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Isolation of Endophytic Actinomycetes From Leaves of Tomato Plants and Their Activities Against Bacterial Speck Disease

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Abstract

The biological control agents of endophytic actinomycetes was investigated to determine whether control of bacterial speck of tomato, caused by *Pseudomonas syringae* pv. *tomato* (*Pst*). From natural infected tomato leaves showing bacterial speck disease symptoms, six isolates of *Pst* (Pst1, Pst2, Pst 3, Pst4, Pst5, and Pst DC3000) were isolated, it proved to be pathogenic and able to infect tomato plants under greenhouse condition, causing speck disease symptoms. From the eighty eight isolates of endophytic actinomycetes recovered from healthy tomato leaves plants, nine isolates were selected and tested for their potential inhibition against bacterial tomato speck disease *in vitro* and *in vivo*. According to16SrDNA analysis the isolates TR-24 and TR-26 of isolated actinomyces proved to be *Streptomyces glaucescens*. While the isolates TR-20, TR-32 and TR-34 were identified as *Streptomyces graminisoli and isolates* TR-25 identified as *Streptomyces flavovirens as well as* isolates TR-33 and TR-35 were identified as *Streptomyces scabies*. Finally isolate TR-36 was identified as *Streptomyces roseochromogenus*. Application of nine isolates of *Streptomyces* by spraying or drenching methods, all *Streptomyces* isolates were able to reduce disease severity of bacterial speck compared to infected control. Minutely, the spore suspensions of TR-24 and TR-26 isolates gave the highest reduction of the disease severity. However, the lowest reduction was achieved by isolate TR-35 compared with other Streptomyces isolates.

Keywords: *Biological control, Bacterial speck, Tomato, Endophytic actinomycetes, Streptomyces.*

Introduction:

Bacterial speck disease on tomato caused by *Pseudomonas syringae* pv. *tomato (Pst)* is an economically important disease in many tomato growing region worldwide (Shenge *et al.* 2007). *P. syringae* pv. *tomato* is seed borne disease (Bashan, 1997) and can spread through tomato transplant fields. As result of infection the bacterial pathogen causes necrotic lesions on the leaves, stems, and fruit of tomato plants (Goode and Sasser, 1980). Fruit lesions are slightly raised and vary in size from tiny flecks to visible lesions approximately 3 mm in diameter (Goode and Sasser, 1980). Infected tomato fruit become unattractive and unsuitable for sale on fresh market or for processing.

The traditional methods for controlling tomato speck disease are not fully effective and are expensive. Chemical bactericides, are routinely used to control this disease. However, excessive dependence on chemical bactericide frequently causes envi-

ronmental pollution and outbreaks of resistant pathogens (Hastuti *et al.* 2012). Therefore, the use of microbe-based biocontrol agents such as endophytic bacteria belonging to the actinomycetes group has been pursued as an alternative replacement or supplement for chemical bactericides. They can be isolated from internal plant tissues after thorough surface-disinfection of the plant tissue, either from herbaceous or woody plants (Taechowisan *et al*. 2003; Cao *et al*. 2004; Inderiati and Franco 2008). Some researchers reported that actinomycetes are capable of suppressing the development of diseases caused by plant pathogenic bacteria or fungi (Hasegawa *et al*. 2006; Lestari 2006). Very few investigations were done on study the use of endophytic actinomycetes as biological control agent against bacterial diseases of plants. However, several researches have tested such endophytic organism against fungal diseases and concluded that the use of such organisms exhibited pronounced % of infection of certain fungal diseases in vitro and decreased the disease incidence in vivo (Inderiati and Franco, 2008; Cao *et al.* 2004; Crawford *et al*. 1993).

Endophytic microorganisms are microbes that colonized inside plant tissues with symptomless to their hosts. The most frequently isolated endophytes are fungi, however, both gram positive and negative bacteria can be found as endophytes (Bacon and Hinton, 2007). The endophytic actinomycetes which are associated with plants also play important role in protection their host from phytopathogenic invasions (Crawford *et al.,* 1993). Several endophytic actinomycetes act as plant growth promoter by producing of phytohormone, indole-3-acetic acid (IAA) or iron chelating molecules (Indananda *et al.,* 2011). Endophytic actinomycetes probably produce antimicrobial metabolites within their host plants, for example *Streptomyces* species, are well-known for their ability to produce biologically active secondary metabolites, particularly antibiotics (Loria *et al*., 1997). Endophytic microorganisms also provide advantages to the host plant by enhancing the physiological activity of the plant or through other modes of action and thus may serve as a source of agroactive compounds, biocontrol agents, or plant growth promoters (Doumbou *et al*., 2001; Shimizu *et al.,* 2009). There are a dearth of using the biological control agents such as endophytic actinomycetes, against tomato speck disease agent, *Pseudomonas syringae* pv. *tomato*. Therefore, the aims of the present work are isolation and identification of actinomycetes isolates from healthy tomato plants and studying the possibility of using actinomycetes species to control of tomato speck disease under greenhouse condition.

Material and Methods:

1-Isolation and identification of pathogen.

Natural infected tomato leaves showing bacterial speck disease symptoms were collected from different localities of Assiut and El-Minia governorates. Samples were collected and keep in dry pages during transformation to the laboratory. Diseased tomato leaves were washed with tab water several times and sterilized in 2% sodium hypochlorite so-

lution for 3 minutes, rinsed twice in sterilized distilled water. Small portion of the diseased tissues were macerated with 5 ml of sterilized 0.05 M potassium phosphate buffer in sterilized pestle and mortar, after 10 minutes a loopful of the resulting suspension was streaked onto Nutrient Sucrose Agar medium (NSA) (contained 5.0 gm peptone, 3.0 gm beef extract, 3.0 gm glucose, 20.0 gm agar, 1litre D.W; pH was adjusted to 7.2) (Dowson, 1957). Plates were incubated at 28°C for 48 h, and then examined for bacterial growth development. The single colony technique was used to obtain pure culture. Single colony of the isolates was subcultured onto NSA in tubes and maintained at 4°C for further studies. Also, the stock cultures of the isolates were stored in -80°C at 10% glycerol for further used. *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) were cultured in 100 ml liquid King's B medium (kindly provided by Prof. Mitsuro Hyakumachi, Head Department of Plant Pathology, Gifu University, Japan). King's B medium constituted of 2 gm protease peptone, 1.5 gm anhydrous K_2HPO_4, 1.5 gm $MgSO_4$,15 ml glycerol, 1000 ml D.W; autoclaved at 121°C for 20 min. (Klement *et al*.,1990).

Bacterial isolates proved to be pathogenic and caused symptoms to tomato plants were identified according to their morphological, cultural and physiological characteristics using the tests described by Lelliott and Stead (1987); Krieg and Holt (1984) and Krieg *et al*. (2005).

2- Pathogenicity test:

2.1 Tomato plant preparation

Pathogenicity of bacterial isolates was tested on tomato cv. Rika. Tomato seeds (*Solanum lycopersicum* var. *pentarosa*) were surface disinfected by immersing in 70 % ethanol for 2 min, washed by sterilized DW, immersed again in 2% Sodium hypochlorite, washed three times with sterilized DW to remove the resident chemicals. Tomato seeds were grown on green containers pots contained sterilized commercial non-soil mix (Napura, Yanmar Co., Ltd., Osaka, Japan) in growth chamber 25°C for 3 weeks 12h/12h light dark cycle (till 3-4 true leaf stage).

2.2 Bacterial inoculum preparation

All obtained isolates were tested for pathogenicity on tomato plants. Bacterial inoculums were prepared as follow: 100 µl of bacterial suspension was cultured in100 ml liquid King's B medium (Klement *et al*.,1990), and incubated at 28°C on a rotary shaker (200rpm) for 24h. Cells were collected by centrifugation at 3000rpm for 10 min, washed twice and resuspended in 10mM $MgSO_4$. Bacterial suspensions were adjusted to a concentration of 2×10^8 CFU/ml using spectrophotometer.

Four-week -old tomato plants were inoculated by spraying each plant with 30 ml of bacterial suspension mixed in 0.01% (v/v) Silwet L-77 (Nihon Unica, Tokyo, Japan) as detergent onto the tomato leaves until run-off. The inoculated plants were kept at 100% relative humidity in darkness for 2 days for disease development. Plants were then transferred to a growth chamber. Five days after pathogen challenge, disease severity was measured for each plant by re-

cording the percentage of total plant leaf surface showing symptoms using a 1 to 5 scale (Yunis *et al*., 1980) with some modification: 0= no lesion, 1=2-5specks together or spread all over the leaf 2=10-20 speck for leaf; 3= more than 20 speck for leaf with some coalescing; 4 = more than 25 and defoliation; 5 dead leaves

Diseases severity was calculated by the following formula:

DS% =AVR1+AVR2+AVR3/HX100

AVR = n1+n2+n3/3

AVR=Average of total spots in each replicate (3 plants)

n = Number of scale in each leaf on one plant/ total number of leaf sample.

H = the highest scale of disease severity. As an untreated control, leaves sprayed with MgSO4 suspension mixed with 0.01% (v/v) Silwet L-77. Three replicates were used for each treatment, and each replicate consisted of three plants. The experiments were repeated twice.

3-Isolation of actinomycetes from healthy tomato plants

Tomatoes seeds (*Solanum lycopersicum* var. *rika*) were surface sterilization as follow, seeds were kept in sterile beaker consists of 70% ethanol for 1-3 min. After that the ethanol sterilized plant parts were kept in beaker consist of sodium hypochlorite (5%) with tween 20 (0.1%) for 5 minutes. Seeds were rinsed with sterilized water for three times. Sterilized tomato seeds were grown in the greenhouse pots (15cm) at 25^{o}C, 12 hours light/daily for two months (mature stage). Healthy tomatoes leaves were collected and kept in the room temperature at 25^{o}C for 24h to avoid the growth of saprophytic fungi. Then, 0.5 gram of tomato leaves was sterilized as follows; leaves were first rinsed in sterilized DW then dipped in a sterile beaker containing 70% ethanol for 1-3 min, then kept in a beaker containing mixture of Sodium hypochlorite (5%) with tween 20 (0.1%) for 5 minutes, and lastly rinsed in sterilized water three times, followed by 70% ethanol for 1 minute. Sterilized plant parts were crushed in 5 ml DW using sterile pestle and mortar, ten serial dilution was prepared using plant suspension (10, 10^{-1},10^{-2},10^{-3}, 10^{-4}, 10^{-5}). 100 µl of each dilution were aseptically spread with sterile glass rod over the surface of Petri dishes containing Humic acid-Vitamin Agar (HVA) medium (Humaic acid 1.0 g, Na2HPO4 0.05 g, CaCo3 0.02 g, Mgso 4.7 H_2O 0.05 g, KCL 1.71 g, FeSO4.7H_2O 0.01g, B-Vitamin10 ml, Nalidixic acid1ml, Cycloheximide 3ml, Trimethoprim 2ml, agar 18 g, 1000ml DW, pH7.2) (Hayakawa and Nonomura, 1987). Plates were incubated at 30^{o} C for three weeks. Individual colonies with characteristics of actinomycete morphology were detected and actinomycete colonies were transferred to a membrane filter (mixed cellulose-ester, pore size, 0.2 µm; Advantec, Tokyo, Japan) placed on the surface of HVA agar medium and incubated at 30°C for further 15 days. After incubation, actinomycetes isolates passed through a membrane filter were transferred to Manitol Soya Agar (MS) (Manitol or glucose 20 g, soy powder 20 g, agar 20 g in 1 liter tap water) (Hobbs *et al*., 1989) and cultured at 30°C until sporulation and then their spores were collected by filtering through a cotton sheet on a

funnel. To disperse the spores evenly, 2 mL of the filtrate were gently sonicated in a sonic water bath for 10 min. The resulting spore suspension was serially diluted with sterilized DW. Small aliquots of the diluted suspensions were spread on Manitol Soya agar (MS) and incubated at 30^{o} C for about 14 days. Visually, selected independent colonies were streaked onto MS agar plates, incubated as described above, and obtained as purified isolates. All isolates were purified by following the same steps. Subsequently, single colonies were individually transferred to fresh MS and incubated at 30°C until sporulation. Spores were harvested and mixed with appropriate amount of 10% (v/v) glycerin solution containing 10% (v/v) dimethyl sulfoxide in eppendorf tubes, and finally stored at–80°C for subsequently used.

4- Antagonistic effect of actinomycetes isolates against *Pseudomonas syringae* pv. *tomato in vitro*

All obtained actinomycetes isolates (88 isolates) were tested for their antibacterial activity *in vitro* against (*Pst*) by a dual culture technique, according to Lee and Hwang (2002) with some modification as follows:

One hundred µl of bacterial suspension of *P. syringae* pv. *tomato* DC3000 (*Pst*) were cultured in 100 ml liquid King's B medium (Klement *et al*., 1990), and incubated at 28°C on a rotary shaker (200rpm) for 24hr. Cells were collected by centrifugation at 3000rpm for 10 min, washed twice and re-suspended in 10mM $MgSO_4$. Bacterial suspensions were adjusted to a concentration of 2 × 10^8 CFU/ml. five ml of the bacterial suspension was inoculated to 100ml of solid King's B after the medium cooled then pour in Petri dishes (9 cm).

Spore suspension (50µl) of each obtained actinomycetes isolates was streaked onto Yeast Starch Agar medium (YSA) (2.0 g yeast extract; 10 gm starch soluble; 15 gm agar; 1 liter D.W) for 7 days for sporulation. Two mycelial plugs of each actinomycetes about 8-mm diameter transferred to one side of Petri dishes (9cm) contained solid King's B medium inoculated with *P. syringae* pv. *tomato* DC 3000. Five replicates of each treatment were used. The plates were then incubated at 30 ± 1°C for 48 h. The inhibition zone width was measured in mm diameter (Waksman, 1961). According to the width of growth inhibition, zone score was recorded as follow; (-) no inhibition zone zero, (+) zone width ranged from 1-5 mm, (++) zone width ranged from 6-10 mm. There are two control treatments in this experiment; positive control (sterilized filter paper disks treated with tetracycline) and negative control (Petri dishes were only inoculated with *P. syringae* pv. *tomato* DC 3000).The experiment was repeated twice.

5- Identification of actinomycetes isolates using Analysis of 16Sr DNA:

5.1 DNA extraction from actinomycetes isolates

Each isolates of actinomycetes were grown on Manitol Soya Agar (MS) for two weeks at 30^{o}C. The independent colonies of actinomycetes about 4mm were picked up with sterilized toothpicks and transferred individually into 1.5 mL centrifuge tubes.

Total DNA was extracted from the specimens using the ISOPLANTII kit (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol. For each isolate to be extracted, two loops of mycelia and spores were scraped from solid growth media and suspended in 100 µl of Prep Man Ultra Sample Preparation Reagent. The sample was vortex for 10-30 sec and heated for 10 min at 100°C in a heat block. Then the sample was cool at room temperature for 2 min. The sample was centrifuge in microcentrifuge at 15.000 rpm for 2 min. 50 µl of the sample supernatant was transfer into new microcentrifuge tube. After this step the supernatant was ready to use in PCR reaction.

5.2 PCR condition

The 16S rDNA region was amplified by polymerase chain reaction (PCR) using a universal primer set of 27f (-AGAGTTTGATCMTGGCTCAG) and 1492r (GGCTACCTTGTTACGA CTT) which can amplify almost the full length of the region (ca. 1.465 bp) (Lane, 1991). Fifty microliters of PCR reaction mixture consisted of 2 µl of DNA template, 4 µl of 2.5 mM deoxyribonucleotide triphosphate (dNTP), 2 µl of each primer described above (1.0 lM final conc.), 0.25 µl of EXTaq Template DNA polymerase (Takara, Otsu, Japan), 5µlof 10 X Ex Taq buffer, and 34.75 mLof sterilized distilled water. The mixture was amplified using a thermal cycler (TaKaRa PCR Thermal Cycler Dice, model TP600, Otsu, Japan) with preheating stage at 94°C for 1 min, and then 30 thermal cycles of 1 min at 94°C for denaturing, 1 min at 55°C for annealing, and 2 min at 72°C for extension. There action was kept at 72°C for 10 min for a final extension. Ten microlitres of the amplifications were analyzed on a 1% agarose gel to confirm whether the PCR products contained the correct size of DNA. Successfully amplified PCR products were purified with the illustra Gen Elute TM PCR Clean-UP Kit (SIGMA-ALDRICH). They were then subjected to sequencing reactions using the thermal cycler with dye-labeled dideoxy terminators of the DYE namic ET Terminator cycle sequencing kit (GE Healthcare). The sequence reaction was conducted with one of 27f, and 517f (5′ATTACCGCGGCTGCTGG 3□) and 517r primers (5′ATTACCGCGGCTGCTGG 3□) (Muyzer *et al*., 1993). For each isolate, the partial sequence of the corresponding nucleotides was determined with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The sequences obtained were manually contiged with the GENETYX v.4.0 software package (Software Development Co., Ltd., Tokyo, Japan). Accession numbers for the sequenced data were obtained from the DNA Data Bank of Japan (DDBJ). The sequence for each isolate was compared with those deposited in the DDBJ/EMBL/Genbank database by BLAST homology (Altschul *et al*., 1997) to evaluate the similarity of actinomycetes taxa.

6-Effect of actinomycetes isolates against tomato speck disease under greenhouse conditions:

Two applications methods of treatments with actinomycetes iso-

lates in tomato plants against bacterial speck disease were evaluated.

6.1 Spraying method

Nine isolates of actinomycetes were selected for their ability to induce systemic resistance in tomato plants against *Pst* DC3000 by spraying of their spore suspension on tomato leaf before inoculated with *Pst* DC 3000 for one week in growth chamber, spore suspension of actinomycetes were prepared as described before.

Challenge inoculation and disease assessment: Nine tested isolates of actinomycetes were used in this experiment. Three replicates were used each replicate contains three plants at 3 weeks old (4-5) leaves stage were first sprayed with each isolates of actinomycetes isolate spore suspension (1×10^7 cfu/ml) 0.02% Silwet L-77(0.01%, v/v; Nihon Unica, Tokyo, Japan). Plants were kept for one week in growth chamber at 25^{o}C 12h/12h light dark cycle. One day before challenge by the pathogen, the plants were placed at 100% relative humidity. The virulent bacterial pathogen *Pst* DC 3000 containing 2×10^8colony forming units/ml (cfu/ml) was sprayed onto the tomato leaves until run-off. The inoculated plants were kept at 100% relative humidity in darkness for 2 days in order to develop the disease. Plants were then transferred to a growth chamber. Five days after pathogen challenge, disease severity was measured for each plant by recording the percentage of total plant leaf surface showing symptoms from 1 -5 using a scale (Yunis *et al*., 1980) as mentioned in pathogenicity test**.**

6.2 Drenching method

Tomato plants were grown in a cell tray filled with sterilized soil at 25°C in a growth chamber under 12 h light:12 h dark conditions for two weeks. After two weeks, the plants were transferred with the sterilized soil mix into 9 cm pots in diameter and grown for further one week. Tomatoes plants at 3 weeks of age (4-5 leaves) stage were treated with 10 ml/100gm soil spore suspension of actinomycetes (1×10^7 cfu/ml) for each replicate. These treated plants were kept for 7 days in growth chamber. One day before challenge, the plants were placed at 100% relative humidity. *Pst DC 3000* inoculation was carried out by spraying it as mentioned before. Five days after pathogen challenge, disease severity was measured for each plant visually by recording the percentage of total plant leaf surface showing symptoms and scored using diseases index scale mention before (Yunis *et al*., 1980). As healthy control, leaves sprayed with MgSO4 suspension mix with 0.01% (v/v) Silwet L-77. While infected control was tomato plants inoculated with *Pst DC 3000.* The experiment was repeated two times for confirmation.

7- Statistical analysis

The experimental design was completely randomized, consisting of three replicates for each treatment. The experiment was repeated at least twice and treatment means obtained were separated using a Duncan's multiple range tests (Duncan's test P> 0.05) (Gomez and Gomez., 1984).

Results:

1. Isolation and Identification of speck disease pathogens

The isolated bacteria were identified as *Pseudomonas syringae* pv. *tomato* (*Pst*) according to their morphological, cultural and physiological characteristics as reported by Krieg and Holt (1984) and Krieg *et al*. (2005).

The five pure bacterial isolates were obtained from naturally diseased tomato plants showed bacterial speck symptoms as well as an isolate of *P.s*.pv. *tomato* DC 3000, kindly provided by Department of Plant Pathology, Gifu University, Japan.

2. Pathogenicity tests

Results in Fig.1 showed that the tested six isolates (*Pst*1, *Pst*2, *Pst* 3, *Pst*4, *Pst*5, and *Pst* DC3000) proved to be pathogenic and able to infect tomato plants under greenhouse condition, causing speck disease symptoms. The symptoms appear on plant surface after five days from inoculation. The symptoms were small chloratic halo spot spread all over the leaves and also appear on stems of tomato plants (Fig.2). The first signs of the disease appeared as moist, light-green spots on the leaves, which later became necrotic dark-brown to black in color and surrounded by a chlorotic halo. These spots in time expanded and coalesced, causing necrosis of a larger leaf area, deformations, stunting, and ultimately death of leaves. The isolates however were varied regarding their disease severity. The results showed that isolate *Pst*DC3000 caused the highest disease severity percent followed by isolates *Pst2, Pst4, Pst5 and Pst1*. However, isolate *Pst3* caused the lowest disease severity percent.

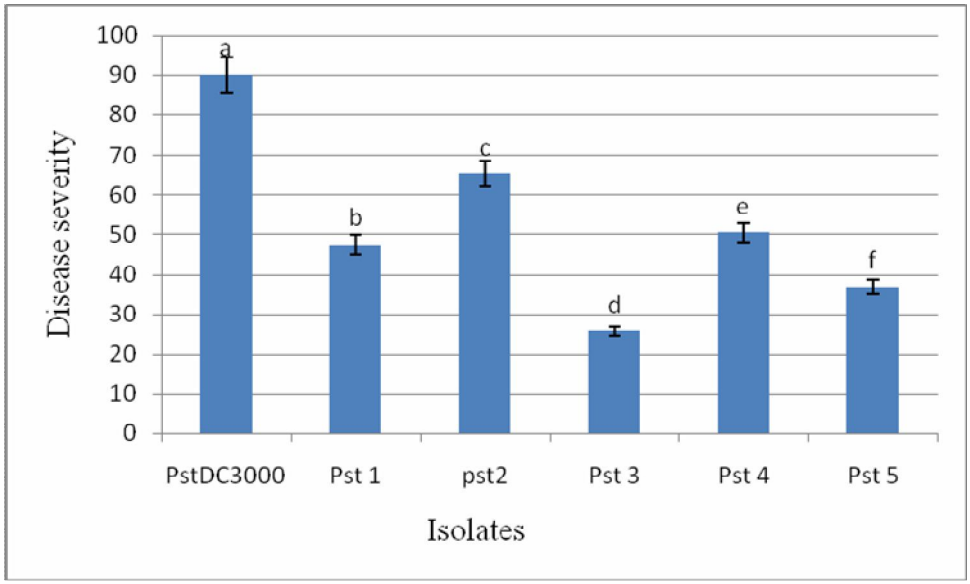

Fig.1. Pathogenicity tests of bacterial isolates and obtained isolate *Pst* DC3000 on tomato plants cv. Rika. The values in the column followed by the same letter are not significantly different according to Duncan's at $P<0.05$.

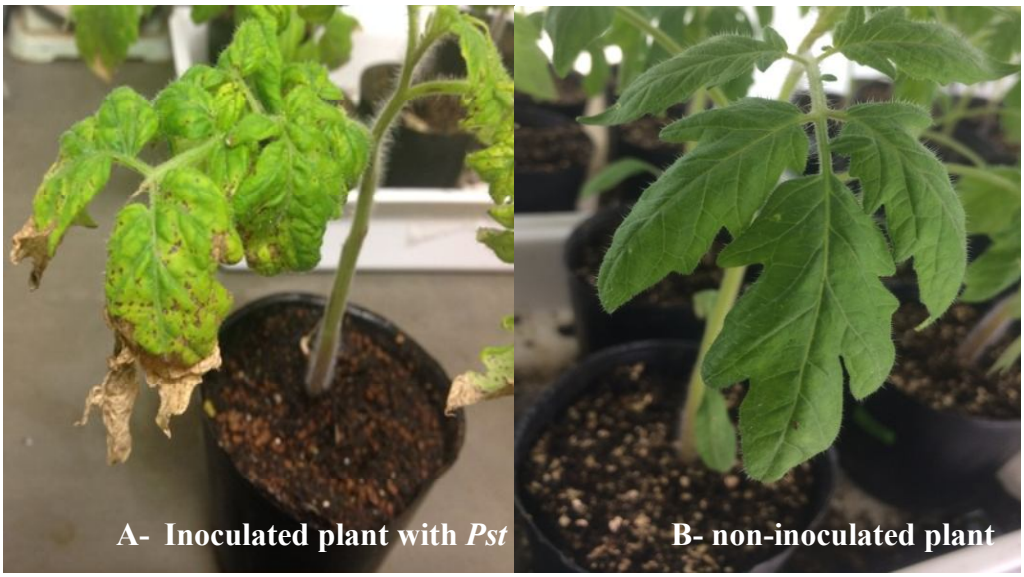

Fig. 2. The symptoms of tomato speck disease appeared after 5 days of challenge by *Pst* DC 3000 under growth chamber condition. A. inoculated plants. B. non-inoculated tomato plants.

3. Isolation of actinomycetes from healthy tomato plants:

A total of 188 isolates were successfully isolated from tomato plants. However, 100 of 188 isolates showed poor mycelial growth and sporulation on the agar media (Fig. 3). Such isolates would not be of practical use as biocontrol agents, and thus they were excluded. Therefore, 88 isolates with active growth were used in the following screening trials.

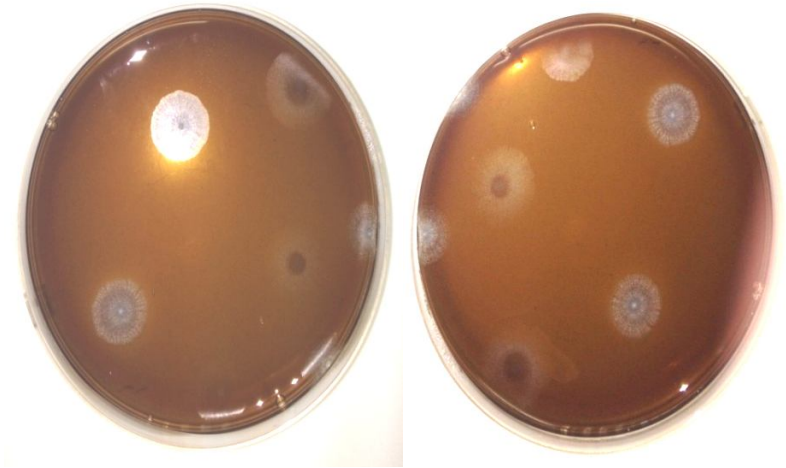

Fig.3. Growth of some actinomycetes isolated from healthy tomato plants and grown on Humic Acid Vitamin B (HV) agar medium.

4. Antagonistic effect of actinomycetes isolates against *Pst* DC3000

According to results displayed in Table (1), there are nine of 88 isolates reacted positively against *Pst* DC 3000. Six isolates named TR-24, TR-26, TR-20, TR-25, TR-32 and TR-33 inhibit growth of *Pst* DC 3000 with area of inhibition zone width from 6-10 mm. However 3 isolates named TR-34, TR-35 and TR-36 could also inhibited the pathogen with inhibition zone width from1-5 mm. The rest of actinomycetes isolates weren't exhibited any ability to inhibit the growth of *Pst* compared with the positive control and negative control. These nine isolates were selected for the others experiments *in vivo*.

Table 1. Antagonistic effect of actinomycetes on growth of *P. Syringae* pv. *tomato* DC3000.

Actinomycetes isolates	Inhibition zone width (mm)
TR-20	6.8 mm (++)
TR-25	7.2 mm (++)
TR-24	8.3 mm (++)
TR-26	8.2 mm (++)
TR-32	6.2 mm (++)
TR-33	6.0 mm (++)
TR-34	3.2 mm (+)
TR-35	2.8 mm (+)
TR-36	2.2 mm (+)

5. Identification of the selected isolates of actinomycetes by 16SrDNA analysis

Data presented in Table (2) indicated that the 16SrDNA analysis revealed that isolates TR-24 and TR-26 were related to one species *Streptomyces glaucescens*, with percent of identity 99%.While the isolates TR-20, TR-32 and TR-34 were identified as *Streptomyces graminisoli.* Isolate TR-25 identified as *Streptomyces flavovirens* with 98% similarity. Isolate TR-33 and TR-35were identified as *Streptomyces scabies* with 95% similarity. Finally isolate TR-36 was identified as *Streptomyces roseochromogenus* with identity percentage 99%.

Table 2: Identification of isolated actinomycetes isolates with 16SrDNA analysis

Isolate name	Identity percent	Identification
TR-20	98	*Streptomyces graminisoli*
TR-24	100	*Streptomyces glaucescens*
TR-25	98	*Streptomyces flavovirens*
TR-26	100	*Streptomyces glaucescens*
TR-32	98	*Streptomyces graminisoli*
TR-33	96	*Streptomyces. Scabiei*
TR-34	98	*Streptomyces graminisoli*
TR-35	95	*Streptomyces scabiei*
TR-36	99	*S.roseochromogenus* subsp.*albocyclini*

6. Effect of two application methods of actinomycetes isolates against tomato speck disease.

Both spraying and drenching treatments on tomato plants with all tested streptomyces isolates were able to reduce disease incidence of tomato speck disease caused by *Pst* DC 3000 compared to infected control (Fig. 4). Using the two applications, the highest disease severity was obtained in infected control plants (93 to 94%). Spraying or drenching of spore suspension of TR-24 and TR-26 isolates were the highest isolates in reduction the disease severity followed by TR-20, whereas the isolates TR-33 and TR- 35 were in between. Isolate TR-34 gave the lowest reduction in the disease severity compared to other streptomyces isolates.

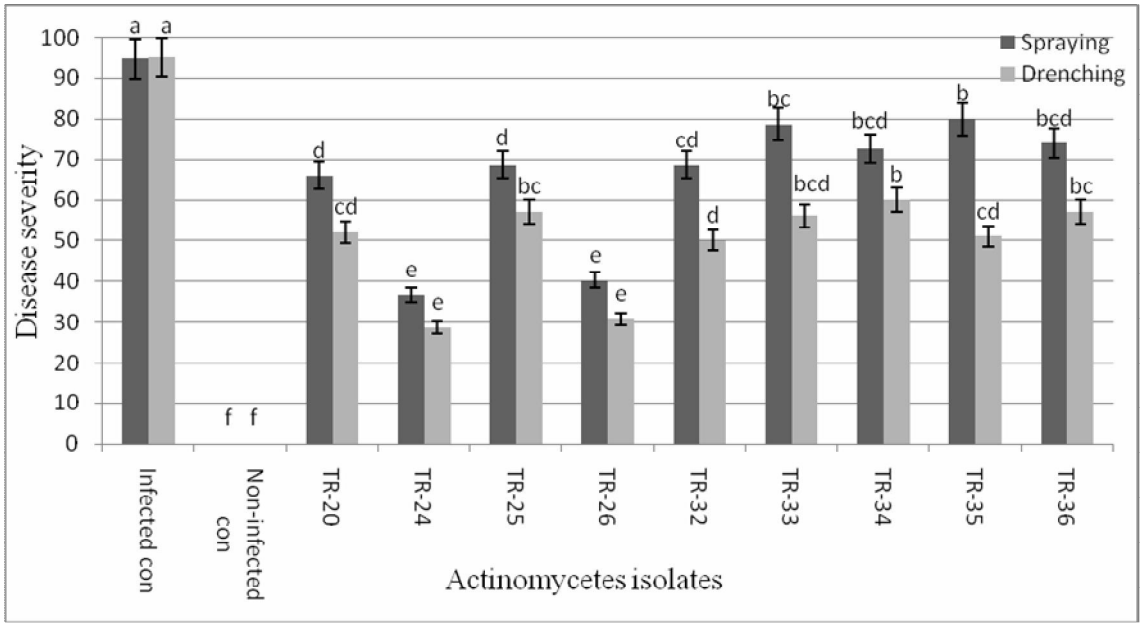

Fig.4. Effect of spraying and drenching treatments with certain Streptomyces isolates on disease severity of tomato speck disease. The values in the column followed by the same letter are not significantly different according to Duncan's at $P<0.05$.

Discussion:

The present work showed that some isolates of *P. syringae* pv. *tomato* were associated with bacterial speck symptoms in tomato field crops under natural condition in Egypt. All isolates were able to infect tomato plants and produce typical symptoms of bacterial speck on tomato plants. These results are in agreement with those reported by (Dawoud, 2012).

All bacterial isolates were identified depends on morphological and physiological characteristics of the isolated pathogenic bacteria. They were clearly indicated that these isolates are *Pseudomonas syringae* pv. *tomato*. These results are agree with those reported by Saad and Hassan, 2000; and Schaad, 1988.

Pathogenicity tests revealed that the *Pst* DC3000 was the most virulent isolate compared to Egyptian isolates. Tomato speck disease is worldwide that favored by low to moderate tem-

peratures and high moisture conditions (Babelegoto *et al*., 1988; and Jones *et al*.,1991). Hence the mild symptoms of Egyptian isolate might be due to the different conditions.

More advanced methods such as induced systemic resistance in tomato plants as biological control against tomato speck disease are in infancy (Bashan, 1997). Although actinomycetes are proved to be more effective in term of plant growth promotion as well as biocontrol activity against several plant pathogens than plant growth- promoting rizobacteria (PGPR) or plant growth-promoting fungi (PGPF) (Conn *et al.,* 2008; Gopalakrishnan *et al.,* 2011; and Misk and Franco, 2011). However, literature on actinomycetes-mediated ISR is sparse (Senthilraja, 2016). Therefore, the present study was done used endophytic actinomycetes as a new biocontrol agent well known to induce systemic resistance to manage tomato plants against the causal agent of tomato speck disease.

A total number of 188 actinomycetes isolates were isolated from the sterilized leaf tissues of healthy tomato plants by the dilution plating method and Humic Acid Vitamin B (HV) medium supplemented with nalidixic acid, trimethoprim and cycloheximide which used as selective medium (Hallmann *et al*., 1997) to suppress growth of such undesirable endophytes (Hayakawa and Nonomura, 1987). Supplementation selective medium with antimicrobial compounds such as nalidixic acid and trimethoprim (Hayakawa and Nonomura, 1987), nystatin (Williams and Davies, 1965), or cycloheximide (Caruso *et al*., 2000) improves selectivity of actinomycetes. Endophytic actinomycetes colonies emerged after 2 weeks of incubation indicated that these isolates were endophytic because actinomycetes grow slowly relative to other bacteria and fungi. Rapidly growing microbes are bacterial and/or fungal contaminants (Shimizu, 2011). It is clear that leaf tissues present a suitable habitat for these actinomycetes. Isolation of endophytic actinomycetes from leaves of tomato is supported by the previous reports (Okazaki, 1995; and Inderiati and Franco, 2008).

In vitro and *in vivo* antagonistic study revealed that 9 of 188 actinomyces isolates reacted positively against *Pst* DC3000 and they have varying degrees of inhibition *in vitro* and *in vivo*. This because these isolates may able to produced spreadable antibiotics with different effect when they interacted with the pathogens *in vitro*. *Streptomyces* species are well-known for their ability to produce biologically-active secondary metabolites, particularly antibiotics (Loria *et al*., 1997; and Samac *et al*., 2003). Similar results on the interaction of endophytic actinomycetes with tomato were obtained by Inderiati and Franco (2008). They considered that although antibiosis might be the most important mechanism, involvement of other mechanisms cannot be denied because of inconsistent correlation between *in vitro* antagonism and *in vivo* biocontrol activity in several isolates.

Morphological characteristics of the *Streptomyces glaucescens* isolate TR-24 under the optical microscopy was in accordance with the described morphology reported for *Streptmyces*

glaucescens isolate originally isolated from soil in Haiderabad/Hindustan in India by Whitman *et al.,* (2012). Thus this is the first report of isolation of *S. glaucescens* form healthy tomato leaves. *S. glaucesce* is very important due to its well-known production of various secondary metabolites like the bacteriocin-like substance laucescin, the antibiotic hydroxystreptomycin, the antimicrobial compound tetracenomycin C and the type-2 diabetes mellitus drug Acarbose (Beyer *et al*., 1996; Rockser and Wehmeier, 2009; Schurter *et al*., 1979). The secondary metabolites produced by the *Streptomyces glaucescens* might contributed to its ability for control tomato speck disease agent *in vitro*. *Streptomyces glaucescens* produces the enzyme tyrosinase and is therefore capable of converting tyrosine to melanin (Lerch and Ettlinger, 1972).

There are variety of techniques have been used for the application of endophytic microbes at the experimental level, such as seed treatments, soil drenches, stem injections, and foliar sprays of microbial suspensions (Hallmann *et al*. 1997). Isolates TR-24 and TR-26 were the most effective isolates and had high significant decrease of tomato speck disease using spraying or soil drenching in growth chamber. When the application techniques changed from spraying to soil drenching (dipping) there are significant differences in reduction percent of the disease incidence. Soil drenching applications are more effective in disease suppression. This may due to soil drenching enhanced induced systemic resistance as a result of colonization of plants roots by certain isolates but spray methods enhanced systemic acquired resistance. These results agree with those reported by (Van Loon *et al*., 1998). They reported that SAR is defined as the expression of hypersensitive response or localized necrotic lesion on host plant upon infection caused by a virulent pathogen in order to arrest further growth of the pathogen, whereas ISR is the enhanced level of defensive responses.

The effectiveness of biological control can be enhanced through the application of different techniques like soaking the roots or seeds before transplantation that accelerated the induction of resistance (Hallmann *et al*., 1997).

Actinomycetes have been and remained the most fruitful source of microorganisms for all types of bioactive metabolite (Doumbou *et al.,* 2001). About 60% of the new insecticides and herbicides reported in the past 5 year originate from *Streptomyces* (Tanaka and Omura, 1993).

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عزل الأكتينوميستس الداخليه من أوراق نباتات الطماطم وفاعليتها فى مقاومه مرض التبقع البكتيرى

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الملخص

تم تقيم قدره عزلات أكتينوميستس لمقاومة مرض التبقع البكتيرى فى الطماطم المتسبب عن بكتيريا سيودوموناس سيرينجا يباثوفار سيرنجي. تم الحصول على سته عزلات بكتيرية *Pst* (Pst1, Pst2, Pst 3, Pst4, Pst5, and Pst DC3000) من أوراق الطماطم المصابة طبيعيا من مناطق مختلفه من محافظتي المنيا وأسيوط في مصر. كانت جميع العزلات قادره على احداث العدوي لنباتات الطماطم وكانت السلالة سيودوموناس سيرينجاي DC3000 الأكثر ضراوة ولذلك تم اختيارها لاجراء تجارب المعمل و الصوبه الزجاجيه. وتم تعريف هذه العزلات وفقا للخصائص المورفولوجية والفسيولوجية والبيوكيميائية على انها بكتيريا سيودوموناس سيرينجاياثوفار سيرنجي. تم اختيار ٩ عزلات من ٨٨ عزله من الأكتينوميستس تم عزلها من سطح أوراق الطماطم السليمه لدراسه تأثيرها فى مقاومه مرض التبقع البكتيرى فى الطماطم. وتم تعريف التسع عزلات من بكتريا على انها لجنس الاستربتومايسس عن طريق الكشف عن تحليل نوع الحامض النووي لكلا منهما باستخدام 16Sr DNA. وكشف تحليل الحمض النووي أن سلالات أكتينوميستس TR-24 وTR-26 كانت تنتمي إلى نوع واحد من ستريبتوميسز غلوسسنس في حين كانت السلالات الأخرى تتعلق بأنواع مختلفة من الستربتوميسيس. بأستخدام طريقه الرش او معامله التربه كانت السلاله TR-24 وTR-26 الأكثر تاثير في القدره علي تثبيط حدوث مرض التبقع البكتيرى في النباتات الحية المختبرة داخل الصوبه الزجاجيه باستخدام رش مستخلص الجراثيم من بكتريا الاستربتومايسس علي أوراق الطماطم او باضافه ١٠% من مستخلص الجراثيم الي التربه المنزرعة بنباتات الطماطم لمده اسبوع قبل العدوي بالبكتريا الممرضه.