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Evaluation of *Trichoderma Harzianum* Biological Control Against *Fusarium Oxysporum* F. Sp. *Melongenae*

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Abstract

The present study was conducted to biocontrol *in vitro* and *in vivo* of *Fusarium oxysporum* that cause Fusarium wilt diseases for eggplant plants by using biological control agent fungus *Trichoderma harzianum*. Fourteen isolates from *F. oxysporum* were isolated and identified from two fields in Iraq. Pathogenicity test indicated that all *F. oxysporum* isolates were pathogenic for eggplant but differed in its level of pathogenicity. Four of the fourteen isolates from *F. oxysporum* were selected depending on their highest pathogenicity for eggplant plants, *F. oxysporum* four isolates F5, F6, F13 and F14 achieved at pre emergence 83.3%, 83.3%, 86.7% and 83.3% and at post emergence 90.0%, 90.0%, 83.3% and 76.7% respectively. *In vitro*, the antagonistic activity evaluation of *T. harzianum* against *F. oxysporum* four isolates revealed that *T. harzianum* was highly significantly inhibited *F. oxysporum* four isolates growth (F5, F6, F13, F14) which recorded the inhibition percentage 87.00, 89.00, 89.00, and 96.33% respectively. Greenhouse (*In vivo*) results of the biocontrol efficiency of *T. harzianum* against *F. oxysporum* showed significant reduction in eggplant Fusarium wilt incidence compared with pathogens control treatments. The pathogen *F. oxysporum* treatments with *T. harzianum* (TF) showed that all the treatments (TF) achieved high germination rate for eggplant seeds compared with control treatment, while the treatment TF13 was significantly superior than all other treatments in number of leaflets, stem height, shoot fresh weight, shoot dry weight, root fresh weight, root dry weight and root size which recorded 8.67, 19.00cm, 20.33gm, 4.73gm, 12.00gm, 1.53gm, 17.67cm^3 respectively.

Keywords: *Fusarium oxysporum*, *Trichoderma harzianum*, Biological control

تقييم المقاومة الحيوية للفطر *Trichoderma harzianum* ضد الفطر *Fusarium oxysporum* f. sp. *Melongenae*

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الخلاصة

اجريت هذه الدراسة لغرض المكافحة الحيوية في ظروف المختبر وفي ظروف البيت البلاستيكي للفطر *Fusarium oxysporum* المسبب لمرض الذبول الفيوزارمي لنباتات الباذنجان وذلك باستعمال العامل

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الاحيائي الفطر *Tricoderma harzianum* . تم عزل وتشخيص 14 عزلة من الفطر *F. oxysporum* من حقلين منفصلين في العراق. اختبار الامراضية لهذا الفطر اظهر ان جميع العزلات كانت ذات امراضية عالية على نباتات الباذنجان لكن بنسب متفاوتة من الضراوة. بعد اثبـــات الامراضية تم اختيار 4 عزلات فقط من الفطر *F. oxysporum* حيث ان هذه العزلات سجلت درجة امراضية عالية على نباتات الباذنجان، العزلات الاربعة الاعلى ضراوة كانت (F5 و F6 و F13 و F14) حيث كانت امراضية هذه العزلات قبل البزوغ (83.3% و83.3% و 86.7% و 83.3%) على التوالي ، اما امراضية هذه العزلات بعد البزوغ كانت على التوالي (90.0% و 90.0% و 83.3% و 76.7%). اختبار التضاد في المختبر بين *T. harzianum* ضد العزلات الاربعة للفطر الممرض *F. oxysporum* اثبت ان الفطر *T. harzianum* ثبط العزلات الاربعة للفطر الممرض *F. oxysporum* ، حيث ان نسبة التثبيط للعزلات (F5 و F6 و F13 و F14) كانت (87.00 و 89.00 و 89.00 و 96.33 %) على التوالي. فعالية العامل الاحيائي ضد الفطر الممرض في ظروف البيت البلاستيكي اظهرت اختزالا كبيرا في مرض الذبول الفيوزارمي لنباتات الباذنجان مقارنة بمعاملة السيطرة التي تحتوي على الفطر الممرض فقط. حيث عند معاملة الفطر الممرض *F. oxysporum* مع *T. harziaunm* (TF) بينت ان جميع المعاملات (TF) حققت نسبة انبات عالية لبذور الباذنجان مقارنة بمعاملة السيطرة ، بينما كانت المعاملة (TF13) متفوقة على باقي المعاملات في عدد الوريقات وطول الساق ووزن المجموع الخضري الرطب ووزن المجموع الخضري الجاف ووزن الجذور الرطب ووزن الجذور الجاف وحجم الجذور حيث سجلت هذه النسب على التوالي لعوامل النمو اعلاه 8.67 و 19.00سم و 20.33غم و 4.73غم و 12.00غم و 1.53غم و 17.67سم^3.

Introduction

Eggplant, *Solanum melongena*, is one of the most important vegetable crops in Iraq which grown in the winter under greenhouse conditions and the summer under field conditions but the productivity of this crop in Iraq is still very low [1, 2]. Fusarium wilt disease is considered one of the most significantly reasons for reduce the eggplant yield in Iraq which causes many economic losses [3]. *Fusarium oxysporum* is the most important soil borne pathogens in Iraq causing Fusarium wilt on eggplant and other solanaceae in the greenhouse and field conditions [4, 5]. Chemical pesticides predominantly work well, but since they are designed to kill living organism they may be caused dangerous problems in humans or other organism, such as contaminate the environment and the food that we eat and this means that pesticide residues may be deposited in our bodies, also sometimes harm other organisms in addition to their target. Another problem in the use of chemical pesticides is that the pests may become resistant to pesticides [6]. So the application of chemical fungicide has been replaced by biological control which is safe to use and environmentally friendly, preventing the pollution and health hazards resulting from the classical uses of chemical pesticides [7, 8]. Biological control is the inhibition of a single pathogen by a single antagonist in a single cropping system by using of biocontrol agents to the infected plant and then inhibited the development of disease by a pathogen. Biocontrol agents are generally bacteria or fungi which separated from the endosphere or rhizosphere. In addition to that, the degree of disease inhibition achieved with biological agents can be identical to that achieved with chemicals [9, 10]. In generally, mechanisms of control done by biocontrol agent either through direct antagonism including hyperparasitism, or indirect antagonism including induce plant growth and competition or mixed-path antagonism including antibiotics, lytic enzymes and some of physical – chemical interactions [11]. Recently, *Trichoderma harzianum* are a successful example of biocontrol agent for inhibiting several plant diseases such as *Fusarium* wilt [12] [13].

The aim of this study is the biological control of the fungus *Fusarium oxysporum*, which causes wilt disease on eggplant plants using *Trichoderma harzianum.*

Materials and Methods

Pathogen Isolation and Identification

The infected eggplant plants were collected from two fields in Iraq. The infected plants were transferred to the laboratory to isolate and diagnose fungal pathogens. The infected plants were diagnosed based on the symptoms that appeared on the vegetative and root, which included yellowing,

drooping and falling of leaves, death of some branches, reddish-brown streaks were visible in the vascular tissues when cut with a knife and finally death of plants. The stems of infected plants were cut at a height of 15 cm above the crown area into small pieces (0.5-1 cm) and sterile by immersing them for three minutes in the sodium hypochlorite solution (1% free chlorine), then washed by immersing them in sterilized distilled water for three times, after that dried with sterile filter paper. Four pieces of the stems were cultured from each infected plant in petri dishes containing autoclaved potato dextrose agar (PDA) and incubated for 4 days at 27±2 Cº. The isolates were purified by taking mycelial plugs with a 5mm diameter from the growing margin and put overhead onto fresh PDA in the center of the Petri dish and incubated for 5 days at 27±2 Cº. *Fusarium oxysporum* was diagnosed based on the morphology of the colony, conidiophores and spores shapes (morphological characteristics) according to [14] and [15], and confirm the diagnosis of isolates being *Fusarium oxysporum* by Assistant Professor Dr. Alaa Mohsen Al-Araji.

Biocontrol agent (BCA) *Trichoderma harzianum*

Trichoderma harzianum isolate was obtained from Assistant Professor Dr. Alaa Mohsen Al-araji (Biology department, Science College, Baghdad University).

Preperation of *Fusarium oxysporum* and *Trichoderma harzianum* inoculum

The pathogen *Fusarium oxysporum* and *Trichoderma harzianum* were cultured on wheat grains medium, which was inoculated with 4-6 mycelial plugs (5mm in diameter) of three days old culture from *Fusarium oxysporum* and *Trichoderma harzianum*, then were shaken gently by hand and incubated for 10 days at 27±2C˚. When the wheat grain seed surfaces were completely covered with fungal mycelium, the flask content was spread out at laboratory temperature and utilized as a pathogenic and biocontrol agent inoculum, and used a rate of 5g / 1kg autoclaved soil mixture per sterile plastic pot were sterilized by formalin 10 % (Barari, H. 2016).

Pathogenicity test of *Fusarium oxysporum*

This test was accomplished to detect the virulence of *F. oxysporum* isolates. Pots containing autoclaved soil mixture were inoculated by pathogen inoculum. The pathogens inoculum were placed at a depth of 1-3cm, then the pots were irrigated and each pot was covered by polyethylene bag, and left for three days. Local eggplant seeds (germination rate of 95%) untreated with fungicide was sown in pots to depth 1cm at a rate of 10 seeds per pot. All pots were watered and transferred to greenhouse. This experiment was carried out in three replicates inoculated with *F. oxysporum*, and another three replicates without the pathogens as a control (only eggplant seeds in soil mixture). These pots were irrigated with water as needed and daily observed through the period of experiment (6 weeks). Two weeks after sowing, the non-germinating seeds were counted for calculating the percentage of dead seedlings before emergence using the formula

$$P = \frac{\text{Number of non germinating seeds}}{\text{Total number of seeds planted}} \text{ X } 100$$

P = the percentage of pre-emergence infected seedlings

While the percentage of post emergence infected seedlings were calculated after 6weeks by the formula:

$$P1 = \frac{\text{The number of infected seedlings}}{\text{Total number of seedlings}} \text{ X } 100$$

P1= the percentage of post emergence infected seedlings

(Barari, H. 2016).

Antagonistic Activity Evaluation

Dual culture technique was used to evaluate in vitro the antagonistic activity of *T. harzianum* against *F. oxysporum*. Mycelial plugs with a 5mm diameter were cut from the growing margin of three days old cultures of the BCA and the pathogen, transferred overhead to fresh PDA petri dishes (9 cm in diameter). The biocontrol plug was placed at the border of the PDA Petri dish, whereas the mycelial plug of the pathogens was placed at the border of opposite side of PDA Petri dish. The space between the pathogen and BCAs was 7cm. The Petri dishes were incubated for 7days at 27±2 Cº. The mycelia growth of pathogenic fungi was monitored. Also the pathogens were cultured on PDA Petri dishes (9cm in diameter) by placing 5mm mycelial plug in the center of the PDA Petri-dishes (control). Percentage of mycelia growth inhibition of the BCAs against the pathogens was measured and calculated after 7days of incubation by using the formula:

Mycelia growth inhibition % = $(G_1 - G_2) / G_1 \times 100$

G_1 = a mean of mycelia radius of the pathogen in Petri dish without BCA (control).
G_2 = a mean of mycelia radius of BCA in Petri dish with the pathogens.
(Barari, H. 2016).

Greenhouse experiment

To evaluate the biocontrol efficiency of *T. harzianum* under greenhouse against virulent isolates of the pathogen *F. oxysporum* causing wilt disease respectively on eggplant plants. At first the autoclaved soil mixture inoculated with *F. oxysporum* inoculum (loaded on wheat grain) 2-3cm in depth and each pot was covered by polyethylene bag. After 2 days the BCA *T. harzianum* inoculum were added 2-3 cm in depth, after that the polyethylene bags were taken off. Local eggplant seeds untreated with fungicide were added, ten seeds were sown per pot at a depth not more than 1cm,. The experiment consisted of three replications per treatment. All treatments randomly distributed under greenhouse conditions (controlled range of temperature 15-35 C°; 10h natural light, irrigation: three times a week). The experiment consisted of the following treatments:

- Treatments of *F. oxysporium** with *T. harzianum*
- Treatment of *F. oxysporium* alone (without BCA) as infected control
- Treatment of healthy control without pathogens or BCA (only eggplant seeds on autoclaved soil mixture).

* *F. oxysporium* included four isolates: F5, F6, F13 and F14.

The daily observation was carried out for each treatment; the percentage of germination rate seedlings after 10 days of sowing was calculated. Also the growing seedlings were thinned to 3 seedlings per pot after 3 weeks of sowing. Ten weeks after sowing the plants were pulled out and shoots were separated from the root and the following parameters were recorded for each treatment:

- The number of leaflets
- Stem height
- Shoot fresh weight
- Shoot dry weight
- Root fresh weight
- Root dry weight
- Root size

Root size was calculated by washed the roots with tap water to remove dust sticking with it, then put on filter paper for 10min to rid it of excess water, after that the fresh weight of root was calculated . The size of root was measured by putting the root into measuring cylinder (500ml) containing 200ml water ,then the size of root was measured through this formula:
Size of root = volume of water with root into cylinder – 200ml

After that the dry weight of roots were calculated by putting it on blotters at first for 15mins , then put into oven 70C° for 48h and calculated the dry weight after that (Barari, H. 2016).

Statistical analyses

All experiments were set up as a randomized complete block design (RCBD), with three replicates per treatment. All data were analyzed using analysis of variance (ANOVA), and means separated using (LSD) tests and alpha values of 0.05 (by computer program called Genstat v. 7.2, Third edition).

Result and Discussion

Pathogen isolation and identification

Fourteen isolates of *F. oxysporum* was isolated from infected eggplant plants from two fields. Purified, and identified depending on morphological characters and microscopically characters [14] [15]. Our result of *F. oxysporum* isolation from the infected eggplant was agreement with [16-18] which isolated the fungus from the stems of the infected eggplant plant Figure- 1.

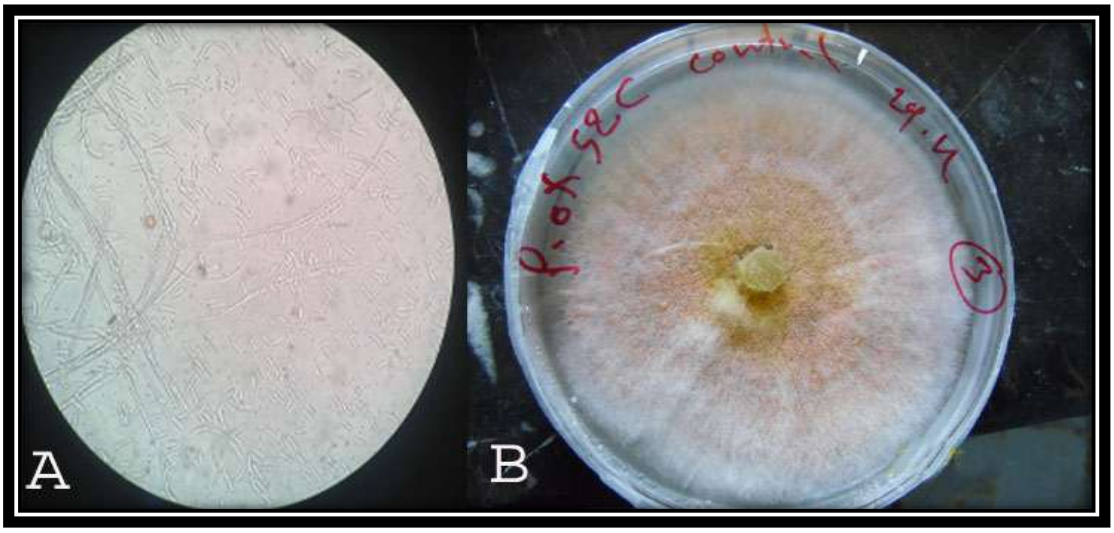

Figure 1- A: *Fusarium oxysporum* mycelium with conidia under microscopic
B: *Fusarium oxysporum* colony

***Fusarium oxysporum* pathogenicity test**

Pathogenicity test results revealed that all isolates of *F. oxysporum* were pathogenic to eggplant plants, but their ability to cause the infection was in varying proportions. The four highly virulence isolates of *F. oxysporum* were F5, F6, F13 and F14 causing infection to eggplant plants pre emergence (83.3%, 83.3%, 86.7% and 83.3%) and post emergence (90.0%, 90.0%, 83.3% and 76.7%) respectively (Table- 1).

Infection processes by *F. oxysporum* were divided into some steps: root recognition, root surface attachment and colonization; penetration and colonization of the root cortex and sometimes hyphal proliferation within the xylem vessels. Then the fungus secretes several cell wall degrading enzymes, such as polygalacturonases, pectate lyases, xylanases, proteases and cellulase, during root penetration and colonization of host plant [19, 20]. Our results were agreement with many studies reported that the pathogenic fungus *F. oxysporum* was highly virulence on eggplant and other solanaceae family [21-23].

Table 1- Pathogenicity test of *Fusarium oxysporum* on eggplant plants under greenhouse conditions (25-30 C$^{\circ}$).

Isolates	Infected seedling by *Fusarium oxysporum* %	
	Pre emergence (P)	Post emergence (P1)
F1	**76.7**	**55.3**
F2	**50.0**	**57.3**
F3	**63.3**	**68.3**
F4	**60.0**	**70.0**
F5	**83.3**	**90.0**
F6	**83.3**	**90.0**
F7	**36.7**	**56.3**
F8	**20.0**	**58.0**
F9	**56.3**	**23.3**
F10	**49.7**	**36.7**
F11	**55.3**	**26.7**
F12	**52.3**	**43.3**
F13	**86.7**	**83.3**

F14	**83.3**	**76.7**
C	**0**	**0**
L.S.D	**P≤ 0.05**	
Between treatments	**18.0**	
Between pre and post	**6.8**	
Between interactions	**25.5**	

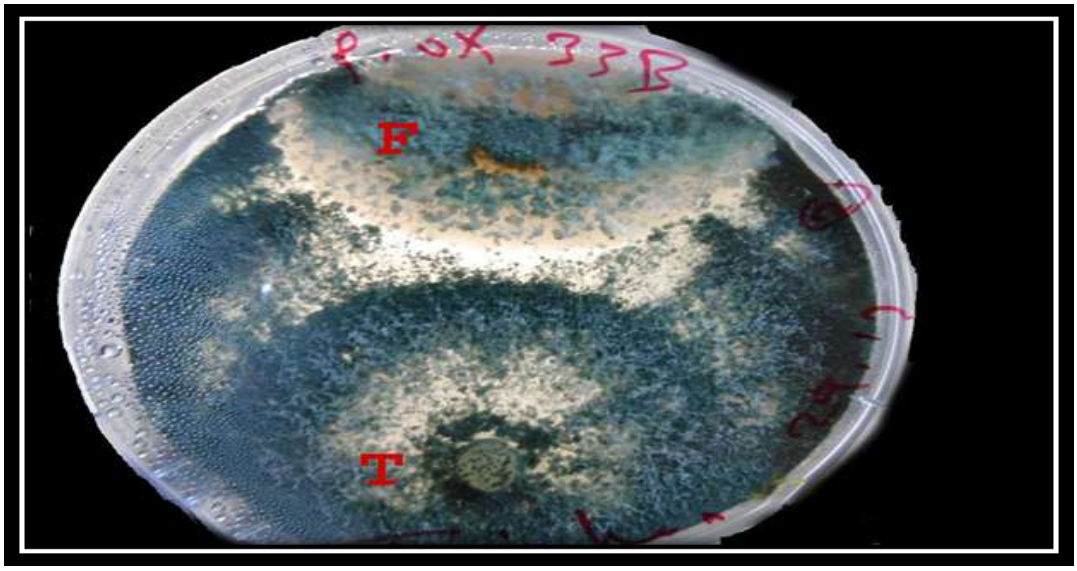

Figure 2- Antagonistic activity by dual culture between *Trichoderma harzianum* and *Fusarium oxysporum* on PDA after 7 days at 27 ± 2C˚ and pH 5.

Antagonistic activity evaluation

The antagonistic activity of *T. harzianum* revealed that this fungus was highly inhibited the growth of *F. oxysporum*, which recorded the inhibition percentage for the four *F. oxysporum* isolates (F5, F6, F13 and F14) as follows (87.00, 89.00, 89.00, and 96.33%) respectively Table- 2 and Figure- 2.

Table 2- Growth inhibition of *Fusarium oxysporum* isolates by *Trichoderma harzianum* on PDA after 7 days at 27 ± 2C˚ and pH 5.

Treatments	Mycelial inhibition of *Fusarium oxysporum* %
TF5	**87.00**
TF6	**89.00**
TF13	**89.00**
TF14	**96.33**
L.S.D	**P ≤ 0.05**
Between treatments	**7.43**

The interaction of *T. harzianum* with *F. oxysporum* mycelia and didn't formed inhibition zone during the antagonistic activity indicated the parasitic activity of *T. harzianum* fungus against the pathogen and this is due to increased production and activity of lytic enzymes such as chitinases, glucanases and proteases [24-27]. These enzymes act to dissolve the host cell walls, which in turn stimulates the production of more enzymes and thus produces a series of physiological changes that stimulate the rapid and direct growth of *Trichoderma* spp. [28].

Table 3- Influence of *Trichoderma harzianum* on pathogenicity of *Fusarium oxysporum* isolates for eggplant growth parameters under greenhouse conditions.

Treatments	Germination rate %	No. of leaflets	Stem height (cm)	Shoot fresh weight (gm)	Shoot dry weight (gm)	Root fresh weight (gm)	Root dry weight (gm)	Root size (cm^3)
TF5	**70.0**	**7.33**	**18.67**	**19.67**	**4.00**	**12.33**	**1.30**	**16.67**
TF6	**63.3**	**8.00**	**18.67**	**19.67**	**4.03**	**11.33**	**1.20**	**16.67**
TF13	**83.3**	**8.67**	**19.00**	**20.33**	**4.73**	**13.00**	**1.53**	**17.67**
TF14	**73.3**	**8.67**	**18.00**	**19.33**	**4.43**	**12.00**	**1.30**	**16.00**
F5	**40.0**	**4.33**	**12.33**	**10.33**	**1.90**	**7.67**	**1.03**	**7.67**
F6	**23.3**	**4.67**	**13.33**	**10.67**	**2.30**	**7.67**	**0.96**	**6.67**
F13	**33.3**	**4.33**	**11.67**	**11.00**	**2.00**	**8.00**	**1.03**	**7.67**
F14	**30.0**	**4.33**	**13.00**	**10.33**	**2.36**	**7.00**	**1.50**	**7.67**
C	**90.0**	**7.67**	**14.67**	**15.00**	**4.46**	**13.67**	**2.03**	**10.00**
L.S.D				**$P \leq 0.05$**				
Between treat	**17.56**	**1.75**	**3.20**	**2.93**	**0.82**	**2.19**	**0.40**	**4.11**

TF= *Trichoderma harzianum* + *Fusarium oxysporum* ; F= *Fusarium oxysporum;* C= control

Greenhouse experiment

The percentage of germination rate seedling of eggplant plants in treatment of soil mixture with *F. oxysporum* pathogen recorded 40.0, 23.3, 33.3 and 30.0% for F5, F6, F13 and F14 respectively compared with healthy control 90.0% (Table-3). But this percentage increased when treated *F. oxysporum* with *T. harzianum* (TF), this percentage which recorded 70.0, 63.3, 83.3 and 73.3% respectively for TF5, TF6, TF13 and TF14. The growth parameters indicate that *F. oxysporum* was highly significantly infected eggplant plants compared with healthy control, for example F5 isolates which the number of leaflets, stem height, shoot fresh weight, shoot dry weight, root fresh weight, root dry weight and root size recorded 4.33, 12.33cm, 10.33gm, 1.90gm, 7.67gm, 1.03gm, and 7.67cm³ respectively, while the TF treatments were higher significantly suppressed this pathogen on eggplant plants and enhanced the plant growth in all tested parameters compared with infected control, and the treatment TF13 was superior than other treatments which recorded 8.67, 19.00cm, 20.33gm, 4.73gm, 12.00gm, 1.53gm, 17.67cm³ for number of leaflets, stem height, shoot fresh weight, shoot dry weight, root fresh weight, root dry weight and root size respectively (Tabl- 3) (Figure- 3).

Trichoderma harzianum significantly inhibited the growth of *F. oxysporum* and enhanced eggplant plant growth; this may be attributed to fact that *Trichoderma* species produce extracellular cellulase and pectinase enzymes that are capable of hydrolyzing the cell walls of pathogenic fungi [29]. According to [30] and [31] this fungus has the enzyme Glucose-methanol-choline oxidoreductases which play significant roles in the antibiosis against plant pathogenic fungi and biological control of plant diseases. The enzyme biosynthesis conception as a mechanism of biocontrol has been expanded to include synergism between enzymes and antibiotics [32]. Moreover, the suppression of *F. oxysporum* growth by *T. harzianum* may be due to direct interaction between them, as in mycoparasitism, which involves physical contact supported with its enzymes and antibiotics [33].

Our result agreed with [34], who found the application of *Trichoderma* native antagonists was effective in suppressing wilt incidence which caused by *F. oxysporum*.

Figure 3-Influence of *Trichoderma harzianum* on pathogenicity of *Rhizoctonia solani* to tomato plants and on tomato plants growth parameters.

Conclusion

Fusarium oxysporum isolates that isolated from two field in Iraq recorded highly pathogenic for eggplant which caused wilt disease in different rates, and the biocontrol agent *T. harzianum* had high efficacy for inhibition this pathogen *in vitro* and enhance the plant growth *in vivo*.

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