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Original Research Article

Effect of the new antagonist; *Aspergillus piperis* on germination and growth of tomato plant and Early Blight incidence caused by *Alternaria solani*

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Abstract

The present work is considered the first record of studying the ability of the new antagonist, *Aspergillus piperis* in decreasing the disease incidence of early blight of tomato plant caused by *Alternaria solani*. The pathogen *A. solani* was isolated from naturally diseased tomato fruits and identified genetically by sequencing of rRNA gene using ITS1 and ITS4 primers. For the field experiment, the toxicity of spore suspension of *A. piperis* on germination of tomato seeds was performed using soaking and irrigation methods where the germination percentage and vigor index were calculated. The results indicated that the irrigation method recorded better results than soaking where the germination percentage was 81.81 % and vigor index was 794.37 related to that of control which recorded 90.9% and 715.83 respectively. The values of vigor index indicated that, the spore suspension of *A. piperis* had induced the plant growth. In the meantime, the spore suspension of *A. piperis* was applied on tomato leaflets by several methods to reduce the incidence of early blight disease. The application of *A. piperis* spore suspension directly on the leaflets exhibited the best result where the percentage of infection was 10.25 % to the control (25 %) after 4 days.

Keywords: Biocontrol agent, *Aspergillus piperis*, *Alternaria solani*, Early blight, Tomato, rRNA, ITS

INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is one of the important vegetable plants worldwide where more than 120 million tons are produced annually (Kimura and Sinha, 2008). Tomato plants are suitable to soil borne fungal pathogens where several genera can infect them causing serious damages to all plant parts especially the edible fruits. Early blight disease caused by *A. solani* is one of serious fungal diseases of foliar and fruits of many vegetable plants. Infections of early blight disease can cause 35-78 % decreasing of fruit yield of tomato plant (Vloutoglou and Kalogerakis, 2000).

There are many effective ways to control early blight disease either chemically or biologically. Various fungicides have been documented in earlier studies (Choulwar and Datar, 1988, Maheswari et al., 1991, Abdel-Mallek et al., 1995), but continuous usage may cause several problems such as toxicity to other organisms, development resistant pathogen and environmental pollution (Varma et al., 2008). For avoiding the hazardous effects of fungicides, the researchers going to apply the bioagents in controlling the plant diseases. Biological control keeping the environment clean and healthy. Biological control of pathogens is based on using living microorganisms, which suppresses or inhibits the pathogen. The most important bio-control agent which is used against many plant pathogens is the fungus *Trichoderma* since 1930 (Gveroska and Zibe-roski, 2011). Later, lot of studies examined the biological

activity of other fungal and bacterial genera as antagonistic agents against plant pathogens. It was founded that, several naturally occurring fungi and bacteria have been identified as bio-control agents such as some species of *Fusarium* and *Aspergillus* (Bandyopadhyay and Cardwell, 2003), *Talaromyces* (Naraghi et al., 2010), *Epicoccum* (Elkot and Derbalah, 2011), *Cladosporium* and *Aureobasidium* (Bisiach et al., 1985), *Bacillus* (Ashwini and Srividya, 2014) and *Pseudomonas* (Weller, 2007).

Aspergillus species are ubiquitous and they generally produce a variety of secondary metabolites exhibiting inhibitory effects on several pathogenic microorganisms (Siddiqui et al., 2004). Recent studies indicate that some species of genus *Aspergillus* have antagonistic effects against some pathogenic microorganisms. *A.niger*, *A. flavus*, *A. terreus*, *A. fumigates* found to control some postharvest diseases of potato caused by *Pythuim ultimum* (Daami-Remadi et al., 2012) and (Abdallah et al., 2014), *F. sambucinum* (Rania et al., 2014) and *Phytophthora* sp. (Abdallah et al., 2015). The *Aspergillus* species using several mechanisms in controlling many fungal pathogens such as; mycoparasitism, competition, mycelial lysis and antibiosis via the synthesis of volatile and/or non-volatile metabolites (Daami-Remadi et al., 2006), (Kaewchai and Soytong, 2010) and (Bhattacharyya and Jha, 2011).

In a previous study of the author (El-Debaiky, 2017) the new antagonist; *A. piperis* exhibited valuable results in controlling some phytopathogenic fungi including *A. solani* in vitro with several antagonistic mechanisms. Therefore, the present study was designed to examine the effects of *A. piperis* in field conditions. Accordingly, its spore suspension activity on seeds germination and the early blight disease incidence of tomato plants caused by *A. solani* were tested.

Experimental Procedures

Tested organisms

Fungi

Culture of *A. piperis* was purchased from the mycological center (AUMC), Assuite University, Assuite, Egypt. While the pathogenic fungus, *A. solani* was isolated by the author from naturally spoiled tomato fruits in sterilized conditions using PDA and incubated at 28±2°C for 7 days. The isolated *A. solani* was deposited in the AUMC with strain number 11660.

The host plant

The field experiments are performed using tomato seeds and plants of *Solanum lycopersicum* L. A prepared compost was used in cultivation process for testing the adverse effects of spore suspension of *A. piperis* on the germination of tomato. For field experiment, a soil mixture of clay and compost (3:1) was used in cultivation.

Molecular identification of *A. solani*

Molecular characterization of *A. solani* was done by sequencing of rRNA gene with the help of Solgent Company, Daejeon South Korea. A small amount of fresh fungal culture was scraped and suspended in 100µl autoclaved distilled water in 2ml sterile vial and boiled at 100° C for 15 minutes. The non-living fungal cells were sent to SolGent Company for rRNA gene sequencing. Fungal DNA was extracted and isolated using SolGent purification bead. Prior to sequencing, the ribosomal rRNA gene (rDNA) was amplified using the polymerase chain reaction (PCR) technique in which two universal fungal primers ITS1 (forward) and ITS4 (reverse) which were incorporated in the reaction mixture. Primers used for gene amplification have the following composition: ITS1 (5' - TCC GTA GGT GAA CCT GCG G - 3'), and ITS4 (5'- TCC TCC GCT TAT TGA TAT GC -3'). The purified PCR products (amplicons) were reconfirmed using a size nucleotide marker (100 base pairs) by electrophoreses on 1% agarose gel. Then these bands were eluted and sequenced with the incorporation of dideoxynucleotides (dd NTPs) in the reaction mixture. The sample was sequenced in the sense and antisense directions using ITS1 and ITS4 primers (White et al., 1990). Sequences were further analyzed using Basic Local Alignment Search Tool (BLAST) from the National Center of Biotechnology Information (NCBI) website. Phylogenetic analysis of sequences was done with the help of MegAlign (DNA Star) software version 5.05.

In vivo experiments

The experiments were designed to find any adverse effects of *A. piperis* spore suspension on the seed germination of tomato, and its activity as biocontrol agent on reducing early blight disease of tomato plants.

Preparation of spore suspension of *A. piperis*

Spores of *A. piperis* were collected by scratching them from 4 days old culture plates in 500 ml distilled water containing 0.2% (V/V) tween 20. The number of spores was calculated by hemocytometer and found to be 3.84×10^6spore/ml. To the prepared spore suspension, 2% (W/V) starch was added as an adhesive.

Figure 1. Seeding tray

Phytotoxic effect of *A. piperis* on the seed germination

The phytotoxic effect of *A. piperis* spore suspension on seed germination of tomato was carried out using special seeding trays (Figure 1) each one consists of 209 holes arranged in 11 × 19 arrays. For this purpose, the intact, dry and clean tomato seeds were selected and surface sterilized in 0.5% sodium hypochlorite for 2 min. After that, they were washed triple by distilled water and let dry in air (Asaduzzaman et al., 2013). Twenty-two seeds were soaked in the previously prepared spore suspension for 10 min. Controls were prepared by soaking seeds separately in distilled water only and in 2% (W/V) starch solution containing 0.2% (V/V) tween 20. After soaking period, all seeds were sown in the seeding tray fill with compost, 5 mm under the surface then irrigated by distilled water. In the meantime, another technique was used by sowing seeds directly in the compost without soaking then irrigated by spore suspension of *A. piperis*. The seeding trays were covered by carton sheet and kept in the dark at room temperature (about 30±2°C) for 72h. After that, the carton sheet was removed, and the seeding tray transferred to open air conditions beyond the direct sunlight and irrigated whatever needed. The shoot length, root length, germination percentage and vigor index of the seedlings were recorded after 6 days. Vigor index (Abdul-Baki and Anderson, 1973) was calculated as follow:

Vigor index = [Mean of root length (cm) + Mean of shoot length (cm)] × percentage of seed germination.

To be realistic and accurate in calculating the obtained results, the germination percentage of the untreated tomato seeds must be detected and taken in mind, where there are many reasons such as genetic mutations, germplasm disorders, etc. which affecting the germination percentage of any plant seeds. To do that, 22 tomato seeds were sown in two rows of the seeding tray full of compost and irrigated only by water then covered by carton sheet as mentioned to the end of the steps.

Activity of *A. piperis* on the disease incidence

For this experiment, 34 days old tomato plants were grown in pots, 15 cm in diameter each one containing 1 Kg of a soil mixture composed of compost and clay (1:3). The soil mixture sterilized in autoclave at 121°C, 1.5 atm. for 20 min then cooled at room temperature. Three elder leaflets of each tomato plant were selected to perform one of the following treatments:

Control

Two equal wounds were made carefully on the upper epidermis of blades of the selected leaflets by a sterilized scalpel. Then, the wounded leaflets were inoculated by mycelial discs of the pathogen (2×2 mm) from 5 days old culture. These discs of *A. solani* were deposited and fixed on the wounds by an adhesive tape which removed once the disease symptoms appeared after 4 days, Figure 2 illustrated that stepwise.

Treatment 1 (T1)

The leaflets were wounded as in the control but without the inoculation by the pathogen. Spore suspension (4×10^6 spores / ml) of 4 days old culture of *A. piperis* was prepared in sterilized distilled water containing 2% tween 20. The spore suspension was spread to the wounded surface of the leaflets by a sterilized spatula.

1. Intact leaflet

2. Wounds were made on the upper epidermis

3. Infection by disc of *A. solani* then put an adhesive tape

Figure 2. The infection steps of tomato leaflet by *A. solani*.

Table 1. Molecular identification of the tested fungus (AUMC11660) and percentage similarities with related strains accessed from the GenBank

Strain No.	Number of nucleotides with primers		Closely related strains accessed from the GenBank				
	ITS1-F	ITS4-R	Name	Strain No./Source	Country	Accession No.	Similarity (%)
AUMC 11660	523	532	*Alternaria solani*	AS-6/tomato leaves	Qalyubia, Egypt	KY412985	100
			Alternaria solani	ASU-4/ tomato leaves	Aswan, Egypt	JF491206	100
			Alternaria solani	OTA-75/tomato seeds	Karnataka, India	KT354939	100
			Alternaria alternata	GRSH6/sinus	Tahran, Iran	KY788023	98
			Alternaria alternata	91_2B2/indoor air of refrigerator	Idrine, Turky	KY859360	98
			Pleospora tarda	==========	=====	AF229481	==
			Pleospora herbarum	==========	=====	NR111243T	==
			Pleospora herbarum	==========	=====	AF229497	==

After 24h, the treated leaflets were inoculated by *A. solani* as mentioned earlier. Three other leaflets remained without inoculation for investigation any adverse actions of the spore suspension of *A. piperis* on the leaflets. All the plants were irrigated by tap water whatever needed.

Treatment 2 (T2)

The leaflets in this treatment were treated with the same manner of the control, but the plants were irrigated by the spore suspension of *A.piperis* instead of water.

After 4 days for the control and treated leaflets, the area of the total blade of the treated leaflets and the infection percentage (infected area / total area of the leaflet × 100) was estimated using graphical method where leaflet image is drawn on the graph paper has small grid size of 1cm. The total number of squares covered totally by the leaflet outline edge was estimated plus the number of squares partially covered by the edge outlines (but more than one half of each square) divided on two according to a modified method mentioned in (Parmar et al., 2015).

All the results were statistically analyzed using the computer programme IBM SPSS Statistics 19.

RESULTS AND DISCUSSION

Molecular identification of *A. solani*

The obtained data from the sequencing process of the rDNA of the tested isolate were deposited in the GenBank with other accession numbers listed in Table 1. The BLAST search for the 18S sequences of the tested

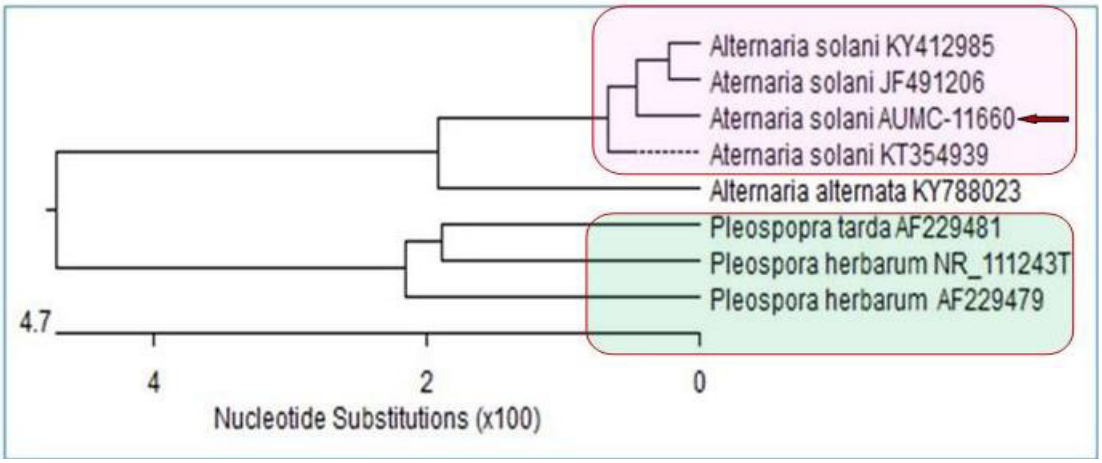

Figure 3. Phylogenetic tree of 18S sequences of *Alternaria solani* isolated in the present study (AUMC-11660) aligned with some related sequences of *Alternaria solani* and *A. alternata*. Species of *Pleospora* (Teleomorph of *Stemphylium*) were included as outgroup species.

Table 2. Toxicity effect of spore suspension of *A. piperis* on tomato seeds germination.

Treatment		Germination percentage (%)	Shoot Length (Mean± SD)	Root Length (Mean± SD)	Vigor Index
Control 1		90.9	5.285± 1.09	2.69± 0.45	724.927
Control 2		90.9	4.67±1.529	2.715±0.774	679.47
Starch Control		81.81	3.955± 1.282	2.788± 0.7	551.64
Treatment 1		77.27	3.447± 1.54	2.994± 0.867	497.69
Treatment 2		81.81	5.5±1.316	4.21±0.915	794.37
Starch Control+ RG		90.91			613.006
Treatment 1+ RG		86.37	-------	-------	556.3
Treatment 2+ RG		90.91	-------	-------	882.73
ANOVA	**F-value**		8.9724	15.8998	
	P		0.0000*	0.0000*	

Control 1. Untreated tomato seeds were cultivated in compost and irrigated with water.
Control 2. Tomato seeds soaked in water.
Starch control. Tomato seeds soaked in 2% starch solution contains 2% tween 20.
Treatment 1. Tomato seeds soaked in spore suspension of *A. piperis*.
Treatment 2. Untreated tomato seeds irrigated by spore suspension of *A. piperis*.
RG. The reduction value in germination percentage of control 1 which equals 9.1%.
P: Probability. *: Significant at P ≤ 0.05

isolate (AUMC11660) indicated its 100 % similarity to two Egyptian and one Indian isolates of *A. solani* (KY412985, JF491206 and KT354939). While it showed 98% similarity with Iranian and Turkish strains of *A. alternata* (KY788023 and KY859360). The present isolate along with the other recorded isolates of *A. solani* and *A. alternata* were subjected to a phylogenetic analysis using available ITS rDNA sequence data downloaded from GenBank. The Phylogenetic tree of 18S sequences indicated that the tested isolate clustered with *A. solani* isolates reported elsewhere (Figure 3). The species of *Pleospora* (Teleomorph of *Stemphylium*) were considered as an out-group strains where they did not cluster with the tested *A. solani* isolate.

Phytotoxic effect of *A. piperis* on the seed germination

Effect of *A. piperis* as a new biocontrol agent on the seed germination and tomato plant parameters was represented in Table 2. There was a normal reduction in the seed germination percentage (RG) equal 9.1 % which was added to recorded values of germination percentage of the other treatments. The vigor index value of the untreated tomato seeds which irrigated by the spore suspension of *A. piperis* (882.73) was higher than the controls (724.927 and 679.47) which indicated that the spore suspension of *A. piperis* induced the growth parameters of tomato plants when added to the soil as

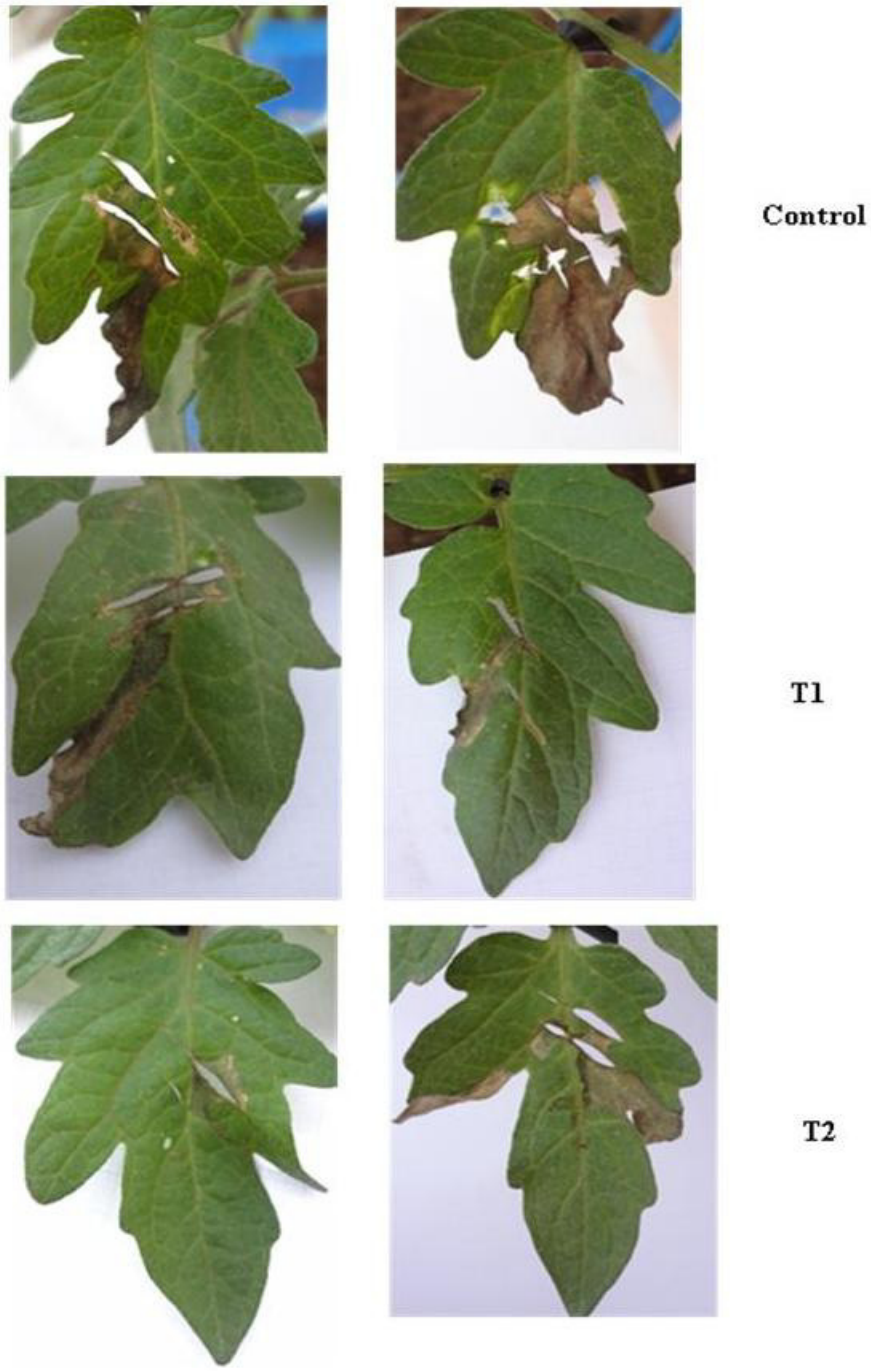

Figure 4. The symptoms of early blight disease on tomato leaflet of control and treatments after 4 days. T1: the spore suspension of *A. piperis* was applied directly on the leaflet while T2: the spore suspension of *A. piperis* was applied to the soil mixture.

irrigation solution.

In contrary, the spore suspension of *A. piperis* showed small phytotoxic effect and reduction in the germination percentage when the tomato seeds were soaked in it where the germination percentage was 86.37%. Also, the vigor index was reduced (556.3) than the control which indicated that; the growth parameters of tomato plants were lowered with prior soaking of the seeds in the spore suspension of *A. piperis*. Table 2 showed that; 2% starch solution containing 2% tween 20 does not

Table 3. The infection percentage on the blade of tomato leaflets

Parameter	C	T1	T2
Total leaflet area cm2 (Mean± SD)	5 ± 0.7	9.5 ± 5.65	6.75± 1.76
Infected area cm2 (Mean± SD)	1.25 ± 0.35	1 ± 0.00	0.75 ± 0.35
Infection percentage (%)	25	10.52	11.11
Reduction in Infection percentage (%)		14.48	13.89

C: Control which infected by *A. solani*.
T1: Treatment 1 where the spore suspension of *A. piperis* was applied directly on the leaflet then infected by *A. solani*.
T2: Treatment 2 where the spore suspension of *A. piperis* was applied to the soil mixture.

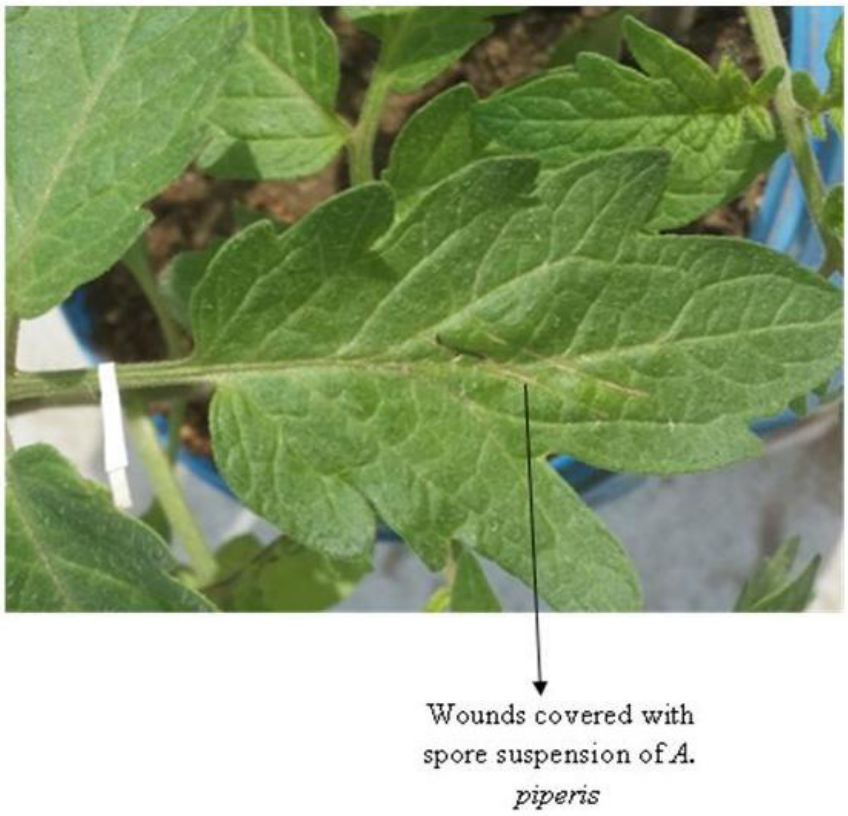

Figure 5. Appearance of the tomato leaflets treated with spore suspension of *A. piperis* after 4 days.

exhibit any adverse effects on the tomato seeds where the germination percentage was 90.91%.

Activity of *A. piperis* on the early blight incidence

The symptoms of early blight disease were observed in all the control and the treatments after 4 days of inoculation the tomato leaflets by *A. solani* in the form of olivaceous green lesions surrounded by yellowing and black colored dead tissues. These lesions characterized by concentric colored which later spread in the surrounding tissues (Figure 4). This is in agreement with (Delahaut and Stevenson, 2004) who recorded similar

symptoms of early blight disease on foliage of tomato plant.

The spore suspension of *A. piperis* exhibited remarked reduction in the infection percentage of early blight disease on the inoculated tomato leaflets compared to the control (Table 3). Where T1 and T2 recorded infection percentage 10.52 % and 11.11 % respectively compared to the control which recorded 25%. Thus, the reduction in the infection percentage in T1 and T2 was 14.48 and 13.89 respectively. Also, it was concluded that; the application of the spore suspension of *A. piperis* directly on the infection site on the leaflets has greater effect in reducing the early blight disease than when added to the soil mixture. These results were in accordance with (Singh et al., 2015) who found that some antagonistic fungi such as species of *Aspergillus* and *Trichoderma* showed marked inhibition of *A. solani* in vitro while, *Trichoderma* reduced the early blight incidence of tomato plant under glass house conditions.

Contrarily, there were no any disease symptoms or adverse appearance on the leaflets of tomato treated with spore suspension of *A. piperis* (Figure 5). This indicated that the spore suspension of *A. piperis* has not ability to infect or parasitize tomato leaflets.

Since this is the first record of using *A. piperis* as biocontrol agent in vivo, so there are no other previous studies concerning *A. piperis* to compare my results with them. But, there are some studies reported the using of some other *Aspergillus* species as biocontrol agents such as; atoxicogenic *Aspergillus* spp. used in biological control of other aflatoxigenic fungi (Tsitsigiannis et al., 2012) and *A. solani* (Atia and Ahmed, 2011).

CONCLUSION

The molecular identification of isolated pathogenic fungus from spoiled tomatoes, using DNA sequencing revealed that it belongs to *A. solani*. Phytotoxic effect of spore suspension of *A. piperis* on the germination of tomato seeds was performed using different techniques. The results investigated that, the application of *A. piperis* spore suspension as irrigation solution to tomato seeds exhibited germination percentage greater than socking the seeds in it. Where, socking process revealed small toxicity to the seeds comparing with the control. The activity of *A. piperis* spore suspension in reducing the early blight incidence on tomato leaflets was tested in vivo. The results revealed that the coverage of wounded upper surface of leaflets reduced the infection percentage of early blight caused by *A. solani* more than irrigation the soil by *A. piperis* spore suspension. Accordingly, I recommended the application of spore suspension of *A. piperis* directly on tomato leaflets to reduce the chance of early blight disease incidence. But, further studies are still needed on the in vivo applications of the new antagonist, *A. piperis* against plant pathogens and its safety of the produced crops.

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