*J. Plant Prot. and Path., Mansoura Univ., Vol.7 (12) ,867–872, 2016*

# Screening Potential of Some Bacterial Species and *Trichoderma hrzianum* Against *Sclerotinia sclerotiorum* on Cucumber.

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## ABSTRACT

Eight bacterial isolates belonged to 4 genera, i.e., *Serratia sp.,*(one isolate) *Bacillus subtilis,* (three isolates) *Bacillus thuringiensis,*(two isolates) *Streptomyces sp.*(one isolate) and *Pseudumonas flourescens*(one isolate) and one fungal isolate (*T. harzianum*) were isolated from cucumber rhizosphere to evaluate their potential as antagonists to Sclerotinia stem and root rot on cucumber. *In vitro*, all isolates resulted in a significant reduction in hyphal growth of pathogenic fungus, *Streptomyces sp.* was more significantly reduced mycelium growth followed by *P. flourescens* and *Bacillus subtilis* (Bs1) (72.22, 68.0 and 62.22 %); respectively. All isolates gave a significant reduction in disease severity on cucumber plants, B2, Bs1, Bs2 and *Streptomyces* sp. isolates gave best reduction in disease severity. All isolates resulted in significant increase in morphological parameters of cucumber plants (stem and root length, foliage and root dry weight) compared to control.

**Keywords:** *Sclerotinia sclerotiorum*; cucumber; Biocontrol; Bacterial species and *Trichoderma harzianum*

## INTODUCTION

Cucumber is among most widely grown vegetables throughout the world (Paris *et al*., 2011). Most of the vegetable crops are cultivated in the plastic house conditions during the winter season, high humidity in the plastic house conditions are favorable for occurrence of plant diseases, especially, Sclerotinia rot diseases is more sever under cool and moist conditions (Purdy, 1979 and Willetts and Wong, 1980).

*Sclerotinia sclerotiorum* (Lib.) de Bary is a serious and a widespread soil borne plant pathogen, it is affecting many susceptible hosts (Gao *et al*. 2014), Sclerotinia Stem and Root Rots or white mold is one of the most dangerous cucumber diseases (Purdy, 1979). Crop rotation and cultural practice are not effective enough in controlling the disease because the wide range of its plant host, the ability to survive as sclerotia (Purdy 1979, Bolan and Hall 1994 and Elkahoui, *et al* 2014).

Low cost and eco-friendly application of biological control method is gaining a highly attention from all methods of control plant diseases, biocontrol using antagonistic fungi and bacteria have important role (Abhiniti *et al*., 2011). *Trichoderma* and *Bacillus* are of the most effective bioagents but very few species have been tested on sclerotinia rots (Singh and Kaur, 2001, Savchuk and Fernando, 2004; Zhang and Fernando, 2004 and Fernando *et al.*, 2007).

Control of Sclerotinia stem and root rots diseases have been studied in numerous researches and effective disease control was found by using fungi (Li GQ, *et al* (2003) and Rodríguez, *et al* (2015)), bacteria (Berry, *et al* (2010) and Abdullah, *et al* (2008)) or biofungicides (Domenech, *et al* (2006) and Zeng, *et al* (2012)) in many crops. The most efficient bacteria used for disease control belonged to the genera Bacillus(Gao *et al*. (2014), Elkahoui, *et al* (2014) Abdullah, *et al* (2008) Monteiro, *et al* (2013) Alvarez *et al*. (2012)), Pseudomonas (Berry, *et al* (2010) and Onaran and Yanar (2011)), Serratia (Onaran and Yanar (2011), El-Tarabily *et al*. (2000) and Kamensky, *et al* (2003)), and at a lesser extent Streptomyces (Onaran and Yanar (2011) and El-Tarabily *et al*. (2000)). Plant growth-promoting rhizobacteria (PGPR) defined as bacteria which colonize plant roots and promote plant growth; it has a highly diversity and used as biocontrol agents against many plant pathogens (Lugtenbergand Kamilova F (2009) Beneduzi *et al* (2012)).

The objective of this study is to evaluate the antagonistic potential of one isolate of *T. harzianum* and 8 bacterial isolates, isolated from cucumber rhizosphere against *S. sclerotiorum* growth and to examine their abilities to suppress *Sclerotinia* stem and root rots disease and to enhance growth of cucumber plants and reduced disease severity under greenhouse condition.

## MATERIALS AND METHODS

**Plant material and growth conditions:**

Cucumber (hybrid Hesham) seedlings were used for all *in vivo* trials. Cucumber seeds were sown in trays (84 holes) and watered then placed under greenhouse conditions tell germination and treatments.

**Isolation of *Sclerotinia sclerotiorum*:**

*S. sclerotiorum* fungus was isolated from cucumber (Hesham hybrid) plants which exhibiting symptoms of root and stem rots collected from El Behaira Governorate according to Zhang and Xue, (2010).

**Pathogenicity test of *S. sclerotiorum* isolates:**

Cucumber seedlings three weeks-old were used to estimate the disease severity of nine isolates of the pathogen according to Baharlouei *et al*., (2011).

**Isolation, identification and preparation of Bacterial isolates:**

Bacterial isolates were isolated from soil rhizosphere of healthy cucumber plants which grown in infested field; they were identified using morphological and biochemical methods.

a loop-full of isolated bacteria was transferred to 10 ml of SDW to make suspension, then added one ml of the suspension to LB broth (300 ml) (peptone1%, yeast extract 0.5% and 1% NaCl) and adjusted to approximately $10^8$cells $ml^{-1}$.

**Soil Samples and Isolation of *Trichoderma harzianum***

Isolate of *T. harzianum* in this study was isolated from soil sample collected from cucumber rhizosphere, then purified on PDA according to Elad *et al* (1982).

***In vitro* antagonistic activity with isolate of *Trichoderma harzianum*:**

Matching method between antagonist and phytopathogen (Dennis and Webster, 1971) was used between isolate of *T. harzianum* against isolate of *S.*

*sclerotiorum* (S.sc.7). Plates containing PDA, inoculated with disks of mycelia on agar from the antagonist and phytopathogen interval 7 cm apart from each other and 1 cm from the edge of the plate. Measures were carried out daily until the meeting of the two mycelia and/or until one of the two fungi were overlaid by the other.

The experiment was replicated three times and measured growth inhibition % (I) = (C-T)/Cx100, where C is mycelial growth in control plate, T is mycelial growth in test organisms inoculated plate and I is inhibition of mycelial growth

**Antagonistic activity with bacterial isolate *in vitro*:**

Antagonistic activity of bacterial isolates against S. sclerotiorum was tested by placed a loop of bacteria in a straight line at the margins of PDA plates, agar disc from the pathogen was placed at the other side of the plate and then incubated at 27 °C for seven days, The percentage growth inhibition was calculated.

**Assessment of antagonistic potential of *T. harzianum* and bacterial isolates in the greenhouse.**

Bacterial isolates and pathogen cultures were prepared as mentioned before. Inoculation was performed on cucumber hybrid Hesham seedlings 20-days-old. In each hole containing a cucumber plant, 30 ml of a bacterial suspension ($10^8$cells /ml) and spore suspension of *T. harzianum* ($3x10^{10}$ spore / ml) were drenched at the collar level.

Pathogen was cultured on PDA medium, then incubated at 25°C for 7 days, 10 PDA Petri dishes (9 cm), full with mycelium growth were macerated using a blender in 1L of SDW, mycelial suspension obtained was used for plant inoculation (Zhang and Xue (2010)).

After one week of bacterial treatment, fungal inoculum (30 ml) was poured to each plant at the same level. Controls were watered with water only. After one day of pathogen challenge, the plants were transplanted into pots (25 cm) (Benchabane *et al* (2000)).

**Treatments in this experiment:**

- Positive control Uninoculated, untreated cucumber plants.
- Cucumber plants inoculated with pathogen only
- Cucumber plants inoculated and treated with each of bacterial isolates or *T. harzianum*.

After two months of inoculation and treatment, the plant height, the foliage and root dry weights were recorded. Disease severity was assessed using 0-5 scale where:

0 = no symptom, 1= 0-25% of root browning, 2 =26-50% of root browning, 3 =51-75% of root browning, 4 =76-100% of root browning, and 5 =plant death.

**Statistical analysis:**

All data were subjected to one way analysis of variance (ANOVA) followed by means separation through least significant difference (L.S.D.) test at $P < 0.05$ level (Snedecor and Cochran, 1980).

## RESULTS

**Bacterial collection source and inoculum preparation:**

Data in Table (1) indicated that isolation from cucumber rhizosphere and identification resulted in 8 bacterial isolates belonged to 4 genera *Serratia sp., Bacillus subtilis, Bacillus thuringiensis, Streptomyces sp.* and *Pseudumonas flourescencs* and one fungal isolate identified as *Trichoderma harzianum*. Figure (1) illustrated bacterial isolates isolated from cucumber rhizosphere.

**Table 1. Isolates isolated from cucumber rhizosphere and their identification.**

| Isolates | Isolate Number | Isolate Name |
|---|---|---|
| *Bacillus thuringiensis* | 3 | B. 1, B. 2 and B. 3 |
| *Bacillus subtilis* | 2 | B.s.1 and B.s.2 |
| *Streptomyces sp.* | 1 | Strep. |
| *Serratia sp.* | 1 | Sera. |
| *Pseudumonas flourescencs* | 1 | Pseud. |
| *Trichoderma harzianum*. | 1 | Tricho. |

1 2 3

**Fig. 1. Bacterial isolates isolated from cucumber rhizosphere, from left:1 *Pseudumonas flourescencs, Serratia sp., Streptomyces sp.* 2 *Bacillus thuringiensis* and 3 *Bacillus subtilis.***

**Pathogenicity Test of *S. sclerotiorum* isolates:**

Table (2) demonstrated that there were nine isolates of *S. sclerotiorum* isolated from cucumber plants exhibited symptoms of sclerotinia stem and root rot, pathogenicity test of different isolates showed that all isolates were significantly more sever on cucumber plants. Isolate Number S.sc.7 was the most virulent isolate in the pathogenicity test.

**Table 2. Pathogenicity test of *Sclerotinia sclerotiorum* (S.sc.) isolates on cucumber plants in greenhouse.**

| Isolates | Disease severity |
|---|---|
| S.sc.1 | 42.65e ±0.84 |
| S.sc.2 | 37.4f ±1.19 |
| S.sc.3 | 56.02d ±1.15 |
| S.sc.4 | 31.25g ±1.43 |
| S.sc.5 | 25.17h ±1.43 |
| S.sc.6 | 65.3c ±1.48 |
| S.sc.7 | 81.4a ±1.71 |
| S.sc.8 | 74.47b ±2.00 |
| S.sc.9 | 23.67h ±1.92 |
| Control (autoclaved soil) | 0.00i ±0.00 |
| LSD | 2.82 |

**Data presented as the means of three replicates ± SD. Different letters refer to significant difference (P≤ 0.05).**

**Antagonistic activity of bacterial isolates and *Trichoderma harzianum in vitro*:**

Data illustrated in figure (2) show that all isolated bacteria, *Serratia sp., Bacillus subtilis, Bacillus thuringiensis, Streptomyces sp.* and *Pseudumonas flourescencs* and *Trichoderma harzianum* fungus significantly reduction the hyphal growth of pathogenic fungus (*S. sclerotiorum*(S.sc.7)) *in vitro*. *Streptomyces sp.* was more significant reduction of mycelium growth

followed by *Pseudumonas flourescencs* and *Bacillus subtilis* (Bs1) (72.22, 68.0 and 62.22 %) respectively compared to control *S. sclerotiorum* (S.sc.7) alone (0.0%). Figure (3) illustrated that zone of inhibition of antagonistic isolates on PDA plats.

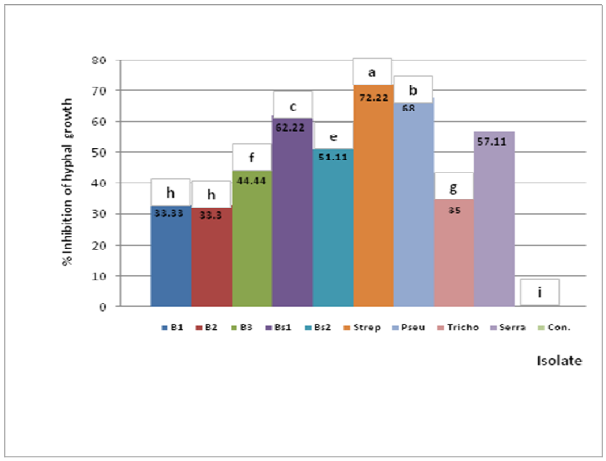


**Fig. 2. % Inhibition of *S. sclerotiorum* (S.sc.7) mycelium% by antagonistic isolates *in vivo*.**

Data presented as the means of three replicates ± SD. Different letters refer to significant difference (P≤ 0.05).LSD= 0.39

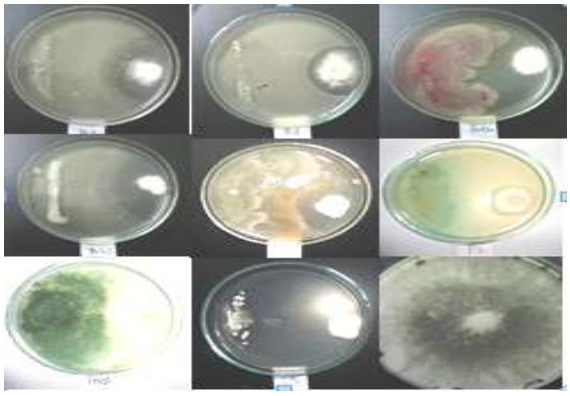

**Fig. 3. Inhibition Zone of antagonistic isolates against *S. sclerotiorum* (S.sc.7) on PDA plates *in vivo*.**

**Assessment of Sclerotinia Stem Rot-suppressive in the greenhouse.**

Data in Table (3) demonstrated that B2, Bs1, Bs2 and *Streptomyces* sp. gave best reduction in disease severity of *S. sclerotiorum* on cucumber plants in greenhouse which efficiency were 100% and followed by *Serratia* sp. (87.77%) and *Pseudumonas flourescencs* (81.22%) and the less isolate efficiency was B1 compared to control inoculated (0.00%).

**Table 3. Suppression of *S. sclerotiorum* by antagonistic isolates and efficacy on cucumber plants in greenhouse.**

| Antagoistic Isolate | %Disease severity | %Efficacy |
|---|---|---|
| *Bacillus thuringiensis* (B.1) | 63.67b ±1.25 | 20.78 |
| *B. thuringiensis* (B.2) | 0.00g ±0.00 | 100.00 |
| *B. thuringiensis* (B.3) | 20.73d ±0.98 | 74.21 |
| *Bacillus subtilis* (B.s.1) | 0.00g ±0.00 | 100.00 |
| *B. subtilis* (B.s.2) | 0.00g ±0.00 | 100.00 |
| *Streptomyces sp.* | 0.00g ±0.00 | 100.00 |
| *Serratia* sp. | 9.83f ±1.03 | 87.77 |
| *Pseudumonas flourescencs* | 15.17e ±0.62 | 81.12 |
| *Trichoderma harzianum* | 60.63c ±1.15 | 24.56 |
| Control (pathogen only) | 80.37a ±1.21 | 0.00 |
| Control (Autoclaved soil) | 0.00g ±0.00 | 100.00 |
| L.S.D | 1.62 | |

**Data presented as the means of three replicates ± SD. Different letters refer to significant difference (P≤ 0.05).**

**Plant growth parameters.**

Data presented in Table (4) demonstrated that all isolates gave a significant increase in morphological parameters of treated plants the most significant isolate in stem length was *Serratia* sp and Bs2 (42.23 and 40.97cm) followed by Bs1 (36.8cm) compared to control inoculated (24.5cm), root length was significantly increased in all isolates, the highly significant isolate was *Serratia* sp. (22.3 cm) followed by B2 (21.43cm) compared to control inoculated (9.67 cm). All isolates showed a significant increase in foliage and root dry weight compared to control.

**Table 4. Effect of bacterial isolates and *T. harzianum* on stem and root length and dry weight of cucumber plants under infection of *S. sclerotiorum* uder greenhouse condition.**

| Antagonistic Isolates | Length(cm) | | Dry weight(g) | |
|---|---|---|---|---|
| | Stem | Root | foliage | Root |
| *Bacillus thuringiensis* (B.1) | 23.30f ±1.43 | 17.60d ±0.50 | 1.37d ±0.03 | 0.70a ±0.08 |
| *B. thuringiensis* (B.2) | 29.00d ±1.10 | 21.43ab ±0.49 | 1.81bc ±0.12 | 0.84a ±0.04 |
| *B. thuringiensis* (B.3) | 25.10ef ±1.02 | 16.47de ±0.62 | 1.68c ±0.13 | 0.82a ±0.10 |
| *Bacillus subtilis* (B.s.1) | 36.80b ±0.88 | 20.33bc ±0.62 | 2.04b ±0.19 | 0.81a ±0.12 |
| *B. subtilis* (B.s.2) | 40.97a ±1.03 | 20.23bc ±1.36 | 2.50a ±0.16 | 0.78a ±0.08 |
| *Streptomyces sp.* | 36.10bc ±1.42 | 20.83abc ±1.03 | 1.78bc ±0.10 | 0.85a ±0.09 |
| *Serratia* sp. | 42.23a ±1.52 | 22.83a ±1.55 | 1.77bc ±0.15 | 0.86a ±0.09 |
| *Pseudumonas flourescencs* | 29.03d ±1.51 | 15.17e ±1.65 | 1.76bc ±0.18 | 0.69a ±0.04 |
| *Trichoderma harzianum* | 34.07c ±1.47 | 18.50cd ±1.22 | 1.79bc ±0.13 | 0.69a ±0.04 |
| Control (pathogen only) | 24.50g ±1.08 | 9.67f ±0.85 | 0.80e ±0.12 | 0.38b ±0.03 |
| Control (Autoclaved soil) | 26.23de ±1.45 | 15.10e ±0.94 | 1.50cd ±0.12 | 0.70a ±0.04 |
| L.S.D. | 2.57 | 2.19 | 0.28 | 0.15 |

**Data presented as the means of three replicates ± SD. Different letters refer to significant difference (P≤ 0.05).**

## DISCUSSION

Fungal soil borne diseases are the most important problems threatening cucumber cropping; application of chemical fertilizers and pesticides has led to health and environmental problems, so searching for alternative control strategies which can ensure competitive yields

while protecting human, plant and soil health are significantly required (Hariprasad and Niranjana (2009)). So there is a widely studied about biocontrol agents to management *S. sclerotiorum* by mycoparasites ,*i.e.; Coniothyrium minitans* and *Sporidesmium sclerotivorum* (Bolton *et al* (2006)), but a few attempts have been made to demonstrate the potential use of biocontrol methods using bacteria to control Sclerotinia diseases (Fernando *et al* (2007) Abdullah, *et al* (2008), and Zhang and Xue (2010)).

In this study, 8 bacterial strains isolated from soils rhizosphere of healthy cucumber plants, belonging to *Serratia sp., Bacillus subtilis, Bacillus thuringiensis, Streptomyces sp.* and *Pseudumonas flourescens* and one fungus isolate (*T. harzianum*) were examined for their potential to suppress the disease and to enhance cucumber growth. *In vitro* experiment, all isolated bacteria, *Serratia sp., Bacillus subtilis, Bacillus thuringiensis, Streptomyces sp.* and *Pseudumonas flourescencs* and *T.harzianum* fungus significantly reduction the hyphal growth of pathogenic fungus (*S. sclerotiorum*(S.sc.7) this is agreement with findings of Whipps (1987) who found the ability of some isolates to acting as biological control agents, i.e.; *Serratia sp., Bacillus thuringiensis, Streptomyces sp.* and *Pseudumonas flourescens*. Also other findings demonstrated that *T. harzianum* had high ability to attack the host fungi with the colonization of the hyphae, so it showed the behavior of a good biocontrol agent by its ability to reduce infections from the beginning and infection progress if it is applied in a suitable time. *T. harzianum* also has the ability to secrete lytic enzymes which gave it the potential to penetrate the cell wall of *S. sclerotiorum* ( Hjeljord and Tronsmo 1998 and Viterbo *et al.* 2002), also it has ability as mycoparasitism, competition (Howell, 1998).

Results in the present study showed a highly significant of zone of inhibition formation on the plate, where biocontrol agents acting during antibiosis mechanism and thus inhibit the pathogen with toxic substances which more effective than other mechanism of action (Leelasuphakul, *et al*., 2008). This mechanism has been reported to inhibit many pathogenic fungi and *S. sclerotiorum* one of it (Ongena and Jacques (2008) and Nagórska *et al* (2007)). Also, Bacillus genus is one of the beneficial bacteria mostly used as biopesticides (Fravel DR (2005)), its mode of action as antagonistic affect pathogen growth, also it has ability to produce a variety of many metabolites acting as antibiosis (Stein (2005) and Chen *et al* (2009)) and have a competitive ability for space and/or nutrients (Nagórska *et al* (2007)).

The bacterial isolates are able to inhibit the sclerotium-forming by the release of protease-resistant and thermo-stable compounds (Príncipe *et al* (2007)). Also, the result agreement with (Zhang *et al* (2008)) who found that *B. subtilis* formed inhibition zones against *S. sclerotiorum*. *B. subtilis* also has ability to reduction mycelia growth of *S. sclerotiorum* and suppress the fungus on sunflower (Zazzerini *et al* (1987)). Also, *B. thuringiensis* has the similar ability as biocontrol agent against *S. sclerotiorum* in many of studies (Gao *et al*. (2014), Duncan, *et al* (2006), Fernando *et al* (2007), Zhang and Xue (2010) and Zeng, *et al* (2012)).

Bacterial antifungal volatiles can diffuse through the soil, to kill sclerotia, which it is preventing them from germinating with a fungicidal effect on sclerotia even under favorable conditions (Alvarez *et al* (2012)).

Greenhouse results revealed that the 8 rhizobacterial strains and *T. harzianum* had reduced disease severity on all inoculated and treated plants compared to control inoculated with pathogen, the most effective isolates in suppressing disease were B2, Bs1, Bs2 and *Streptomyces* sp. which gave the best reduction in disease severity of *S. sclerotiorum* on cucumber plants in greenhouse which efficiency were 100% and followed by *Serratia* sp. and *P. flourescens*, on the other hand, the less isolate efficiency was B1 compared to control inoculated. As agree with Ryu *et al.* (2003) who found that the percentage of healthy plants were significantly higher compared to control plants. Similar results are presented by *B. subtilis* on chirpine seedlings where result in reduction in root rot diseae caused by *M. phaseolina*, also it was increased root and shoot dry weight, compared to control (Singh *et al* (2008)).

Also the result demonstrated that plant growth parameters i.e. plant height and root and stem dry weight were significantly increased, these results are in agreement with results that ensuring competitive yields while protecting plant health and soil (Domenech, *et al* (2006), Xue *et al* (2013) and Bellishree *et al* (2014)). Biocontrol agent is equipped with several characters which promotes plant growth as reduced fungal growth, hormone (IAA) production and ability of competition (siderophores) and to the ability to solubilize the phosphate (Saharan and Nehra (2011) and Saraf *et al* (2014)).

## CONCLUSION

The present study provides vigorous evidence that cucumber rhizosphere soils contain various isolates from *T. harzianum*, *Bacillus subtilis*, *Bacillus thuringiensis*, *pseudomonas flourescens* and *Serratia* sp., with plant growth-promoting and disease-reduction ability. With additive studies it could be useful as bio-fertilizers or biofungicide.

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## فحص قدرة بعض العزلات البكتيرية و التريكودرما ضد فطر الاسكليروتينيا على الخيار.

**كريمة جابر حلمى**

**قسم أمراض النبات – كلية الزراعة جامعة عين شمس.**

تم اختبار ثمانية عزلات بكتيرية تنتمى إلى ٤ أجناس بكتيرية *Serratia sp., Bacillus subtilis, Bacillus thuringiensis, Stryptomyces sp., Pseudumonas flourecence* وفطر الترايكوديرما والتى تم عزلها من ريزوسفير الخيار لتقييم قدرتها على مكافحة فطر الاسكليروتينيا مسبب عفن الساق والجذر على الخيار. تم اختبار قدرة هذه العزلات فى المعمل فوجد خفضها للنمو الميسليومى للفطر الممرض انخفاض معنوى. أعطت كل العزلات انخفاض كبير في شدة المرض على نباتات الخيار بالمقارنة بالكنترول. أظهرت العزلات زيادة كبيرة في الصفات المورفولوجية لنباتات الخيار ( طول الساق و الجذر، الوزن الجاف للمجموع الخضرى والجذرى) مقارنة بالكنترول.