inhibited by increasing the levels of antagonistic fungus. *B. subtilis* is stable in soil as spores, and this is advantageous for the use of this bacterium as a biocontrol agent mainly because of the spore stability and ease of handling (Kugler et al., 1990). The biocontrol of sugar beet damping-off diseases caused by *Pythium* spp. has been successfully applied using *B. subtilis* (Schmidt et al., 2004). In addition, *B. subtilis* used to be a bio-agent against many genera of plant pathogenic fungi (Karunya et al., 2011; Abo-Elnaga, 2006). Fluorescent *Pseudomonas* which has a great importance in nature is ever-present in agricultural soils and well adapted to growing in plants rhizosphere. It possesses many traits that make them

well suited as biocontrol and growth-promoting agents (Anitha & Dass, 2011; Weller, 2007). *P. fluorescens* isolates effectively controlled many plant fungal pathogens (Bryk et al., 2004; Kazempour, 2004). Different isolates of *P. fluorescens* produced salicylic acid with a different concentration in their culture media which is responsible for inducing resistance against different plant pathogens (Aly et al., 2002). Guava decline is one of the major fungal diseases threatening guava production in Egypt. There are many agricultural practices, e.g. chemical and biological ones can be used to manage this serious disease. The main objective of this study was to investigate the effect of different biological agents, including bacteria and fungi, on guava decline disease.

## Materials and methods

**Isolation and identification of pathogens and biocontrol agents:** Plant samples of guava trees showing typical decline symptoms were collected from 39 fields from Alexandria (3 counties), El-Behera (5 counties) and Matruh (1 county) governorates, Egypt. The plant samples included 300 from roots, 106 from main stems, 195 from branches, 218 from leaves and 146 from fruits. In addition, 980 samples were collected from the soil. The isolations of fungi from diseased roots, stems and leaves were carried out according to Ashour & Saber (2003). Biocontrol agents were recovered from guava roots and soil samples. Fungal cultures were then purified by either single spore isolation or hyphal tip techniques (Chohan et al., 2011). The purified fungal isolates were

identified to the genus level by using the morphological characteristics according to Soni & Sharma (2014) and Leslie & Summerell (2006). The pure cultures of the isolated fungi were kept on PDA slants at 4°C. The isolation and purification of bacterial bioagents were carried out according to Maleki *et al.* (2010) and Vijayalakshmi et al. (2012). The isolated bacteria were identified morphologically and biochemically according to Bergey's Manual of Systematic Bacteriology (Sneath & Halt; 1986). Koch's postulates were achieved for the recovered fungi isolated from diseased guava trees.

**Effect of fungal bioagents on radial growth of pathogens *in vitro*:** Mycelial discs (6 mm in diameter) obtained from actively growing colonies of the three pathogenic fungi and four *T. harzianum* isolates were used to conduct the dual culture tests. Each dual culture PDA plate was divided into two halves, one half contained a disc of a pathogenic fungus and the other half contained a disc of *T. harzianum* isolate. Plates were incubated at 30°C. The control plates has two mycelial discs of tested pathogen. The radial growth of tested pathogens in treated and control plates were recorded after 5 days from incubation. The mycelial growth inhibition percentage of pathogens was calculated by using the following equation (Grondona et al., 1997):

$$\text{Inhibition (\%)} = \frac{R_2 - R_1}{R_2} \times 100$$

Where:  $R_1$  is the radial growth of the pathogen in dual culture with the antagonist (cm),  $R_2$  is the radial growth of the pathogen in control plates (cm).

**Effect of volatile compounds of *Trichoderma* isolates on the radial growth of pathogens *in vitro*:**

The method by Ajith & Lakshmidevi, (2010) was applied to test volatile compounds emitted from four isolates of *T. harzianum* on radial growth of the tested pathogens. The two bottom portions of Petri plates containing PDA were inoculated with a 6 mm disc of pathogen and antagonist, respectively. Both inoculated bottom plates were placed facing each other and sealed with cellophane adhesive tape. Petri plate containing PDA without antagonist served as control. Five plates were prepared for each treatment beside the control treatment. Observations on the radial growth of the tested fungus were recorded after 5 days of incubation at 30°C. Colony diameter of the tested fungus was recorded.

**Effect of bacterial bioagents on growth of pathogens *in vitro*:**

Glycerol agar (GA) and Nutrient agar (NA) media were used to obtain pure cultures of *P. fluorescens* and *B. subtilis*, respectively. Potato dextrose agar and glycerol agar media were mixed and PH was adjusted to 7 to form an antagonistic medium to test the *in vitro* interactions between virulent isolates of guava decline fungi and *P. fluorescens* and *B. subtilis*. The antagonistic medium was poured into sterilized petri dishes 9 cm in diameter. Two straight parallel lines were drawn each at 1.5 cm from dish edge. On the medium tracing those two lines, a full loop of the tested bacterial suspension was streaked. The Petri plates were incubated at 28°C for 24h before the fungus inoculum was introduced in a central position between the two lines.

The inoculated Petri dishes were incubated at 30°C for 5 days. The antagonistic effect was determined by measuring the longest and shortest free growth zone between the antagonistic bacteria and the tested isolates of decline disease fungi. The reaction was scored as: (+++) high inhibition, (++) moderate inhibition (+) slight inhibition and (-) no inhibition zone (Khattab, 2004).

**Greenhouse experiments:** To prepare inocula of tested fungi, Erlenmeyer flasks containing 100 g of barley were autoclaved at 121.6°C for 20 min. After cooling, five disks (6 mm diameter) of seven days old culture of each tested fungus were dropped into each flask under sterilized condition. The flasks were kept at 30°C for two weeks. *T. harzianum* T4 was grown on 100 ml potato dextrose broth medium in 250 ml Erlenmeyer flasks for two weeks at 30°C. Fifty grams of the produced mycelium were blended with 500 ml sterilized water and spore suspension was then adjusted to  $14 \times 10^6$  spores/ml (Ganesan *et al.*, 2007). Soil infestation was achieved by mixing the inoculum of the tested fungus with the upper layer of the soil at the depth of 20 cm at the rate of 15 g of mixed pathogenic fungi/kg soil and left for one week before transplanting. Each bag of *T. harzianum* treatment was drenched by 50, 100 and 150 ml of the prepared *T. harzianum* suspension at the time of transplanting guava seedling 1.5 years old. The plastic bags containing the soil-pathogen inoculum mixture without *T. harzianum* were served as the control. Guava decline symptoms were recorded after nine months of treatments, at three months interval periods. Plants were

uprooted after 18 months post treatments. Disease severities, plant height, total dry weights of shoots and roots and chlorophyll content were recorded. The recorded growth parameters included plant height in cm (measured from the soil surface to the highest point of the plant) and dry weights in gm of shoots and roots. For plant biomass, the samples were oven dried separately at 60°C till full dryness. Photosynthetic pigments; Chlorophyll a, Chlorophyll b, and Carotenoids were extracted from the fresh leaf according to the method of Arnon, (1949). The developed color was measured at three wavelengths 470, 645 and 662 nm using spectro22 colorimeter (Labomed, Inc, U.S.A). Amounts of pigments were calculated according to Costache *et al.*, (2012) simultaneous equations as follows: Chlorophyll a ( $\mu\text{g/ml}$ ) =  $11.75 A_{662} - 2.350 A_{645}$ , Chlorophyll b ( $\mu\text{g/ml}$ ) =  $18.61 A_{645} - 3.960 A_{662}$ , Carotenoids ( $\mu\text{g/ml}$ ) =  $(1000 A_{470} - 2.270[\text{Chl a}] - 81.4 [\text{Chl b}]) / 227$ .

**Statistical analysis:** All the experiments were conducted with five replicates for each treatment and arranged in a randomized complete block design. The quantitative data obtained were analyzed by the statistical analysis of variance with SAS software (SAS institute, 1988) and the level of significance was determined by LSD comparisons at the 5% probability level.

## Results

**Isolation and identification of guava pathogenic fungi:** Seven fungi namely, *F. oxysporum*, *F. solani*, *F. culmorum*,

*Pythium* sp., *Macrophomina phaseolina*, *Sclerotinia sclerotiorum*, and *R. solani* were isolated from both guava roots and soil samples. Out of these 7 fungi, *F. oxysporum* and *R. solani* were the most abundant. However, the isolation from guava branches, twigs, leaves and fruits resulted in 11 fungi namely, *F. oxysporum*, *B. theobromae*, *Alternaria alternate*, *A. solani*, *Aspergillus niger*, *Cercospora* sp., *Cladosporium* sp., *Curvularia* sp., *Colletotrichum gloeosporioides*, *Helminthosporium* sp. and *Phytophthora* sp. The most abundant fungi from guava aerial parts were *F. oxysporum* and *B. theobromae*. Generally, the most abundant fungi, *F. oxysporum*, *B. theobromae* and *R. solani*, were selected for achieving Koch's postulates. Based on the Koch's postulates, the most virulent isolate of *F. oxysporum*, *B. theobromae* and *R. solani* were selected for further pathogenicity and antagonistic studies. Disease symptoms were recorded on different parts of guava plants. The roots of infected trees showed blackening and rotting regions. The bark of main stems and lateral branches of infected trees was splitted. Moreover, wilting and dieback of lateral branches terminal tips were observed. Infected trees showed yellowing, curling and drying leaves leading to severe shedding and eventually the whole plant dried and died. The transverse sections from infected stems showed blackening or browning vascular tissues.

**Isolation and identification of bioagents:** Bioagents isolates were recovered from soil samples collected from the 39 visited fields. Four isolates of *T. harzianum* (T1, T2, T3, and T4)

were isolated and identified using the morphological characteristics according to Soni & Sharma (2014) and Leslie & Summerell (2006). Based on morphological and biochemical characteristics, the two isolated bacterial bioagents were belonging to *B. subtilis* and *P. fluorescens*.

**Effect of fungal bioagents on radial growth of pathogens *in vitro*:** In dual culture test, *T. harzianum* isolates showed significant inhibitory effects on the radial growth of the three tested pathogenic fungi (Table 1). Moreover, *T. harzianum* T4 showed the highest inhibitory effect on *F. oxysporum* (88.44%), *B. theobromae* (62.44%) and *R. solani* (66.55%).

**Effect of volatile compounds of *Trichoderma* isolates on the radial growth of pathogens *in vitro*:** In this experiment, the effect of volatile metabolites produced by four isolates of *T. harzianum* had an inhibitory effect on the growth of the three tested pathogenic fungi. After five days of incubation, volatile compounds from *T. harzianum* T4 exhibited maximum growth inhibition to *R. solani* (63.33%), *F. oxysporum* (58.44%) and *B. theobromae* (26.67%). Whereas, *Trichoderma* isolate T2 showed least growth inhibitions of tested fungi, *R. solani* (9.56%), *F. oxysporum* (7.11%) and *B. theobromae* (11.33%). On other hand, *Trichoderma* isolates T1 and T3 had no effect on the radial growth of the three pathogenic fungi (Figures 1 and Table 2).

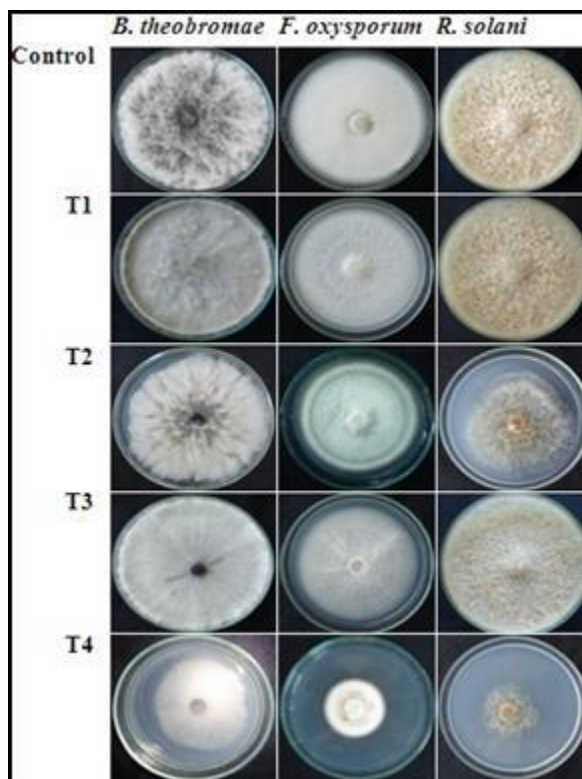


Figure 1: Effect of volatile compounds of *T. harzianum* (T1, T2, T3 and T4) isolates on the radial growth of *B. theobromae*, *F. oxysporum*, and *R. solani* grown on PDA medium.

Table 1: Effects of four isolates of *T. harzianum* on the radial growth of *B. theobromae*, *F. oxysporum*, and *R. solani* isolated from diseased guava plants.

Trichoderma isolates (B)	Fungi (A)						
	<i>B. theobromae</i>		<i>F. oxysporum</i>		<i>R. solani</i>		Mean (B)
	Radial growth(cm)	Inhibition (%)	Radial growth(cm)	Inhibition (%)	Radial growth(cm)	Inhibition (%)	
Control	9.00	00.00	9.00	00.00	9.00	00.00	9.00
T1	5.12	43.11	3.20	64.44	5.08	43.56	4.47
T2	5.54	38.44	2.72	69.78	4.20	53.33	4.15
T3	5.06	43.78	2.54	71.78	4.50	50.00	4.03
T4	3.38	62.44	1.04	88.44	3.01	66.56	2.47
Mean(A)	5.62	-	3.70	-	5.15	-	-

L.S.D<sub>0.05</sub> of (A×B) = 0.14 Fungi (A) = 0.06 Trichoderma isolates (B) = 0.08Table 2: Effect of volatile compounds from four isolates of *T. harzianum* on the radial growth of *B. theobromae*, *F. oxysporum*, and *R. solani* grown on PDA medium.

Trichoderma isolates (B)	Fungi (A)						
	<i>B. theobromae</i>		<i>F. oxysporum</i>		<i>R. solani</i>		Mean (B)
	Radial growth(cm)	Inhibition (%)	Radial growth(cm)	Inhibition (%)	Radial growth(cm)	Inhibition (%)	
Control	9.00	-	9.00	-	9.00	-	9.00
T1	9.00	00.00	9.00	00.00	9.00	00.00	9.00
T2	7.98	11.33	8.36	7.11	8.14	9.56	8.10
T3	9.00	00.00	9.00	00.00	9.00	00.00	9.00
T4	6.60	26.67	3.74	58.44	3.30	63.33	4.50
Mean (A)	8.32	-	7.82	-	7.69	-	-

L.S.D<sub>0.05</sub> of (A×B) = 0.21, Fungi (A) = 0.09, Trichoderma isolates (B) = 0.12

**Effect of bacterial bioagents on growth of pathogens *in vitro*:** To study the antagonistic effect of the studied bacteria *i.e.*, *B. subtilis* and *P. fluorescens* were tested against the three pathogenic fungi after five days of incubation. The inhibition zone between the tested bacterium and the pathogenic fungus was measured by the dual culture method. Data in Table 3 revealed that *B. subtilis*

caused the highest suppression in case of *R. solani* and *B. theobromae* with an inhibition zone of 1.08cm and 0.71cm, respectively. While, the least effect was indicated in case of *F. oxysporum* (0.68cm). On the other hand, testing of *P. fluorescens* significantly inhibited the growth of *B. theobromae*, *F. oxysporum* and *R. solani* of 0.42cm, 1.18cm and 1.45cm respectively (Figure 2).

Table 3: Effects of *P. fluorescens* and *B. subtilis* on the radial growth of *B. theobromae*, *F. oxysporum* and *R. solani*\*.

Treatments (A)	Fungi (B)			
	<i>B. theobromae</i>	<i>F. oxysporum</i>	<i>R. solani</i>	Mean (A)
<i>Pseudomonas fluorescens</i>	(+) 0.42*	(+++) 1.18	(+++) 1.45	1.02
<i>Bacillus subtilis</i>	(++) 0.71	(++) 0.68	(+++) 1.08	0.82
Mean (B)	0.57	0.93	1.27	-

L.S.D<sub>0.05</sub> of (A×B) = 0.11, Treatments (A) = 0.07, Fungi (B) = 0.08. \* Inhibition zone per cm, High inhibition (+++) = (1.5-1.0), Moderate inhibition (++) = (0.9 - 0.5), Slight inhibition (+) = (0.4 - 0.1).



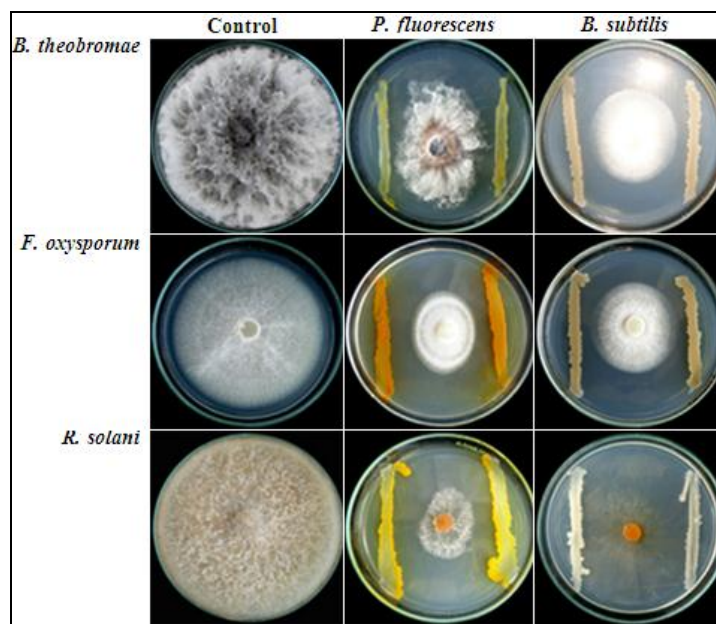


Figure 2: Effect of *P. fluorescens* and *B. subtilis* on the radial growth of *B. theobromae*, *F. oxysporum*, and *R. solani* grown on PDA medium amended with glycerol agar.

**Greenhouse experiments:** The efficacy of *T. harzianum* T4 at the rate of 50, 100 and 150 ml,  $10^7$  spores/ ml, in the form of soil drench treatment for controlling guava decline disease was evaluated under greenhouse condition. Data (Table 4) illustrated that all tested concentrations of *T. harzianum* T4 significantly reduced guava decline syndrome comparing with the control. In addition, the results show that *T. harzianum* treatment at 150ml/ Kg

soil gave the highest reduction in disease severity. Concentrations of photosynthetic pigments, Chlorophyll a (Chl. a), Chlorophyll b (Chl. b), and Carotenoids in leaves of treated infected guava plants were high comparing with the healthy control. Increasing concentrations of photosynthetic pigments resulted in increasing of plant height and dry weight of shoots and roots (Table 4).

Table 4: Effect of different densities of *T. harzianum* T4 isolate, in the guava rhizosphere, on the disease severity, plant growth and the leaf content of photosynthetic pigments under healthy and decline disease complex conditions.

Treatments	Disease severity	Plant characters			Photosynthetic pigments (mg/g fresh weight)		
		Height (cm)	Dry weight of shoot (gm)	Dry weight of root (gm)	Chl. a	Chl. b	Carotenoids
Healthy/ 0	00.00	70.40	68.47	31.79	11.22	7.21	4.39
Infected/ 0	90.00	40.20	18.68	7.53	0.70	0.37	0.16
Infected/ 50 ml/bag	64.40	48.40	31.66	12.5	2.99	1.68	0.94
Infected/ 100 ml/bag	49.26	51.20	36.57	14.0	6.69	3.50	2.70
Infected/ 150 ml/bag	31.30	71.30	60.89	27.80	12.00	8.10	5.10
L.S.D <sub>(0.05)</sub>	3.95	6.93	2.59	2.93	0.68	0.86	0.75



## Discussion

Biological control involves the use of beneficial organisms, their genes, e.g. chitinases and glucanases, and/or products, such as metabolites, that reduce the negative effects of plant pathogens and promote positive responses by the plant. Biocontrol agents, *B. subtilis*, *P. fluorescens* and four isolates of the fungus *T. harzianum* were evaluated against the fungi under study by measuring the radial growth and by studying the effect of bio-agents on the dry weight of fungi using antibiosis test in Petri dishes. Results showed significant antagonistic effect. Disease suppression, as mediated by biocontrol agents, is the consequence of the interactions between the plant, pathogens and the microbial community (Sivan & Chet, 1986). Thus, biological control is being considered as an alternative or a supplemental way of reducing the use of chemicals in agriculture (Compant et al., 2005). Isolates of *Trichoderma* spp. have been described as potential biocontrol agents against several soil-borne plant pathogens *i. e.*, *Rhizoctonia*, *Sclerotinia*, *Botrytis* and *Fusarium* (Latunde, 1991; El-Abbasi et al., 2003). By using *T. harzianum* (*in vitro* or *in vivo*) as biocontrol agent for guava decline fungi, *B. theobromae*, *F. oxysporum* and *R. solani*, showed significant suppression of the radial growth and the biomass of guava decline fungi especially *T. harzianum* T4 isolate that showed high ability in their inhibition as well as the secretion of volatile inhibition substances. These results are in accordance with results of Abdel-Kader et al. (2002); Srivastava et al. (2009). Using filtrates of *T. harizianum* T4, *P.*

*fluorescens*, *B. subtilis*, *A. niger*, *A. flavus* and *Penicillium* spp. as biocontrol agents against guava decline fungi *in vitro* exhibited significant suppression of its growth. These results were similar to that Gupta et al., 2009 and Madhanraj *et al.*, 2010. Using of *B. subtilis*, radial growth of *R. solani* and *F. oxysporum* was significantly inhibited in comparison to *B. theobromae*. In comparison with *B. theobromae* and *F. oxysporum*, *R. solani* was significantly inhibited using *P. fluorescens*. Effect of *T. harzianum* T4 secretion on the dry weight of guava decline fungi at 75% concentration had a significant decrease in comparison to other secretions at the same concentration. These results were confirmed for *B. subtilis*, *P. fluorescens* in an antagonistic test using potato dextrose agar and glycerol agar (PDA+GA) medium in petri dishes. The efficacy of *T. harizianum* T4 at 150ml/Kg soil gave the highest reduction in disease severity. Concentrations of photosynthetic pigments in leaves of treated infected guava plants were high comparing with the healthy control. Increasing of concentrations of photosynthetic pigments resulted in increasing of plant height and dry weight of shoots and roots. In conclusion, the application of biocontrol agents (e.g. *T. harzianum*, *B. subtilis* and *P. fluorescens*) either themselves or their bio-products (volatile or antimicrobial compounds) decreased significantly guava decline disease and improved the health of guava by increasing plant height, dry weight of roots and shoots, and total pigments. However, more *in vitro* and *in vivo* studies are required to establish an effective biocontrol strategy to manage guava decline disease.

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