World Applied Sciences Journal 19 (1): 89-99, 2012
ISSN 1818-4952;

DOI: 10.5829/idosi.wasj.2012.19.01.2765

# Molecular Studies on the Biocontrol Effect of *Trichoderma viride* and *Bacillus subtilis* on *Fusarium oxysporum* and *Rhizoctonia solani* Infected Tomato Plants

[1]Elsayed E. Hafez, [2]Mahmoud M. Balbaa, [3]Sanaa S.A. Kabeil, [2]M.A. El-Saadani and [1]Seham A. Ahmed

[1]Department of Molecular Plant Pathology, Arid lands Cultivation Research Institute,
City of Scientific Research and Technology Applications, New Borg El-Arab City, Alexandria, Egypt
[2]Departermnt of Biochemistry, Faculty of Science, Alexandria University, Egypt
[3]Department of Protein Research, Genetic Engineering and Biotechnology Research Institute (GEBRI),
City of Scientific Research and Technology Applications New Borg El-Arab City, Alexandria, Egypt

**Abstract:** Tomato is the second most important vegetable cropnext potato in Egypt and all over the world. Tomato attacked by several serious diseases, *Fusarium oxysporum* cause wilt disease and *Rhizoctonia solani* casu tomato root rot. Six bacterial and ten fungal isolates were isolated from different parts of infected plant with root rot disease and plant rhizosphere as well. Both of bacterial and fungal isolates were used for antagonistic activity test against *F. oxysporum* and *R. solani*. Only one bacterium and one fungal isolates revealed a high activity against the two pathogenic strains. For the highest antagonistic microorganisms identification both of 16S rRNA and ITS genes were amplified and sequenced, sequence analysis revealed that the bacterial isolate is *Bacillus subtilis* and the fungal isolate is *Trichoderma viride*. On the other hand, *Fusarium oxysporum* and *Rhizoctonia solani* were identified by microscopic examination. The efficiency of *B. subtilis* and *T. viride* on the plant defense against wilt and root rot diseases was examined when both organisms applied on tomato plants infected with *F. oxysporum* and *R. solani*. In pot experiment, *B. subtilis* and *Trichoderma viride* were effective in reducing tomato wilt and root rot disease incidence. The used biological control agents induced plant defense system moreover: The bioagents *T. viride* and *B. subtilis* activate pathogenesis-related proteins synthesis before the pathogen invades the host plant which has a direct impact on decreasing the ability of pathogen to cause wilt and root rot diseases.

**Key words:** *Bacillus subtilis* · *Trichoderma viride* · Biological control · *Fusarium oxysporum* · *Rhizoctonia solani*

## INTRODUCTION

Tomato (*Lycopersicon esculentum* L.) is considered one of the most important economic vegetable crops in Egypt [1]. Montealegre *et al.* [2] reported that tomato plants are subjected to attack by several soil borne fungal pathogens, which cause serious diseases as root rot and wilt. Plant diseases, especially root-rot diseases, cause significant losses in agricultural production every year. These losses can result in reduced food supplies, poorer-quality of agricultural products, economic hardship for growers and processors and, ultimately, higher prices [4, 5]. Diseases caused by *Fusarium* spp. are important limiting factors in the production of tomato. Several types of diseases associated with these pathogenic fungi have been identified, including *Fusarium* wilt; foot rot and crown root rot diseases [6]. One of the most damaging soil-borne pathogens of tomato is *F. oxysporum* f.sp. *radicis-lycopersici* (FORL), which causes *Fusarium* crown and root rot (FCRR) [7]. *Fusarium* root rot has considerably increased in Egyptian soils. It causes severe and high damage in tomato plants especially those grown in old soils. Also, *R. solani* which was gained the reputation of being a widespread, destructive and versatile plant pathogen capable of attacking a wide range of hosts (250 plant species)[8],

**Corresponding Author:** Sanaa S.A. Kabeil, Department of Protein Research, Genetic Engineering and Biotechnology Research Institute (GEBRI), City of Scientific Research and Technology Applications New Borg El-Arab City, Alexandria, Egypt. E-mail: sanaa5769@yahoo.com.

causing seed decay, damping-off, stem canker, root rot, fruit decay and foliage disease [9]. Biological control of several phytopathogens is based on the presence of suppressive soils where several biocontrol microorganisms belonging to *Trichoderma*, *Pseudomonas* and *Bacillus* genera are detected [10]. *Trichoderma* spp. has proved to be useful in the control of phytopathogens affecting different crops [11].

In this regards, tomato plants treated by *Bacillus subtilis* only and/or *Trichoderma harzianum* have shown biocontrol activity against damping-off and root rot diseases and gave high yield of tomato [12].The beneficial effects of biological control have stimulated research on this topic and also the commercial use of several *Trichoderma* species for the protection and growth enhancement of a number of crops. Seed-coating of broad bean with *Trichoderma viride* and *Bacillus subtilis* effectively controlled the Fusarium root rot caused by *F. solani* [13]. The direct interaction between *Trichoderma* spp. and the pathogen is called mycoparasitism. *Trichoderma* spp. is antagonistic to or hyperparasitic on a wide array of fungi and effectively reduce diseases caused by several soil-borne plant pathogens [14]. Mycoparasitism is considered to be an important mechanism of biological control and probably depends on the production of lytic enzymes including chitinases, glucanases and proteases. These enzymes often have antifungal activity individually and are synergistic in mixtures or with antibiotics [15, 16]. Chitinases from *Trichoderma* inhibited *in vitro* spore germination and tube elongation of a variety of fungi, which contain chitin as a major cell wall component [17]. Moreover, the correlation between the production of chitinlytic enzymes and the suppression of fungi containing chitin as the main cell wall constituent has been demonstrated for many *Trichoderma* species [18]. The fungus *T. virens* (*Gliocladium virens*) was reported to be a mycoparasite of the cotton seedling pathogen, *R. solani* Kühn [19], *T. lignorum* as a wheat-bran preparation, conidial suspension, or seed coating greatly decreased the number of infested seeds by *R. solani* as well as damping-off percentages and hence controlling the fungal disease [20].

The main objective of the current study was to evaluate the Egyptian isolates *T. viride* and *B. subtilis* as biocontrol control agents against wilt and root rot diseases of tomato and examine their effect on the tomato plant defense system.

## MATERIALS AND METHODS

**Isolation of Microbes Used in this Study:** *Fusarium oxysporum* and *R. solani* were isolated from root rotted and wilted tomato plants cultivated in Alexandria, Beni-Swiff, El-Dakahlya and El-Fayoum governorates in Egypt. They were microscopically identified on the basis of cultural and microscopic characteristics. Pathogenicity of the Obtained isolates to induce rot and wilt diseases of tomato plant was estimated according to Sneh *et al.* [21] under green house conditions. The isolate was maintained on PDA medium and stored at 4°C. *Trichoderma* isolates and *Bacillus* spp were isolated from tomato plant rhizosphere and identified microscopically. All microbial isolates were identified using and. All microbial isolates were identified using different molecular techniques RAPD PCR, 16S rRNA and ITS gene at the Molecular Plant Pathology Department, Arid lands Cultivation Research Institute, City of Scientific Research and Technology Applications, Alexandria, Egypt.

**Fingerprinting for the Tested Bioagents Bacterial and *Trichoderma* Isolates:** Genomic DNA bacterial isolates was extracted from overnight culture using the Wizard ® Genomic DNA purification Kit (Promega, USA) according to the manufactures instructions. Also, the fungal genomic DNA was extracted using Wizard® DNA Purification Kit (Promega, USA).

**A) RAPD-PCR Analysis for the Bacterial Isolates:** 4ix random primers were used for typing bacterial isolates. The primer sequence illustrated in Table 1. RAPD-PCR reaction was carried out in a 25 µL, 2.5 µL 10 x buffer with $MgCl_2$, 2 µL 2.5 mM dNTPS, 1 µL of 10 pmol primer, 50ng DNA template and 0.2 µL (2 units/µL) Taq DNA polymerase. The PCR amplification conditions consisted of denaturation at 95°C for 5 min, then 40 cycles as follows: 30 sec at 95°C for denaturation, 1 min at 30°C for annealing for 1 min and 1 min at 72 °C for elongation.

**B) For *Trichoderma* Isolates:** Six random primers were used for *Trichoderma* isolates. The primer sequence illustrated in Table1. The reaction mixture and reaction conditions as previously mentioned.

**Cluster Analysis of RAPD Assay:** RAPD banding patterns of the bacterial isolates and *Trichoderma* isolates were examined and the bands were scored, with the data coded as a factor of 1 or 0, representing the presence or

Table 1: Primers sequence used in the amplification of 16S rRNA gene and ITS region.

| Primer | Primer sequence 5`-`3 | Annealing (°C) |
|---|---|---|
| 16S(F) | AGG AGG TGA TCC AAC CGC | 58°C |
| 16S(R) | AAC TGG AGG AAG GTG GGGAT | |
| A9B7 | GGTGACGCAGGGGTAACGCC | 28 |
| Bacteria | ATA TGA CGT GTC TGC TCC | 30 |
| D2 | TAC CGT CAT TAT CTT CCC CAAA | 32 |
| D1 | AAA GAT GGC ATC ATC ATT CAA C | 28 |
| 18S | CTT CCG TCA ATT CCT TTA AG | 30 |
| A7A10 | AGGAGG TGA TCC AAC CGC | 30 |
| I | CGC TGT CGC C | 28 |
| RRPD10 | GAG AGC CAA C | 30 |
| ITS(1) | TCCGTAGGTGAACCTGCGG | 54°C |
| ITS(4) | TCCTCCGCTTATTGATATGC | |

absence of bands, respectively. Cluster analysis was used to produce dendrograms showing estimates of the distance values and to analyze the genetic relatedness among the bacterial isolates and *Trichderma* isolates. The dendrogram based on the similarities was derived from Unweighted Pair Group Method using Arthemetic Average (UPGMA).

**PCR Amplification of 16S rDNA and ITS Region:** For the bacterial isolates 350 bp of the 16S rRNA gene and 600 bp for *Trichoderma* ITS regions were amplified using the specific primers. The primer sequence illustrated in Table 1. 25 µL, 2.5 µL 10x buffer with MgCl, 2 µL2.5 mM dNTPS, 2 µL of 10 pmol forward primer, 2 µL of 10 pmol reveres primer, 50ng DNA template and 0.2 µL (2 units/µL) Taq DNA polymerase. The PCR amplification conditions consisted of denaturation at 95°C for 5 min, then 35 cycles as follows: 30 sec at 95°C for denaturation, 1 min at 30°C for annealing for 1 min and 1 min at 72°C for elongation. The PCR reactions were performed under the following conditions: 34 cycles of denaturation at 95°C (5 min), annealing temperature for both 16S and ITS illustrated in Table 1 and extension at 72°C (1min). A 350-bp product was amplified using the following primers.

**Screening of the Efficacy of the Isolated Bioagents against *F. oxysporum* and *R. solani*:** The antagonistic effect of the tested biocontrol agents against *F. oxysporum* f. sp. *radicis-lycopersici* and *R. solani* was examined. *Trichoderma* species, *R. solani* and *F. oxysporum* were cultured on Plates containing sterilized PDA medium for 7 days at 28-30°C. Then, a disc (0.5 cm in-diam.) of the antagonistic fungal colony was cut and placed opposite to the colony of the pathogen. Similar feature was carried out for bacterial test, a streak of the bacterial strain was placed on PDA plates at 30°C for 24h and then a mycelial disc (0.5cm) of the tested fungi was placed onto PDA plates at 0.5 cm distant from the bacterial colony. Three replicates were prepared in each experiment. Inoculated plates were incubated at 25°C until the fungal growth of the control plates reached the edge of the plate. The growth and reduction in mycelial growth of the pathogenic fungus was calculated [18].

**Efficiency of two Antagonistic Biocontrol Agents under Greenhouse Conditions:** A pot experiment was carried out to examine the *in- vivo* efficiency of bioagents against *F. oxysporum* and *R. solani* under greenhouse conditions. Antagonistic effect of *Trichoderma viride* and *Bacillus subtilis* on disease incidence of pathogenic fungi were tested on tomato cultivar (Castle Rock obtained from Agriculture Center Research, Alexandria, Egypt). A pot experiment was designed under greenhouse conditions using plastic pots (15 cm diameter) containing reasonable weight of sterilized soil clay. Soil was infested with *F. oxysporum* and *Rhizoctonia solani* before tomato planting. Pots were irrigated for 3 days before tomato planting and addition of biocontrol agents. The experiment included the following treatments: 1) non inoculated soil (control), 2) inoculated soil (with pathogenic fungi) (treated with *F.* oxysporum only, 3) soil treated with a combination of *F. oxysporum* + *B. subtilis*, 4) soil treated with *F. oxysporum*+ *T. viride,* 5) soil treated with *R. solani* only, 6) soil treated with *R. solani* +*B. subtilis* and 7) and finally soil treated with *R. solani*+ *T. viride*. To prepare virulent *Fusarium oxysporum* and *Rhizoctonia solani* inocula for infesting soil, 50 mL of sterile PDB medium in 250 mL Erlenmeyer flasks were inoculated with 5 mm plugs taken from a PDA Petri plates cultures (7days-old). Flasks were incubated without shaking in the dark at 25°C for 1 week. Mycelial mats, on the surface of the medium were then homogenized at 894 xg for 2 min in sterile water and mixed with the soil at the ratio of 20 mL of the homogenized mycelial mats to one pot (15cm diameter), 3 days before tomato planting. For inoculation of *Trichoderma viride* [T5] into soil, prepared by the same method and 20 mL of the homogenized mycelial mats added to pots, at tomato planting time. For inoculation of *B. subtilis* [B1] into soil, the bacterium was inoculated into nutrient broth medium and incubated overnight at 30°C with constant shaking at 200 rpm and then 20 mL of the cell suspension was added to each pot at tomato planting time. For each treatment, 3 pots were prepared and three plants were planted with using control for each treatment. Three pots containing three plants each were used as replicates for each particular treatment.

Table 2: Sequence of primers used in the real-time PCR.

| Primers | Primer sequence 3??5? | Annealing (°C) |
|---|---|---|
| Chitinase1(F) | TTC GTG GAG TGC ATC GGT | 60 |
| Chitinase 1(R ) | ATS TAC GCA ATA | |
| | ATA AGT ATT AGA | |
| Cellulase (3) | GAY GAR CAN GAR CAY | 60 |
| Cellulase (4) | TAN GCN CNN NCC NGG RTT | |
| Endogluconase 1(F) | TCC GGG GTA TGT | |
| | TAT GGA AGA | 60 |
| Endogluconase1(R) | CGG CGT CTG TTA | |
| | TGG AGG AAA | |
| Defensin F | CTT ATC AGA TCT | |
| | CAA TGG AGA AAT C | 60 |
| Defensin R | CAA TGT AAC TTA | |
| | AAG TGC CTA ATT ATG | |

Check treatment were pots containing infested soil only with either *F. oxysporum* or *R. solani* as three replicates for each. Diseases assessment and vegetative growth measurements and Enzymes activity are missed. The expression of some defense genes using Real Time PCR

**RNA Isolation and cDNA Synthesis:** Total RNA was extracted from plant tissue using GStract™ RNA isolation kit [22] Guanidium Thiocynate according to the manufacture method. Reverse transcription reaction was performed using oligo dT (USA). The 25 µL cDNA synthesis reaction consist of; 2.5µL (5x) buffer with $MgCl_2$, 2.5µL (2.5 mM) dNTPs, 1µL (10 pmol) primer, 2.5µL RNA (2mg/mL) and 0.5 unit reverse transcriptase enzyme. PCR amplification was performed in a thermal cycler (Applied Biosystems (ABI), USA) programmed at 42°C for 1h, 72°C for 10 min (enzyme inactivation) and the product was stored at 4°C until be used.

**Quantitative Estimation of Cellulase, Chitinase, Endogluconase and Defensin Gene Using Real Time PCR:** For samples analyzed, the RT-PCR reaction consists of, 12.5µL of 2x Quantitech SYBR® Green RT Mix Fermentas, Germany), 1µL of 25 pm/µL forward primer (Table 2), 1 µL of 25 pm/µL reverse primer, 2 µL cDNA (100 ng) 9.25 µL of RNase free water. Samples were spun before loading in the Rotor's wells. The real time PCR program was performed as follows: initial denaturation at 95°C for 10 min.; 40 cycles of 95°C for 15 sec.; annealing at 60°C for 30 sec and extension at 72°C for 30 sec. Data acquisition performed during the extension step. This reaction was performed using Rotor-Gene6000system (Qiagen, USA).

**Data Analysis:** Comparative quantification analysis was done using Rotor-Gene-6000 Series Software based on the following equation [22]:

Ratio target gene expression (Experimental/control) = Fold change in target gene expression (except /control)

Fold change in reference gene expression (except control)

**Statistical Analysis:** The significance of various treatments was evaluated by one way analysis of variance (ANOVA) showed that all the bacterial and *Trichoderma* isolates had high significant antagonistic effect ($P \leq 0.001$) on linear growth of *Rhizoctonia solani* and *Fusarium oxysporum* f.sp. *radicis-lycopersici*.

## RESULTS AND DISCUSSION

**Fingerprinting for the Bacterial and *Trichoderma* Isolates Using the RAPD -PCR:** With bacterial isolates the highest amplification was with the primer 16S (R) which generated 6 monomorphic bands and 57 polymorphic bands in total 63 RAPD patterns with the percentage of polymorphic 90.47% followed by the A9B7 which generated 6 monomorphic bands and 51 polymorphic band in total 57 RAPD patterns with 89.47% polymorphic. The approximately size of the largest fragment amplified was in the range of 1300 bp to 1200 bp, that was the primer 16s (R) and the smallest easily recognizable fragment amplified was approximately less than 100 bp of the isolates that was with the primer RAPD7. The obtained pattern for each isolate was scored for the presence or absence of each band. The presence or absence band among the isolates was called a band polymorphic, while it is a monomorphic band if it was present in all isolates.

**Identification for the Most Potent Bioagnet Bacterium:** The 350 bp region of the 16S rRNA gene were amplified for all the six bacterial isolates used to study the genetic differentiation between the six bacteria isolates. Approximately 350 bp. was amplified using universal primer 16SF and 16SR. PCR amplicon with specific forward 16S rRNA (350) primers of the six bacterial isolates were subjected to DNA sequence analysis. Sequence analysis revealed that the obtained nucleotide sequence is *Bacillus subtilis* and the isolate was deposit in gene bank under the accession number GU393320.

**Fungal Fingerprinting Using RAPD-PCR:** RAPD-PCR that used genomic DNA of *Trichoderma* isolates as a template with the five primers showed constantly different banding patterns with reproducible polymorphic bands that variable in size and number. The highest amplification

was with the primer RAPD 10 which generated 1 monomorphic band and 89 polymorphic bands in total 90 RAPD patterns with the percentage of polymorphic 98.88% followed by the A9B7 which generated 7 monomorphic bands and 87 polymorphic bands in total 94 RAPD patterns with 92.55% polymorphic.

**Identification for the Most Potent Bioagnet Fungal Isolate:** The DNA template of ITS regions (ITS1, ITS4) in rDNA of the fungi was amplified by PCR using the universal primers ITS1/ITS4. ITS gene was amplified for *Trichoderma* isolate which show high biocontrol activity (*Trichoderm viride* T5). Approximately 600 bp was amplified and subjected to DNA sequence analysis. And the resulting nucleotide sequence was aligned in NCBI gene bank and take accession number HQ438699.

**Evaluation of *Trichoderma* and Bacterial Strains for Antagonistic Activities against *F. oxysporum* and *Rhizoctonia solani in vitro:*** The antagonism in dual cultures and the interaction between the screened *Trichoderma*, bacterial isolates and the tested pathogens were the first explored points to evaluate their mycoparasitic activity. Initially results of studding the mycoparasitic activity using dual culture interactions showed that most of the investigated *Trichoderma* isolates were able to suppress the radial growth of the pathogens against *F. oxysporum* and *R. solani* on Petri dishes containing PDA medium. Tables 3 and 4 show that the *Trichoderma* isolates succeeded in reducing the radial growth of *F. oxysporum* and *R. solani* where *T. viride* (T5) was the most aggressive isolate with growth inhibition reached (79.6%) and (76.6 %) respectively.

Moreover, *B. subtilis* (B1) was more active bacterial isolate for reducing the radial growth of *F. oxysporum* and *R. solani* with growth inhibition reached 63.16% and 76.195% respectively comparing with control from *F. oxysporum* and *R. solani*. This result came in agreement with the antagonistic activity of *T. harzianum* and *T. longibrachiatum in vitro* against *R. solani*, the cause of cotton seedlings damping-off disease, in dual culture and found that all isolates of *Trichoderma* spp. showed highly significant antagonistic interaction with *R. solani* [23, 24]. Also a laboratory experiment on the comparative antagonistic performance of *T. harzianum* and *T. viride* against *Rhizoctonia solani* and found that, *T. harzianum* exhibited maximum (75.55%) mycelial growth inhibition of *R. solani* followed by *T. viride*, which showed (65.93%) mycelial growth inhibition of the pathogen [24]. Also, this is accordance with all the biological agents include *T. viride*, *T. harzianum* and *T. virens* inhibited the growth of *Fusarium oxysporum* f. sp. *ciceris* in dual culture technique. Growth inhibition ranged from 14.8% with *T. virens* to 43.4% with *T. harzianum* [24]. Similarly, the study of the direct interaction on PDA medium of remote interaction between *F. oxysporum, radicis-lycopersici* and *T. harzianum* revealed that the latter inhibited mycelial growth of the pathogen by more than 65% compared to the control [25].

**Efficiency of two Antagonistic Biocontrol Agents under Greenhouse Conditions:** Antagonistic effect of *B. subtilis* and *T. viride* against *in vivo* growth of phytopathogenic fungi revealed that *B. subtilis* and *T. viride* have the ability in reducing disease incidence and severity levels on tomato plants (symptoms severity, number of dead

Table 3: Effect of bacterial isolates on the radial growth of *Rhizoctonia solani* and *F. oxysporum*

| Identified the isolates from 1-6 | Linear growth (cm) of *R. solani* | Growth reduction % of *R. Solani* | Linear growth (cm) of *F. oxysporum* | Growth reduction % of *F. oxysporum* |
|---|---|---|---|---|
| B1 | 3.37±0.57 | 76.195 | 3.70±0.26 | 63.16 |
| B2 | 3.27±0.25 | 44.14 | 2.77±0.25 | 55.33 |
| B3 | 3.67±0.15 | 50.23 | 2.83±0.29 | 54.49 |
| B4 | 2.87±0.32 | 40.38 | 1.80±0.26 | 42.86 |
| B5 | 1.57±0.06 | 21.76 | 1.70±0.26 | 34.69 |
| B6 | 3.07±0.15 | 41.44 | 0.80±0.26 | 19.05 |

Each value is the mean of three replicate ± standard deviation.

All results are highly significant at P=0.001 using one way analysis of variance (ANOVA).

The statistical analysis performed according to the following equation:

R= [(A-B)/A] x100

Where:

R=percentage of growth reduction

A=Mycelial growth of the pathogenic fungus

B=Mycelial growth of the pathogenic fungus towards the antagonistic fungus

Table 4: Effect of *Trichoderma* isolates on the radial growth of *R. solani* and *F. oxysporum*

| Identified the isolates from 1 | Linear growth (cm) of *R. solani* ($R^2$) | Growth reduction (%) *R. solani* ($R^2$) | Linear growth (cm) of *F. oxysporum* | Growth reduction (%) *F. oxysporum* |
|---|---|---|---|---|
| Identified the isolates from 1 | 2.43±0.06 | 67.12 | 1.53±0.47 | 61.75 |
| Identified the isolates from 1 | 2.90±0.10 | 60.81 | 1.40±0.20 | 58.82 |
| Identified the isolates from 1 | 2.20±0.44 | 69.86 | 1.40±0.26 | 60 |
| Identified the isolates from 1 | 2.23±0.51 | 50.44 | 0.17± 0.21 | 62.25 |
| Identified the isolates from 1 | 1.53±0.06 | 79.6 | 0.70±0.26 | 76.6 |
| Identified the isolates from 1 | 1.63±0.46 | 77.36 | 0.17± 0.21 | 62.25 |
| Identified the isolates from 1 | 2.50±0.26 | 44.44 | 1.70±0.30 | 51.42 |
| Identified the isolates from 1 | 2±0.10 | 72.22 | 1.70±0.30 | 51.42 |
| Identified the isolates from 1 | 2.7±0.35 | 64 | 1.80±0.26 | 59.90 |
| Identified the isolates from 1 | 2.20±0.36 | 65.8 | 1.53±0.31 | 56.28 |

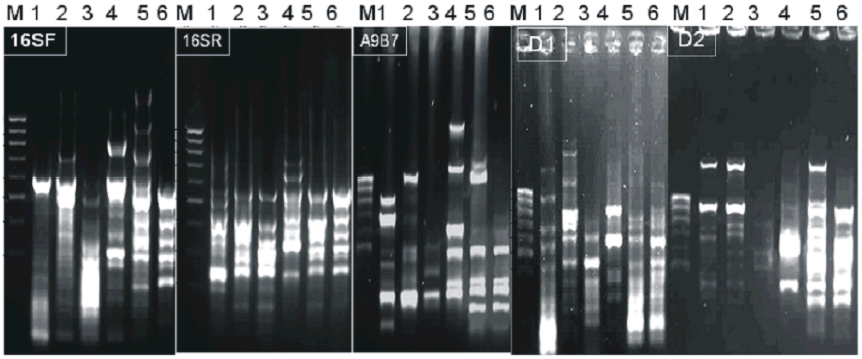


Fig. 1: RAPD-PCR for the 6 bacterial isolates using five different arbitary primers. Lanes, M: 1kbp DNA marker ranged from 1000bp to 100bp. Lanes from 1to 6 indicated for the six examined bacterial isolates. Primer name as indicated in the right corner of each picture.

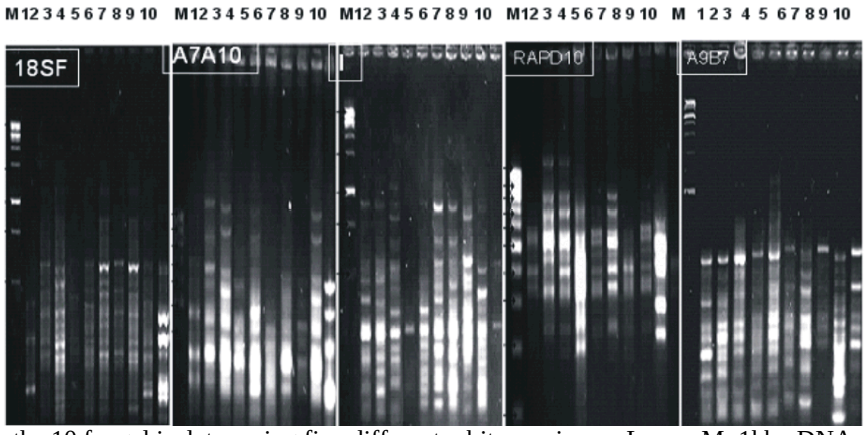


Fig. 2: RAPD-PCR for the 10 fungal isolates using five different arbitary primers. Lanes, M: 1kbp DNA marker ranged from 1000bp to 100bp. Lanes from 1to 6 indicated for the six examined bacterial isolates. Primer name as indicated in the right corner of each picture.

plants and growth rate) infected with *F. oxysporum* f. sp. *lycopersici* and *Rhizoctonia solani*. But the disease incidence and severity was detected on the tomato plants cultivated in soil infected with *F. oxysporum* f. sp. *lycopersici* and *R. solani* without adding the bioagents *B. subtilis* and *T. viride*. Where *T. viride* and *B. subtilis* stimulated the growth of tomato plants (the plant height, number of leaves, symptomless appearance and etc) compared to the control of tomato plants infected with *F. oxyspoum* f. sp. *lycopersici* and *R. solani* as a plant pathogen and control of healthy plant. This may be due to plants react to pathogen attack by the induction of a battery of defense responses suggesting that protective mechanisms may have complementary roles in the overall

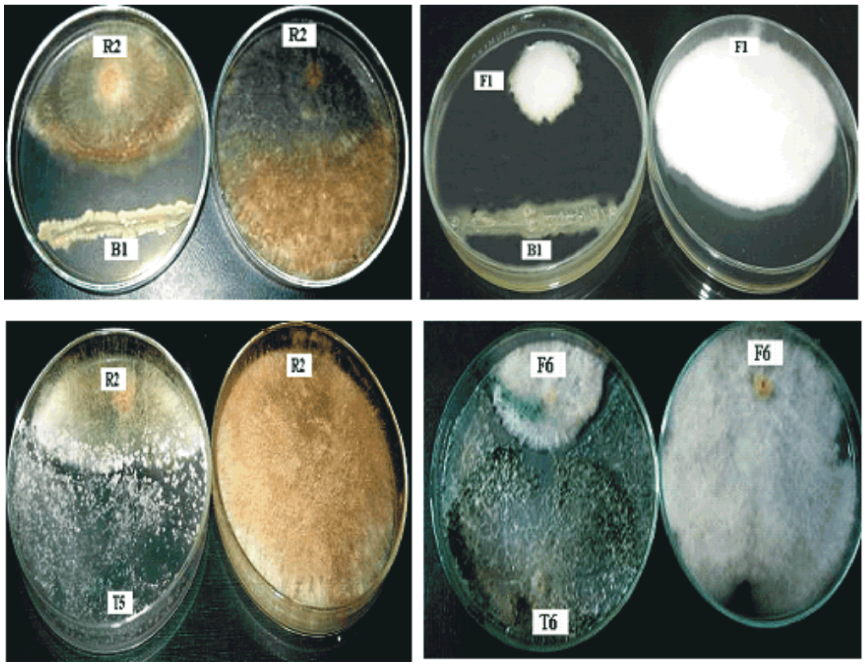


Fig. 3: Antagonistic effect of the tested *T.viride* (T5) and *B. subtilis*(B1) on the linear growth of *F. oxysporum* (F1)and *R. solani* (R2) in dual culture test on PDA medium after seven days of incubation at 25°C.

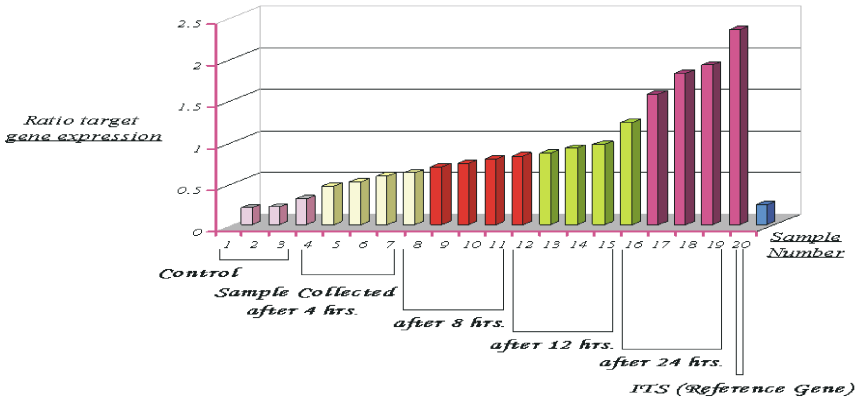


Fig. 4: Histogram of quantitative estimation of cellulase gene expression during amplification of (cellulase) defense gene.

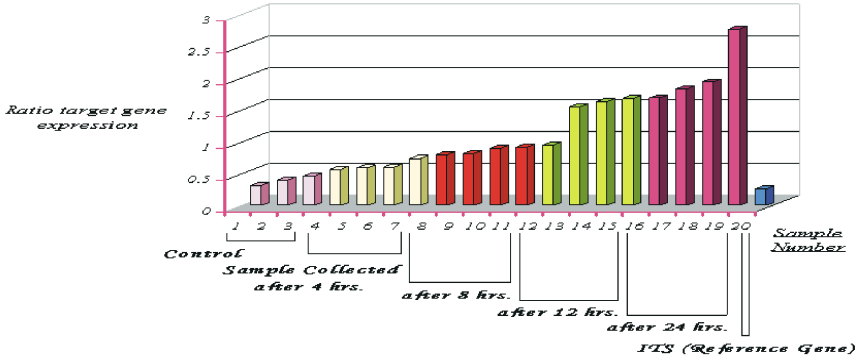


Fig. 5: Histogram of quantitative estimation of chitinase gene expression during amplification of chitinase defense gene.

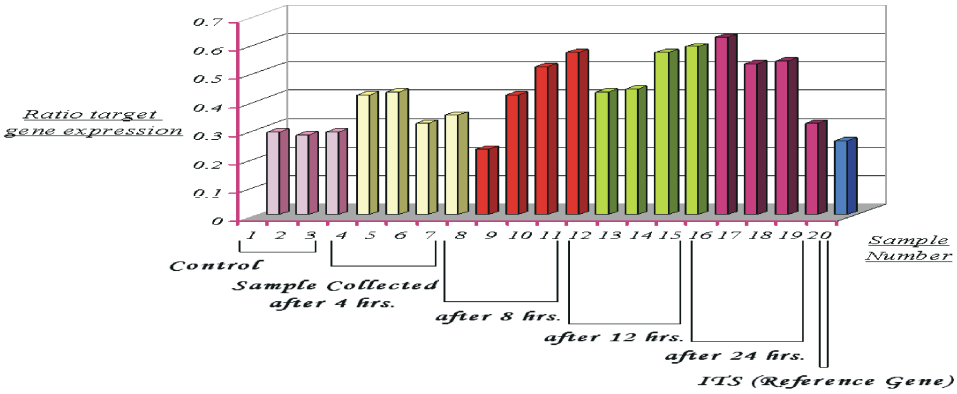


Fig. 6: Histogram of quantitative estimation of defense gene expression during amplification of defense gene.

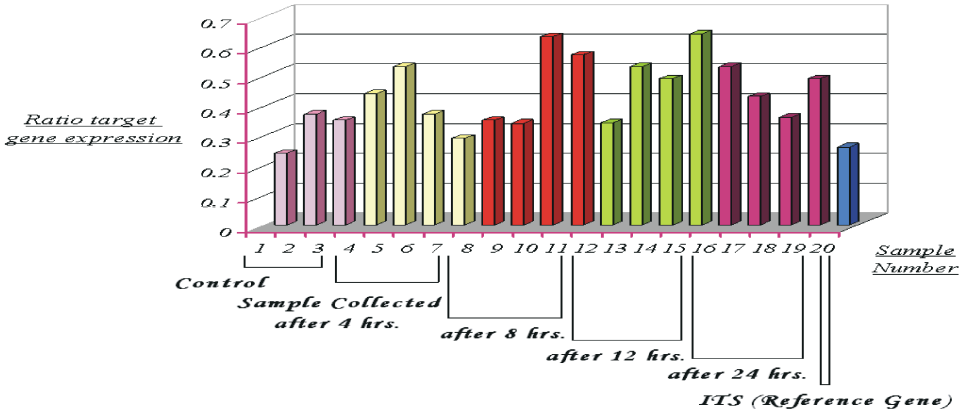


Fig. 7: Histogram of quantitative estimation of endogluconase 1 gene expression amplification of endogluconase 1 gen

expression of disease resistance [26]. Plant defense response to non-specific facultative pathogens takes place in four important phases. The first phase of fungal infection occurs when the fungal propagule makes contact with the surface of a plant structure (lodicules, lemmal tissue and ovary base of the spikelets). Even before penetration has taken place, signaling mechanisms within the affected and surrounding cells detect the presence of the fungus [27, 28]. When a breach in cell wall/membrane occurs, a second phase is established, where generalized stress response/infection recognition occurs. These responses include activation of genes in several pathways that can combat spread of the pathogen [29]. Examples include the synthesis of phytoalexins, that is, compounds that are toxic or fungi static and synthesis of lignin to strengthen cell walls and consist of enzymes that function to degrade fungal cell walls, including $-glucanases and chitinases [30]. A defense response in the plant is often mounted primarily in response to the soluble cell wall degradation products which act as elicitors. A particularly well-studied aspect of plant resistance is the pathogenesis-related (PR) protein response [31]. PR proteins produced in response to pathogen attack, as well as in response to treatment with elicitors and a biotic factor. Real-time PCR, which combines the advantages of conventional PCR with quantitative capability [31] is, at present one of the most sensitive and accurate method for the detection and quantification of gene expression [32]. Its high sensitivity allows the quantification of rare transcripts and small changes in gene expression [33]. In this study real-time PCR was used to detect PR proteins in response to pathogen attack, as well as in response to treatment with elicitors and biotic factors (*Trichoderma* and *Bacillus*). Chitinase genes are differentially regulated in response to development or by colonization of plant tissues by micro-organisms [34] and as well as the cellulase gene. Where the relative amounts of mRNA detected from target genes (cellulase, chitinase, endogluconase and defense gene) present in samples collected post inoculation for each treatment by *T. viride* and *B. subtilis* at time (4, 8, 12 and 24h) were compared with the amount of mRNA that in control of *F. oxysporum* f. sp. *radicis-lycopersici* and *R. solani* and the results

normalized to ITS gene (reference gene) show high level of gene expression. Using 6 sets of arbitrary primers combinations, arbitrary degenerate primers (cellulase1), (cellulase2), (cellulase3), (Chi 15), (Ns2) and (endogluconase 1F) were used in fingerprinting and demonstration the genetic variation between the examined bacterial and fungal isolates. The major observation was an increase in genetic variations between control and treated samples. In addition, many down-regulated genes (turned off) and up-regulated genes (turned on) were observed in both samples treated with *T. viride* and *B. subtilis* when the samples collected at different times after addition of bioagents.

The relative amounts of mRNA detected from target genes (cellulase, chitinase, endogluconase and defense gene) present in plant samples collected post inoculation for each treatment by *T. viride* and *B. subtilis* at time intervals (4, 8, 12 and 24h) were compared with the amount of mRNA that in control *F. oxysporum* and *R. solani* and the results normalized to ITS gene (reference gene or house keeping gene). Where the highest level of gene expression (gene copy number) of cellulase gene was in tomato plants treated with *T. viride* than the other treated with *B. subtilis.* Moreover, the level of gene expression was high at the tomato plants infected with *F. oxysporum* and treated with *T. viride* and tomato plants infected with *R. solani* and treated *T. viride* where it was (1.921) and (2.35) after 24 h, respectively when compared with the tomato plants contain control *Fusarium* which was (0.208) and control *Rhizoctonia* which was (0.31). Meanwhile, the level of chitinase gene expression was high after 24 hr post inoculation where the level of gene expression was (1.932) in tomato plants infected with *F. oxysporum* and treated with *T. viride*. Also the level of chitinase gene expression was (2.76) with plants infected with *R. solani* and treated with *T. viride*. While the lowest level of gene expression obtained in case of plants infected with *F. oxysporum* and treated with *Bacillus subtilis* and *Rhizoctonia solani* and treated with *B. subtilis* was 0.567 and 0.587 compared to *Fusarium* and *Rhizoctonia* control which was (0.394, 0.456). Also the quantitative estimation of defense gene expression in plants infected with *F. oxysporum* and treated with *B. subtilis* was (0.623), while the level of defense gene expression in plants infected with *R. solani* and treated with *Bacillus subtilis* was (0.53) these after 24h from inoculation of *B. subtilis*. And the level of defense gene expression also increase in tomato plants treated with *Trichoderma viride* where, the level of gene expression show high level of defense gene after 12 hr in tomato plants infected with *Fusarium oxysporum* and treated with *T. viride* was (0.57). And it was (0.59) in tomato plants infected with *R.solani* and treated with *Trichoderma viride* compared with *Fusarium* and *Rhizoctonia* control which was 0.28 and 0.29. Meanwhile, the quantitative estimation of endogluconase PR gene were estimated where, the level of endogluconase 1 gene expression was (0.63) in tomato plants infected with *F. oxysporum* and treated with *T. viride* and was 0.57 in plants infected with *Rhizoctonia solani* and treated with *T. viride* after 8h. While, the level of gene expression in plants infected with *Fusarium oxysporum* and treated with *B. subtilis* was 0.53 and was 0.43 in plants infected with *R. solani* and treated with *B. subtilis* after 12h compared with *F. oxysporum* and *R. solani* control which was 0.37 and 0.35.

From the aforementioned results, it could be conclude that the dual treatment with *B. subtilis* and *T. viride* has a significant and more feasible to control root rot and wilt disease pathogens *in vitro* and decrease the symptoms of the disease (data not presented) comparing with the individual treatments, because of their potentialities to produce plant growth promoting substances [35, 36] and induce PR proteins in response to pathogen attack, as well as in response to treatment with elicitors. The plant systemic resistance was induced due to the presence both of *Trichoderma* isolates and *Bacillus* isolates as bioagents. On the other hand many microorganisms activate pathogenesis-related protein synthesis before the pathogen invades the host plant [37]. The induction of plant resistance mechanisms by different *Trichoderma* strains has been shown to enhance the production of defense-related metabolites in the plant such as enzymes involved in the response to oxidative stress and pathogensis-related proteins [38, 39].

## REFERENCES

1. FAO, 2008. FAOSTAT database. Food and Agriculture Organization of the United Nations, Rome, Italy.http://faostat.fao.org.
2 Montealegre, J.R., R. Herrera, J.C. Velasquez, P. Silva, X. Besoain and L.M. Perez, 2005. Biocontrol of root and crown rot in tomatoes under greenhouse conditions using *Trichoderma harzianum* and *Paenibacillus entimorbus*. Additional effect of solarization. Electronic Biotech., 8: 249-257.
3. Srinon, W., K. Chuncheen, K. Jirattiwarutkul, K. Soytong and S. Kanokmedhakul, 2006. Efficacies of antagonistic fungi against *Fusarium* wilt disease of cucumber and tomato and the assay of its enzyme activity. J. Agric. Technol., 2: 191-201.

4. Papavizas, G.C., 1985. *Trichoderma* and *Gliocladium*: biology, ecology and potential for biocontrol. Ann. Rev. Phytopathol., 23: 23-54.
5. Monte, E., 2001. Understanding Trichoderma: between biotechnology and microbial ecology. Int. Microbiol., 4: 1-4.
6. Kucharek, T., J.P. Jones, D. Hopkins and J. Strandberg, 2000. Some diseases of vegetables and agronomic crops caused by Fusarium in Florida. Florida Cooperative Extension Service/Institute of Food and Agriculture Sciences/ University of Florida. Circular, 1025.
7. Jarvis, W.R., 1988. *Fusarium* crown and root rot of tomatoes. Phytoprotection, 69: 49-64.
8. Marten, M., M. Guizzardi and G.C. Pratella, 1996. Biological control of gray mold in pears by antagonistic bacteria. Biol. Control, 7: 30-37.
9. Truter, M., 2005. Etiology and alternative control of potato rhizoctoniasis in South Africa. M.Sc. thesis, Fac. Nat. and Agric. Sciences, Pretoria Uni., 36: 87-93.
10. Huang, C.J., T.K.Wang, S.C. Chung and C.Y. Chen, 2005. Identification of an antifungal chitinase from a potential biocontrol agent *Bacillus cereus* 28-9. J. Biochem. Mol. Biol., 38: 82-88.
11. Soytong, K., W. Srinon, K. Ratanacherdchai, S. Kanokmedhakul and K. Kanokmedhakul, 2005. Application of antagonistic fungi to control anthracnose disease of grape. J. Agric. Technol., 1: 33-42.
12. Zaghloul, R.A., A. Hanafy Ehsan, N.A. Neweigy and A. Khalifa Neamat, 2007. Application of biofertilization and biological control for tomato production.12$^{th}$ Conference of Microbiology; Cairo, Egypt, (18-22) March, pp: 98-212.
13. Yehia, A.H., S.A. EI-Hassan and A.H. EI-Bahadli, 1982. Biological seed treatment to control Fusarium root-rot of broad bean. Egypt. J. Phytopathol., 14: 59-66.
14. Gindrat, D., 1979. Biocontrol of Plant Diseases by Inoculation of Fresh Wounds, Seeds and Soil with Antagonists In: Soil-Borne Plant Pathogens (B. Schippers and W. Gams, Eds.), Academic press, New York, pp: 537-551.
15. Di Pietro, A., M. LOrito, C. K. Hayes, R. M. Broadway and G.E. Harman, 1993. Endochitinase from *Gliocladium virens*: Isolation, characterization and synergistic antifungal activity in combination with gliotoxin. Phytopathology, 83: 308-313.
16. Lorito, M., G.E. Harman, C.K. Hayes, R.M. Broadway, A. Tronsmo, S.L. Woo and A. Di Pletro, 1993a. Chitinolytic enzymes produced by *Trichoderma harzianum*: Antifungal activity of purified endochitinase and chitobiodase. Phytopathology, 83: 302-307.
17. Miigheli, Q., L. Gonzalez-Candelas, A. Camponogara and D. Ramon-Vidal, 1998. Transformants of *Trichoderma longibrachitum* over expressing the B-1, 4-endogluconase gene egl1 show enhanced biocontrol of *Pythium ultimum* on cucumber. Phytopathology, 88: 673-677.
18. Broñovà, J., 2004. Mycoparasitic Fungi Trichoderma spp. in plant protection. Plant Protect. Sci., 40: 63-74.
19. Dennis, C. and J. Webster, 1971b. Antagonistic properties of species-groups of Trichoderma. II. Production of volatile antibiotics. Trns. Br. Mycol. Soc., 57: 25-39.
20. Aziz, N.H., M.Z. El-Fouly, A.A. El-Essawy and M.A. Khalaf, 1997. Influence of bean seedling root exudates on the rhizosphere colonization by *Trichoderma lignorum* for the control of *Rhizoctonia solani*. Bot. Bull. Acad. Sin., 38: 33-39.
21. Sneh, B., B Lee and O. Akira, 1991. Identification of *Rhizoctonia* species. The American Pytopathological Society, St. Paul, Minnesota, USA. pp: 129.
22. Rasmussen, R., 2001. Quantification on the Light Cycler. In: Rapid Cycle Real-time PCR, Methods and Applications (S. Meuer, C. Wittwer and K. Nakagawara, Eds.), Springer Press, Heidelberg, pp: 21-34.
23. Asran, A.A., K.A. Abd-Elsalam, M.R. Omar and A.A. Aly, 2005. Antagonistic potential of *Trichoderma* spp. against *Rhizoctonia solani* and use of M13 microsatellite-primed PCR to evaluate the antagonistic genetic variation. Zeitschrift - für - Pflanzenkrankheiten - und - Pflanzenschutz, 112: 550-561.
24. Gagwar, R.K., R.K. Prajapati and K. Kumar, 2004. Evaluation of fungal antagonists against *Fusarium oxysporum* f.sp. *ciceris*. Ann. Plant Protect. Sci., 12: 444-445.
25. Hibar, K., D.M. Remadi, H. Khiareddine and M. El-Majoub, 2005. *In vitro* and in vivo antagonistic effect of *Trichoderma harzianum* against *Fusarium oxysporum* f.sp *radicis lycopersici*. Biotechnol. Agron. Soc. Environ., 9: 163-171.

26. Zhou, N., T.L. Tootle and J. Glazebrook, 1999. Arabidopsis PAD3, a Gene Required for Camalexin Biosynthesis, Encodes a Putative Cytochrome P450 Monooxygenase Plant Cell, 11: 2419-2428.
27. Collings, B.D. and A.J. Slusarenko, 1987. Plant gene expression in response to pathogens. Plant Mol. Biology, 9: 389-410.
28. Graham, T.L. and M.Y. Graham, 19991. Cellular coordination of molecular responses in plant defense, Mol. Plant Microbe In., 4: 415-427.
29. Seetharaman, K., R.D. Waniska and L.W. Rooney, 1996. Physiological changes in sorghum antifungal proteins. J. Agric. Food Chem., 44: 2435-244.
30. Darnetty, J., J.F. Leslie, S. Muthukrishnan, M. Swegele, A.J. Vigers and C.P. Selitrennikoff, 1993. Variability in antifungal proteins in the grains of maize, sorghum and wheat. Physiol. Plantarum, 88: 339-349.
31. Selitrennikoff, C.P., 2001. Antifungal proteins. Appl. Environ. Microbiol., 67: 2883-2894.
32. Simpson, D.A., S. Feeney, C. Boyle and A.W. Stitt, 2000. Retinal VEGF mRNA measured by SYBR Green I fluorescence a versatile approach to quantitative PCR. Mol. Vis., 6: 178-183.
33. Giulietti, A., L. Overbergh, D. Valckx, B. Decallonne, R. Bouillon and C. Mathieu 2001. An overview of real-time quantitative PCR: Applications to quantify cytokine gene expression. Methods, 25: 386-401.
34. Kim, B.R., H.Y. Nam, S.U. Kim, S.I. Kim and Y.J. Chang, 2003. Normalization of reveres transcription quantitative-PCR with housekeeping genes in rice. Biotechnol. Lett., 25: 2869-1872.
35. Salzer, P., A. Bonanomi, K. Beyer, R. Vogeli-Lange, R.A. Aeschbacher, J. Lange, A. Wiemken, D. Kim, D.R. Cook and T. Boller, 2000. Differential expression of eight chitinase genes in *Medicgo truncatula* roots during Mycorrhiza formation. Nodulation and pathogen infection. MPMI, 13: 763-777.
36. Bochow, H., S.F. El-Sayed, H. Junge, A. Stavropoulou and G. Schmiedeknecht, 2001. Use of *Bacillus subtilis* as biocontrol agent. IV. Salt stress tolerance induction by *Bacillus subtilis* FZB 24 seed treatment in tropical vegetables field crops and its mode of action. *Z. PfiKrankh.PflSchuez*, 108: 21-30.
37. Morsy, Ebtsam, M., 2005. Role of growth promoting substances producing microorganisms on tomato plant and control of some root rot fungi. Ph.D. Thesis, Fac. of Agric. Ain Shams Univ. Cairo.
38. Harman, G.E., C.R.Howell, A. Viterbo, I. Chet and M. Lorito, 2004. *Trichoderma* species-opportunistic, virulent plant symbionts. Natl. Rev. Microbiol., 2: 43-56.
39. Harman, G.E., 2000. Myths and dogmas of biocontrol: Changes in perceptions derived from research on *Trichoderma harzianum* T-22. Plant Dis., 84: 377- 393.