Journal of Plant Protection and Pathology

Journal homepage: www.jppp.mans.edu.eg
Available online at: www.jppp.journals.ekb.eg

Biological, Histological and Pathological Studies of Tomato Wilt Disease Caused by *Fusarium oxysporum* f. sp. *Lycopersici*

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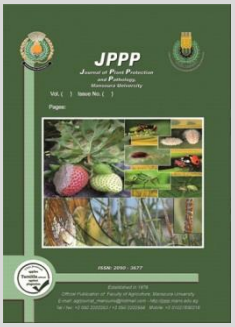

ABSTRACT

Tomato wilt disease caused by *Fusarium oxysporum* f.sp. *lycopersici* is one of the most destructive diseases, and causes significant yield losses in Egypt. In the present study, the antifungal activities of some essential oils (clove, garlic and thyme) at various concentrations (10, 15 and 20 %) in addition to different Trichoderma isolates (*Trichoderma harzianum*, *Trichoderma hamatum* and *Trichoderma viride*) and three different concentrations of urea (0.5 , 1 and 1.5%) was investigated against the pathogen (FOL).. The results revealed that the all tested essential oils reduced the growth and spore population of FOL significantly. The highest pathogen reduction was recorded with clove oil followed by garlic oil either under laboratory or green-house conditions. Contrary, application of urea fertilization at concentrations of 1% and 1.5% increased significantly both growth mass and sporulation capability of FOL comparing to the control. The highest percentage (٩٤.2%) of FOL growth inhibition under *in vitro* condition was recorded with *Trichoderma harzianum*, followed by *Trichoderma viride* (90.8%). Similar results were recorded under greenhouse conditions. Furthermore, the results showed that the all tested oils, Trichoderma isolates and urea fertilization improved the growth criteria of tomato plants comparing to the control plants. On the other hand, the histological investigation proved that *Trichoderma harzianum* and clove oil were the best treatments where there weren't histological changes in the external cortex, epidermal cells, endodermis and vascular vessel.

Keywords: Fusarium wilt *Trichoderma harzianum. Trichoderma viride* Clove oil. Histopathology

INTRODUCTION

Tomato (*Lycopersicon esculentum* Mill.) belongs to the family Solanaceae is one of the most important vegetable crops worldwide (Barari 2016). It consider the second most important vegetable crop next potato in Egypt and all over the world (Hafez *et al*., 2012). Many serious pathogens attack tomato plants and cause a significant suppression in tomato production and productivity. *Fusarium oxysporum* f.sp. *lycopersici* (FOL) is one of these main pathogens of both greenhouse and field grown tomatoes. Fusarium wilt of tomato is a very difficult disease to treat because the fungus attacks plants through the roots and spreads within the vascular tissues growing internally through the cortex to the stele led to plant death (Bowers and Locke 2000).

Biocontrol agents have been used as an eco-friendly alternative method to control the disease instead of chemical application. Recently, it has been reported that some essential oils of medicinal plants have a strong effective antifungal potential (Bokhari and Perveen, 2012; Neela *et al*., 2014; Enespa and Dwivedi, 2014) against storage fungi, foliar and soilborne pathogens (Bowers and Locke 2000). Among these oils, clove, thyme and garlic offer a variety of functions for plants together as they protecting themselves of cold or heat, repelling or attracting insects and using chemical ingredients in them as defence equipment. Moreover, plant oils have good results *in vitro* and *in vivo* studies for their antifungal effects on mycelial growth and sporulation against FOL fungus.

Trichoderma spp such as *T.harzianum , T. viridi and T. hamatum have an* ecological importance as a biocontrol agents in the field and consider from common saprophytic fungi inhabitants in the rhizosphere of soil with no negative effect on the environment and nontoxic to human health (Boureghda and Bouznad 2009, Kareem *et al*., 2016) . It can improve growth parameters of plant, crop productivity, and induce both localized and systemic resistance to many plant pathogens (Kareem *et al*., 2016). *Trichoderma viride* and *Trichoderma harzianum* have been found to induce resistance in host plants against *Fusarium oxysporum* f.sp. *lycopersici* leading reducing disease incidence from 100 to 7.69%(Mohd Rajik *et al*., 2012). Harman *et al*., (2004) observed that the ability of *Trichoderma* isolates to protect plants against wilt disease and its induced systemic resistance in tomato plants which back to colonization of root with *Trichoderma* isolates that can increase levels of defence-related plant enzymes and pathogenesis-related proteins (PR).

Nitrogen as a form of urea strongly affects plant health, the physical, chemical and biological characteristics of soil, in addition to supply and form affect plant defence and disease suppression by the regulation of intracellular concentrations of cell sugars and amino acids, which provide nutrients for invading fungi (Mur *et al.*, 2016). Various studies reported that Nitrogen supply affects pathogenicity of *F. oxysporum* especially with tomato *(*López-Berges *et al*., 2010). On the other hand some studies have shown increased severity of Fusarium wilt with increasing supply of nitrogen. Increasing susceptibility to

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DOI: 10.21608/jppp.2021.198993

disease in plant back to deficiency stress of nitrogen, and increased nitrogen application has been shown to decrease disease severity in a field planted with faba bean (Dong *et al*., 2013) and a hydroponic trial with cucumber (Zhou *et al*., 2017). Contrary, other studies concluded that no relation between nitrogen concentrations and suppression of Fusarium wilt of tomato (Hoffland *et al*., 2000). This may indicate that increasing the concentration that the plant can uptake, further nitrogen fertilizing has no benefit, or may even be harmful. Further research is needed to identify the stander level of nitrogen in each system, as well as understand better the effects of nitrogen form on *F. oxysporum* f.sp. *lycopersici.*

The aim of the present work was to study the inhibitory effects of different concentrations of some essential oils, urea fertilization and some isolates of *Trichoderma* on tomato wilt disease caused by *F. oxysporum*.sp. *lycopersici*. and investigate the histopathological features on treated tomato plants to infer size the mechanisms involving in tri-interactions between tomato plants, FOL and some promising plant oil extracts as well as beneficial biocontrol agents.

MATERIALS AND METHODS

Isolation, purification and maintenance of pathogen and tested fungi:

Both isolates of Fusarium oxysporum f.sp. lycopersici (FOL) as a pathogen and Trichoderma spp. isolates as biocontrol fungi used in this work were naturally isolated from healthy and diseased tomato plants showing typical symptoms of tomato wilt disease expressed as root rots, vascular discoloration and plant wilting. These isolates were collected randomly and separately from agricultural field of Kafr El-sheikh governorate. Tissue bits were surface sterilized with 3 % sodium hypochlorite for 4 minutes and washed with sterile distilled water. Then, they were grown on Potato Dextrose Agar (PDA) medium supplemented with streptomycin sulfate (250 mg/ml) (w/v) and incubated at 25°C for 14 days. The pathogen (FOL) and biocontrol fungi were purified by hyphal tip technique (Ho and Ko, 1997) and identified on the basis of cultural and morphological characters as micro and macro conidia (Suresh et al., 2011 and Nasr- Abdul-Rahman, 2014) at Botany Department, Faculty of Agriculture, Menoufia University. F. oxysporum and Trichoderma isolates were selected for this study according to (Booth, 1971; Nelson, 1983). The concentration of spore suspension was adjusted to 106 spores mL-1 using slide of hemocytometer (Elad and Baker, 1985), the isolates were then stored at 3 °C for further studies.

Pathogenicity test:

Pathogenicity test of the isolated pathogen was conducted according to (Jasnic et al., 2005). Inoculum were prepared by growing each isolate on sterilized Barley sand medium (75 g barley + 25 g sand + 100 ml water); using 1000 ml flasks. Flasks which incubated 25°C for 14 days and shacked daily to allow more fungal growth. Clay loam soil was autoclaved twice at 121°C for an hour and mixed with the inoculated Barley sand medium .Soil infestation was conducted separately at the rate of 3% of soil weight, potted soil was irrigated every second day for a week to allow the fungal distribution into the soil. Tomato seedling (Cv. Kesma) 14 days old were planted. . The sowing plants were investigate every week for the wilt disease. After 30 days, the plants were picked up and longitudinal sections through roots and stem bases were obtained to examine the browning vesicles of each plant.

***In vitro* experiments**

Preparation of plant oils concentrations and determination of its effect on mycelial growth and sporulation of FOL:

Plant oils were obtained from El-Gomhouria Company for oils, Cairo, Egypt. To obtain 10, 15 and 20% concentrations of plant oils (clove, garlic and thyme) it were emulsified with 3% (v: v) tween 20 and mixed with PDA medium (Fontes et al., 2018). Petri dishes contained oils were inoculated with 5 mm disc of FOL 7days old and incubated at 25 °C for 7 days.

Preparation of plant urea concentrations and its determination of its effect on mycelial growth and sporulation of FOL:

Urea was diluted with 3% (w: v) distilled water mixed with PDA medium to obtain 0.5, 1 and 1.5% concentrations. Petri dishes contained urea were inoculated with 5 mm disc of FOL 7days old and incubated 25 °C for 7 days.

Preparation of *Trichoderma* spp. and its effect on mycelial growth and sporulation of FOL:

The in-vitro experiment was laid out in completely randomized design (CRD) with three isolates of Trichoderma spp., T. harzianum, T. viride and T. hamatum were individually tested for their efficacy against FOL pathogen. The observation of Dual culture method was followed according to (Devi et al., 2015). Five mm disc of FOL was placed on PDA medium one cm away from the edge of the plate, separately. Trichoderma spp 5 mm disc was placed at opposite side of the Petri plate. Three replicate plates for each treatment maintained and incubated at 25±2°C. Inhibition percent over control was calculated (Sundaramoorthy and Balabaskar, 2013) as the formula:

$$\% \text{ GI} = (a - b / a) \times 100$$

Where,

GI is the percent inhibition over control, a: is the growth of tested pathogen in absence of antagonist (mm) and b: is the growth of tested pathogen against antagonist (mm). Control plates had the pathogen disc 5mm in the middle. Petri dishes were incubated at 25±2 °C for 7 days and examined daily.

***In vivo* experiments**

Inoculum Preparation and Soil infestation:

For inoculum preparation, all the fungal isolates were cultured in sterilized Barley sand medium (75 g barley + 25 g sand + 100 ml water); using 1000 ml flasks for 15 days at 25° C, and shacked every second day to allow more fungal growth. The experiment was carried out in a greenhouse and soil infestation was conducted separately at the rate of 3% of soil weight. Pots were irrigated every second day for a week to allow the fungal distribution into the soil.

Control of tomato wilt disease:

To control Fusarium wilt of tomato in the greenhouse, four-week old tomato seedlings were planted in plastic pots (15 cm diameter; 20 cm height) containing soil treatments . The treatments included: (a) healthy control (no fungus); (b) fungal infested control; (c) infested soil amended with Trichoderma isolates; Trichoderma hamatum,Trichoderma harzianum and Trichoderma viride (d) infested soil amended with plant oils ; Clove, Garlic and Thyme oils at the

concentrations of 10, 15, 20 % (e) infested soil amended with urea fertelization at the concentrations of 0.5, 1 and1.5 %. Plants were arranged following a completely randomized design and irrigated with tap water as it needed. At 60 days the inoculated plants were first visually assessed for symptoms and soil samples of previous treatments were collected regularly and the populations of the fungus were assessed in vitro using the soil dilution method (Aneja 2005). Variation in symptom developments were observed in all the treatments.

Determination of disease severity:

Wilt severity from tomato seedlings was recorded after 90 days according to a following scale . This was based on the wilt severity rated as follows;

(% of shoot wilted, using a scale of 0–5 where, 0 = no symptoms, 1 = one leaf wilted (1–25%), 2 = 2 or 3 leaves wilted (26–49%), 3 = half plant wilted (50–74%), 4 = all leaves wilted (75–100%), 5 = dead plant).

Determination of plant growth parameters:

The average of root length, root fresh weight, root dry weight, Plant height and No. of branches per plant were recorded at the end of experiments.

Biochemical Analysis

Determination of Chlorophyll A and B in leaves of tomato plants

To estimate Chlorophyll A and B in leaves (60 days after transplanting) one gram of fresh leaf material was cut and ground with a clean pestle and mortar. 20ml of 80% acetone and 0.5gm MgCO3 powder was added to this homogenized leaf material. The homogenate was centrifuged at 500 rpm for 5 minutes. The final volume of supernatant were re-extracted by adding 80% acetone. The color absorbance of the solution was estimated by a spectrophotometer 645 and 663 nm in the UV-spectrophotometer. The content of chlorophyll was expressed as mg/g of extract (ge) according to the following equations (Lichtenthaler, 1987):

$$\text{Ch. A} = (12.7 \times A_{663} - 2.7 \times A_{645}),$$
$$\text{Ch. B} = (22.9 \times A_{645} - 2.7 \times A_{663}).$$

Where,

Ch. A and Chl. B are the chlorophyll A and chlorophyll B, A is absorbance.

Determination of total phenol content in roots tomato plants

Total phenol content (TPC) was performed after60 days after transplanting according to Folin-Ciocalteu method described by (Magalhaes *et al*., 2010). 3 ml of the extracts were dissolved in absolute methanol with distilled water to it 0.5 ml folin ciocalteau reagent (1:1 with water) and 2 ml Na2 CO3 (20%) were added to the extracts. Following an incubation for 30min at room temperature, then cooled and absorbance was measured at 650 nm using known concentrations of catechol (blank). Total phenol content (TPC) was expressed as mg catechol equivalent of phenol/g of sample.

Histological studies:

For fixation : Samples were fixed in FAA solution (85% alcohol to 70% , 10% formalin and 5% acetic acid) for 48 h, dehydrated in an ethyl alcohol(EtOH) series (70, 80, 90, 96% and absolute EtOH) for 24 h each and rinsed in 100% terbuatanol for 24 h. Sections of plants were immersed in a mixture of paraffin : terbutanol 1:1 w/v and then in pure paraffin at 60°C, for a 24h period per pass. Finally, the blocks containing the samples were sectioned in a rotational microtome (Conn 1953).

For the staining process: different techniques were tested: safranin - fast green, periodic acid - Schiff reagent (PAS) (Bancroft and Stevens 1982), with the modification of including a contrast with fast green for 2 seconds; and astra blue - basic fuchsin double staining (Kraus *et al*., ,1998).

For microscopic studies : Sections were observed using a light microscope (Eclipse 50i; Nikon Instruments Inc., magnification ×40 to ×100) and photographed using a digital optical device connected to a computer through the control unit DS-U2 (Nikon Instruments Inc.). Inoculated plant samples were compared with non-inoculated control samples.

Statistical analysis:

Experiments were performed in completely randomized design. Mean values were compared by the least significant difference (LSD) testing at p = 0.05. Duncan's multiple Range test at p = 0.05 was used to compare means. All statistical analyses were conducted using Costate, Statistical Software (Gomez and Gomez 1984).

RESULTS AND DISCUSSION

Nine isolates of *Fusarium oxysporum* f.sp. *lycopersici* (FOL) were isolated from Kafr El-sheikh governorate. All the obtained isolates were pathogenic to healthy tomato plants, where isolate No. 4 was used in the further stdies as it showed the highest virulence potential and produced more spores than the others. Three isolates of *Trichoderma* spp. were identified as *Trichoderma harzianum*, *Trichoderma hamatum* and *Trichoderma viride* according to (Bisett, 1991).

***In vitro* experiments**

Effect of plant oils, *Trichoderma* spp. And urea concentrations on the growth and sporulation of FOL:

The recorded mean fungal radial growth (mm) and sporulation of *Fusarium oxysporum* f.sp. *lycopersici* for each treatment presented in Fig1, 2 and 3 which indicate that all different concentrations of tested plant oils and biocontrol agents showed significant reduction in both of growth and sporulation potential of the pathogen . Results in (Fig1) showed that clove oil was the most effective oil where it inhibited the mycelial growth and number of spores of FOL at the concentration of 20% followed by 15 and 10 %, respectively. Whereas, at 20% concentration of Thyme oil inhibited the mycelial growth and sporulation of FOL by 53.73% and 74.11%, respectively. The moderate results were obtained by Garlic oil which recorded 62.5% and 76.85% growth reduction at 10 and 15 % conc. and gave 100% Growth reduction at the concentration of 20%. It is of logic that increasing oil concentrations gives more significant effect in reducing the fungal growth and sporulation. Such results were observed by (Eman M. *et al.*, 2020), and these results are in harmony with those obtained by (Ohunakin and Bolanle, 2017) Antimicrobial activity of Clove oil against *F. oxysporum* could be due to the main chemical components of it which are eugenol, acetyl eugenol, iso-eugenol and caryophyllene (Rahimi *et al.*, 2012). Such compounds can be responsible for the antibacterial and antifungal properties of the essential oil (Akthar *et al.*, 2014).

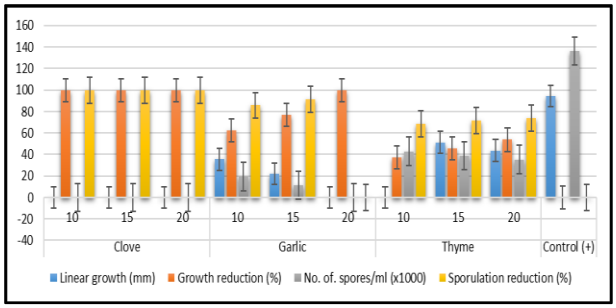


Fig 1. Effect of different concentrations of some plant oils on the growth and sporulation of *Fusarium oxysporum* f.sp. *lycopersici* .

Results in (Fig 2) showed that all tested *Trichoderma* isolates inhibited fungal radial growth (mm) and sporulation of *Fusarium oxysporum* f.sp. *lycopersici.* There were significant differences among these isolates. In this respect, *T. harzianum* and *T.viride* recorded the greatest action while *Trichoderma hamatum* gave the least efficiency. The growth reduction recorded 77.51% and 65.18% with application of *T. harzianum* and *T.viride* ,respectively. However *Trichoderma hamatum* recorded 43.82%. These results are in agreement with previous studies (Lakshman Prasad *et al*., 2016; Andleeb Zehra *et al*., 2017and Mwangi *et al*., 2019). The inhibitory effect of these isolates of *Trichoderma* against FOL may be due to production of an antagonistic substance which lysis cell wall components of the pathogen and help the antagonists to penetrate the host hyphae and grow on it as a hyperparasite (Papavizas 1984) and/or prevent the continued growth of pathogen through cloning of their hyphae around the hyphae of the pathogens (Adekunle *et al*., 2002).

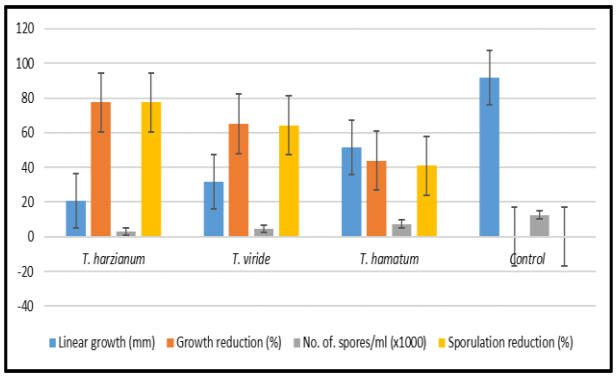


Fig. 2. Effect of different *Trichoderma* spp. isolates on the growth and sporulation of *Fusarium oxysporum* f.sp. *lycopersici*

The obtained results showed that there were reduction in all growth and sporulation of FOL in application of urea at concentration 0.5% as growth reduction (%) and number of spores/ ml which recorded 31.58 and 7.85 respectively compared to the other concentrations 1% and 1.5 % which recorded 23.69 , 8.57 and 4.06 , 9.47 respectively Fig (3). It is of logic that increasing the concentration of urea gives less significant effect in reducing the fungal growth and sporulation. Such results are confirmed by (Carlini 2008 and Follmer 2004) who cleared that application of urea at a standard rate can affect on the fungus growth, and it exert a toxic effect on fungi and certain insects which is independent of the urease activity.

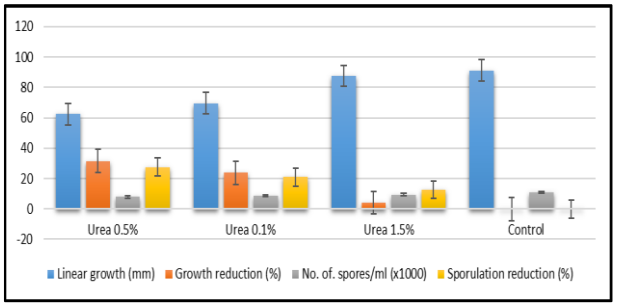


Fig. 3. Effect of different concentrations of urea on the growth and sporulation of *Fusarium oxysporum* f.sp. *lycopersici* .

***In vivo* experiments**

Effect of plant oils, *Trichoderma* spp. And urea concentrations on FOL spore population in the soil:

Data in Fig (4) indicate that the application of tested plant oils to the artificially infested soil with FOL decreased the population of the pathogen's spore; significantly in comparison with control. Clove oil gave the best results at the highest concentrations (20%), however, at the lowest concentration of either garlic or thyme (10%) the spore population was 2.64 and 6 spore at 80 days after infestation respectively. Increasing the concentration of the plant oils resulted more reduction of FOL spore population whereas it was increased by time in control treatment (FOL only).. Our findings are in agreement with those obtained by (Sharma *et al*., 2017 and Eman M. *et al.*, 2020). They confirmed that clove oil provided a high inhibition after 60 and 80 days as it has the ability to inhibit radial growth, spore germination and to reduce the dry weight of *Fusarium oxysporum* f. sp. *lycopersici.*

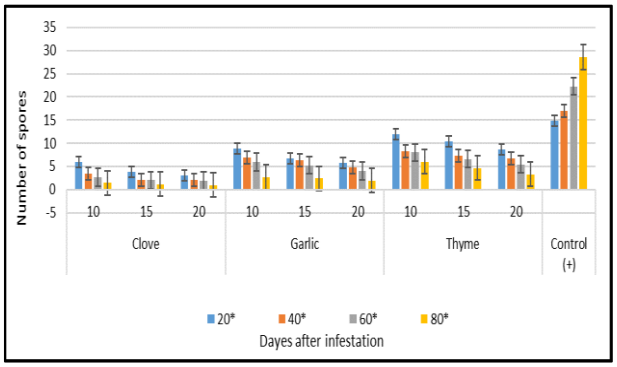


Fig 4. Effect of different concentrations of some plant oils on spore population / l gm soil infested with FOL.

Results shown in Fig (5) clear that the application of selected antagonists (*T. harzianum* ,*T.viride* and *T.hamatum*) had significant efficiency in suppressing spore population in the infested soil ; in comparison with control treatment (pathogen). The best tested biocontrol agents in reducing spore population were *Trichoderma harzianum* and *T. viride* which recorded 1.99 and 3.33 spore / ml as a mean. On the other hand, *T. hamatum* was the least effective one in reducing spore population by4.32 spore / ml. Such results are recommended by (Nourozian *et al*.,2006). The effect of action of reducing spore population was attributed to different antibiotics, antifungal metabolites or secrete hydrolytic enzymes by *Trichoderma* spp. (Keswani *et al.*, 2014 and Basco *et al.*, 2017).

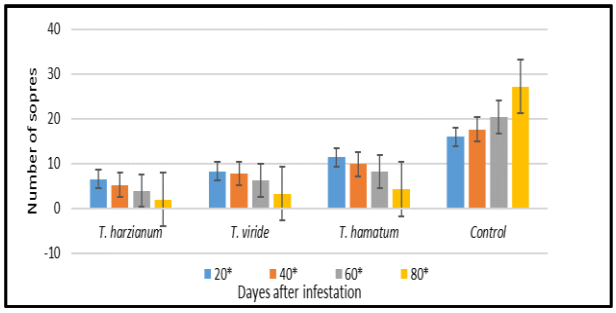


Fig 5. Effect of different *Trichoderma* spp.isolates on spore population / l gm soil infested with FOL.

Results present in Fig (6) clear that at the concentration of 0.5%, urea significantly inhibited development on spore population in the infested soil. The results recorded 5.55 spore population / l gm of FOL, comparison with control (pathogen) which recorded 24.74 spore population / l gm at 80 days after infestation. Increasing the concentration of urea resulted more development of FOL spore population by time (Hemissi *et al*., 2018) . These results are in harmony with those obtained by (Ma *et al*., 2004) who reported that the mode of the toxic action by urea may be more complex, The addition of urea to the soil at low concentration increases the concentration of nitrogen in that soil, which may enhance the activity of soil microorganisms antagonistic to a pathogen. On the other hand, (Martin *et al.*, 1991) showed that an increase in nitrogen rates to the soil significantly increased the occurrence of Fusarium foot and root rot disease in some crops.

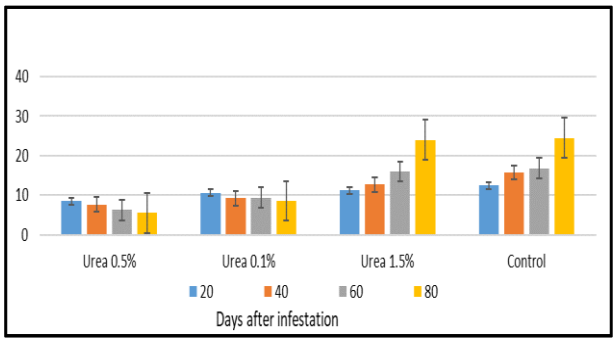


Fig 6. Effect of different concentrations of urea on spore population / l gm soil infested with FOL.

Effect of different concentrations of some plant oils, *Trichoderma* spp. and urea concentrations on disease severity under greenhouse conditions:

Results shown in Fig (7) indicate that application of the plant oils at all used concentrations significantly decreased disease severity % of tomato wilt disease compared to control (pathogen) treatment. Clove oil gave the best results at the highest concentration (20%) followed by garlic oil at the same concentration. They recorded 6.9 and 9.8 % disease severity of tomato wilt respectively. The antifungal activity of the essential oils against FOL was also reported by (La Torre *et al.*, 2016 and Eman M. *et al.*, 2020). They confirmed that increasing concentration of the clove oil can inhibit the incidence and severity of tomato Fusarium wilt under greenhouse conditions compared to control. In addition, Hamad *et al*., 2015 demonstrate that clove oil applied on guava seedlings roots completely inhibited *F. oxysporum.*

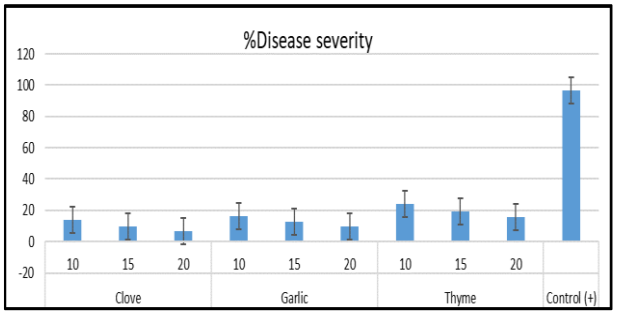


Fig 7. Effect of different concentrations of plant oils on disease severity

Under greenhouse conditions, separate application of the three isolates of *Trichoderma*, *T. harzianum*, *T.viride* and *T.hamatum*,as selected from *in vitro* tests, to the soil infested with FOL , reported a reduction of disease severity percentage. Results present in Fig (8) indicated that all isolates of *Trichoderma* had effective in suppressing wilt disease of tomato compared to control . The application of *T. harzianum* to the infested soil had the lowest disease severity percentage 5.8% followed by *T.viride* which had moderate percentage of disease severity 9.2% compared with control (soil infested with pathogen) which recorded 97.8%. Such results were also observed by (Tsegaye *et al.*, 2018 and Kareem *et al.*, 2016).

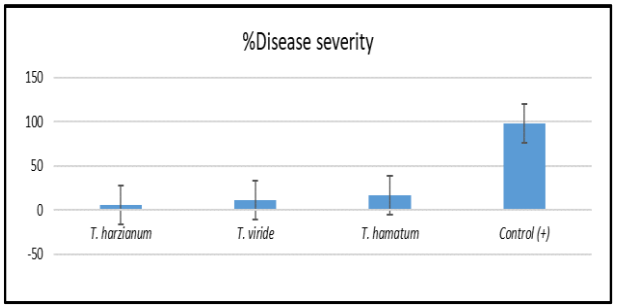


Fig 8. Effect of *Trichoderma* spp. isolates on disease severity

Results shown in Fig (9) clear that an artificial inoculation significantly increased disease severity compared to the control. The application of urea at the concentration of 0.5% to the infested soil with FOL decreased the disease severity significantly, in comparison with control. It considered the best treatment from all urea concentrations treatments. It recorded 78.2 % on reduction the severity of wilt and root rot diseases on tomato plants under greenhouse conditions at 60 days after transplanting in soil infested with FOL. This result attributed to the application of urea fertilization at stander rate increase the plant defence and disease suppression by the regulation of intracellular concentrations of cell sugars and amino acids, which provide nutrients for invading fungi (Mur *et al.*, 2016). On the other hand, application of urea at the concentrations of 1 and 1.5 % to the infested soil with the pathogen significantly increased disease severity, as it recorded 89.7 and 94.7% respectively of disease severity in comparison with control + (pathogen) which recorded 96.3%. These results are in agreement with (Lemmens *et al.*, 2004), who decided that high nitrogen rates (up to 80 kg/ha) significantly affected Fusarium head blight development in wheat.

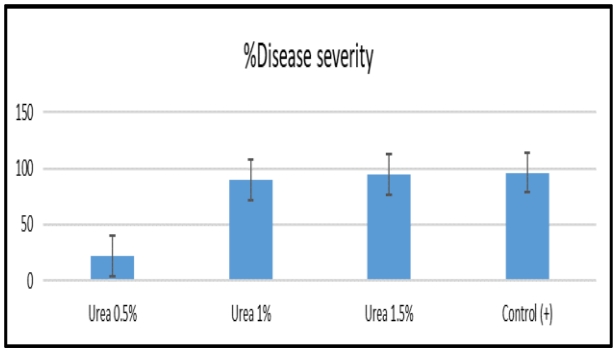


Fig 9. Effect of different concentrations of urea on disease severity

Effect of different concentrations of some plant oils, *Trichoderma* spp. and urea concentrations on plant growth parameters

Plant growth parameters were recorded at 90 days from the date of seedling transplanting. The average of root length, root fresh weight, root dry weight, Plant height and No. of branches per plant were recorded. Results given in Figs (10, a and b) reported that all Plant growth parameters were significantly increased in response to the application of different oils to the infested soil.

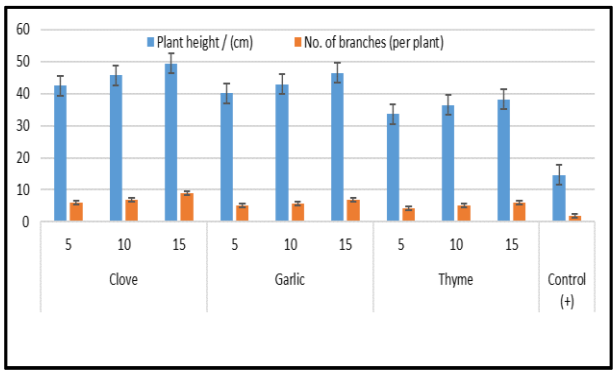


Fig. 10, a. Effect of different concentrations of some plant oils on Plant height and No. of branches per plant.

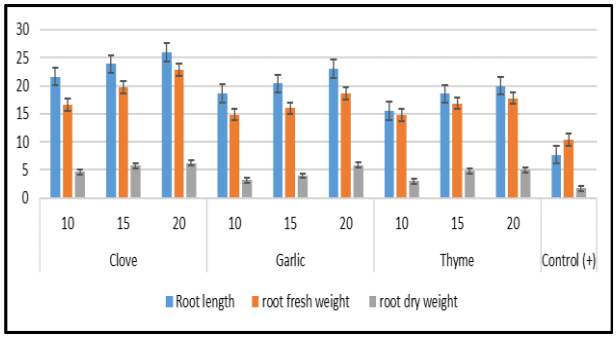


Fig. 10, b. Effect of different concentrations of some plant oils on root length, root fresh weight and root dry weight.

The best results were observed when clove oil was applied by concentration 0.5 % followed by garlic oil which improved all Plant growth parameters. On the other hand, under in *vivo* conditions, *Fusarium oxysporum* f.sp. *lycopersici* treatment (pathogen) adversely affected the Plant growth parameters leading to decrease fresh and dry weights of root (Lamour *et al*., 2012). Such results were recommended by the abovementioned authors.

In this study, the average plant height, No. of branches per plant were also increased in response to the application of *T.harzianum and T. viride* to the infested soil. It recorded 54 and 41 cm of the average plant height, 8cm and 7 branches / plant in comparison with 15cm and 3 branches / plant in control plants (+) Fig (11 ,a). And the same trend were obtained with root length, root fresh weight and root dry weight Fig (11, b). Similar results were obtained by (Dubey *et al*., 2007; Khatabi *et al*., 2012) who reported that *Trichoderma* spp. attack the pathogen and improve the plant growth and yield by enhancing the growth hormones .Data in Fig (12 ,a) and Fig (12 ,b) clear that all Plant growth parameters were increased significantly with the increase of all urea application regardless the inoculation with FOl. the highest growth parameters were obtained in plants supplied with the highest concentration of urea 1.5 % regardless the pathogen inoculation. (Hemissi *et al.*, 2018*)*

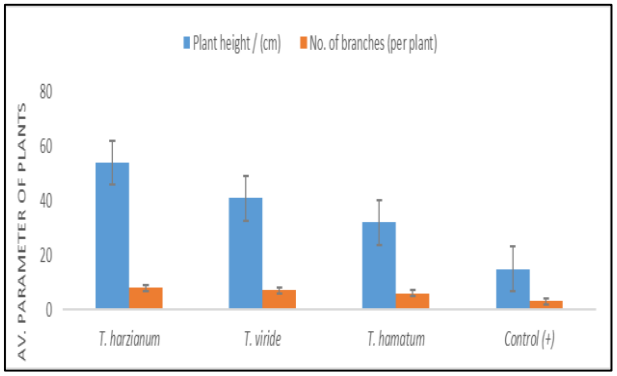


Fig 11, a. Effect of different *Trichoderma* spp. isolates on plant height and number of branches per plant.

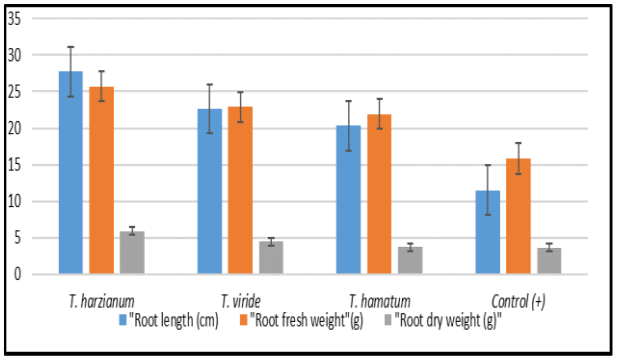


Fig 11, b. Effect of different *Trichoderma* spp. isolates on root length, root fresh weight and root dry weight.

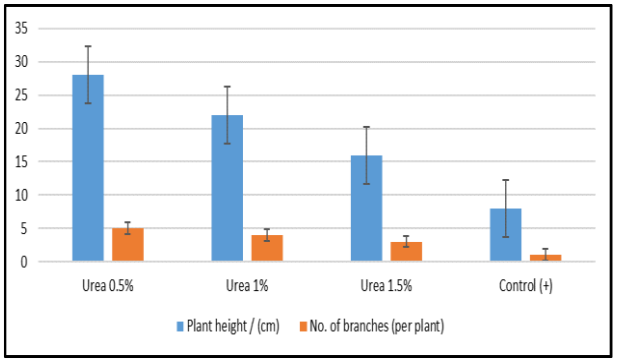


Fig. 12, a. Effect of different concentrations of urea on plant height and number of branches

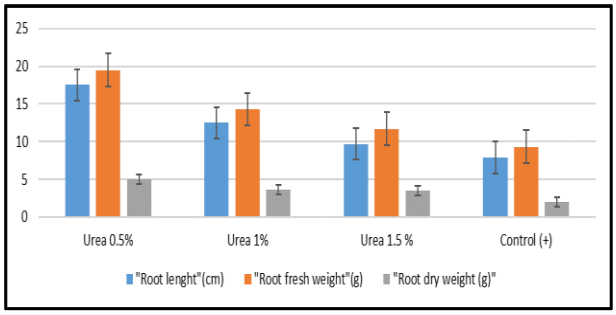


Fig. 12, b. Effect of different concentrations of urea on root length, root fresh weight and root dry weight.

Chemical analysis

Effect of different concentrations of some plant oils, *Trichoderma* spp. and urea concentrations on Chlorophyll A and B in tomato plants leaves:

There were differences obtained between all inoculation treatments at 60 days after transferring seedlings, Results shown in Fig. (13, a) indicate that a lower amount of chlorophyll A and B were recorded with a pathogen (control +) treatment only compared with the other treatments. Where in *Fusarium oxysporum* f.sp. *lycopersici* treatment the Chl. A and Chl. B contents were recorded 1.8 and 1.2 mg/ge respectively, which consider the lowest amount of chlorophyll content in leaves tomato plant treatments . On the other hand, the Chlorophyll A and B contents increased with an increase in the concentration of plant oils compared to healthy plants (control -). As clove oil gave the best results where obtained at the highest concentration (20 %), 5.7 and 4.4 mg/ge respectively, 5 and 3.8 mg/ge respectively at the concentration (15 %), followed by 4.2 and 3 mg/ge respectively at the highest concentrations (10 %). Results shown in Fig. (13, b) illustrate that all tested *Trichoderma* spp. isolates had significant effects in increasing chlorophyll A and B contents in comparison with control (-) and (+) treatments. *Trichoderma harzianum* and *T. viride* were the best tested biocontrol agents increasing chlorophyll A and B contents which recorded 4.4 and 3.6 mg/ge respectively and 3.1 and 2.6 mg/ge respectively. On the other results shown in fig. (13, c) clear that Chlorophyll A and B contents decreased with an increase in the concentration of urea fertilization compared to control (healthy plants).

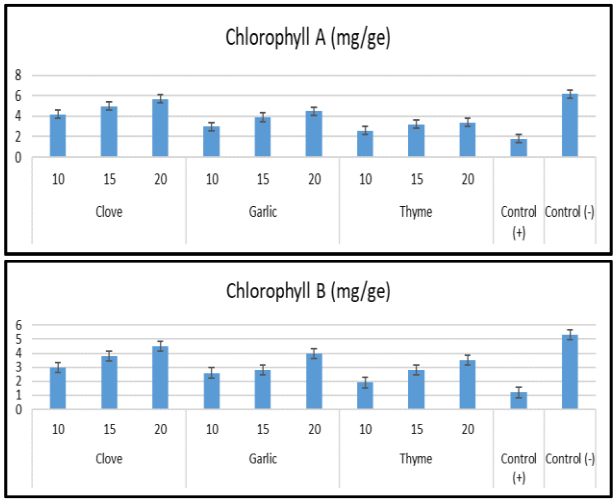


Fig 13a. Effect of different concentrations of some plant oils on Chlorophyll A and B contents in tomato plants leaves.

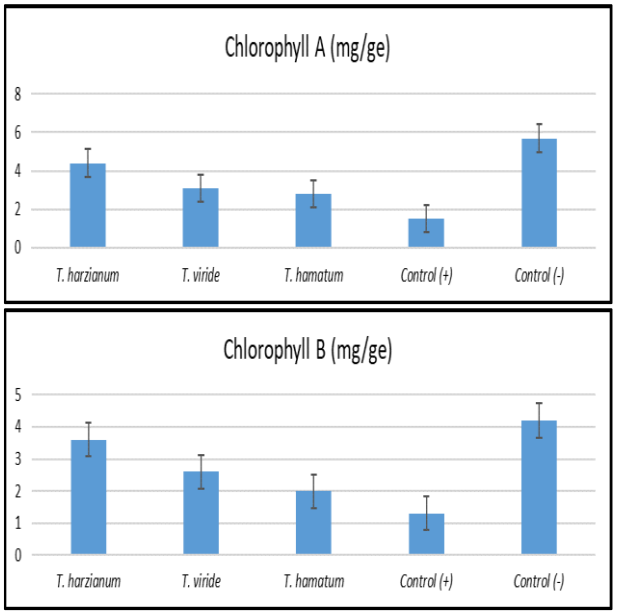


Fig. 13b.Effect of different *Trichoderma* spp. isolates on Chlorophyll A and B contents in tomato plants leaves.

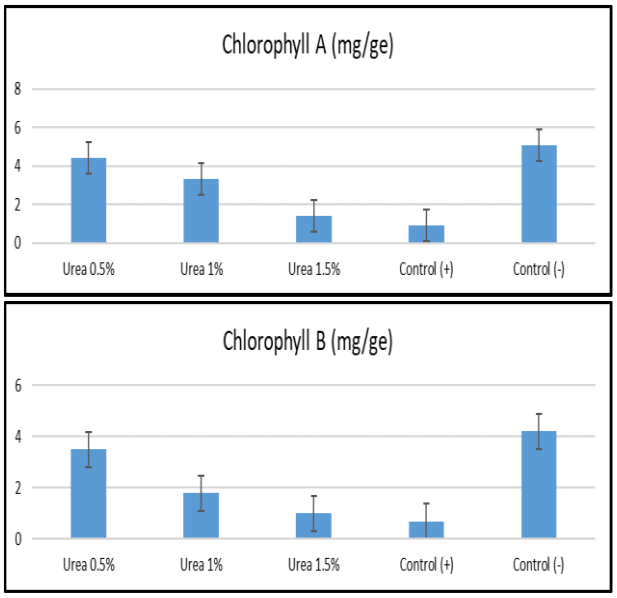


Fig. 13c. Effect of different urea concentrations on Chlorophyll A and B contents in tomato plants leaves

At concentration 0.5 of urea fertilization there was the best result which recorded 4.4 and 3.5 mg/ge respectively. While Chlorophyll A and B contents recorded 3.3 and 1.8 3.5 mg/ge respectively in urea treatment at 1% followed by 1.4 and 1 mg/ge respectively in urea treatment at 1.5%.

Effect of different concentrations of some plant oils, *Trichoderma* spp. and urea concentrations on total phenol contents:

Total phenol contents are consider from the important components in plant. These are essential for plant growth and reproduction. All phenolic derivatives are natural antioxidants that exist in all parts of the plant and it have a great role of antibiotics and natural pesticides (V. K. Gupta and S. K. Sharma. 2014). Figure (14) indicate the total phenol contents of roots tomato plant in all treatments.

The pathogen (control +) treatment exhibited the highest phenolic contents with value of 22.7 mg GAE/ge compared with healthy plants (control -). Values of total phenol contents of roots tomato in other treatments ranged by 12.3 7 mg GAE/ge to 19.5 mg GAE/ge. All these values were lower to those reported by pathogen. The difference between previous results could be explained by defense responses in root tissues and these result was well defined by Zvirin *et al.*, (2010) and the role of phenolic compounds in defence mechanisms in *F. oxysporum*-host is well known (Panina *et al.*, 2007).

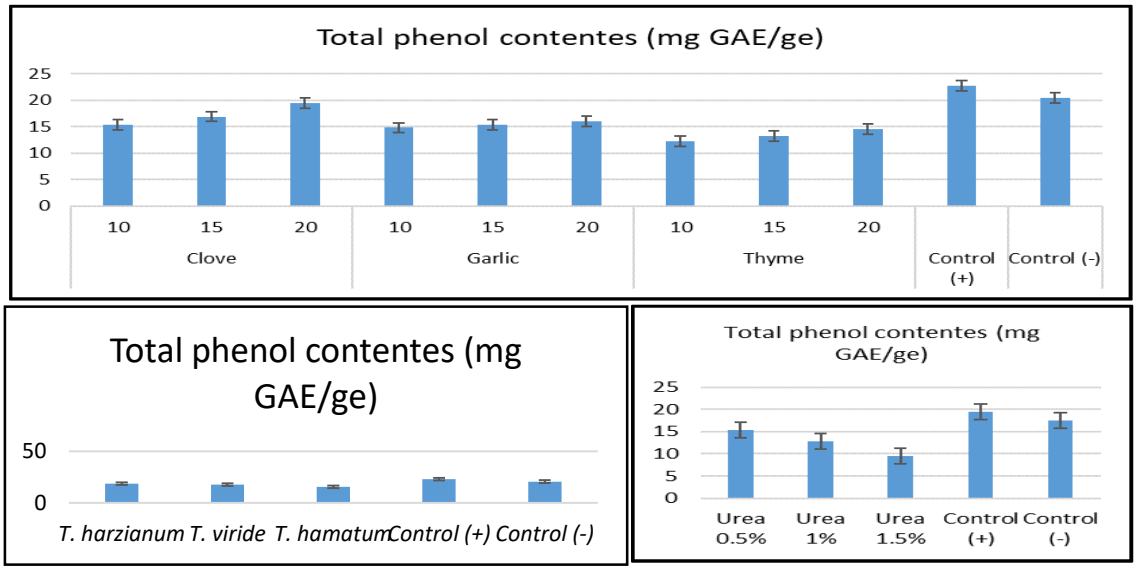


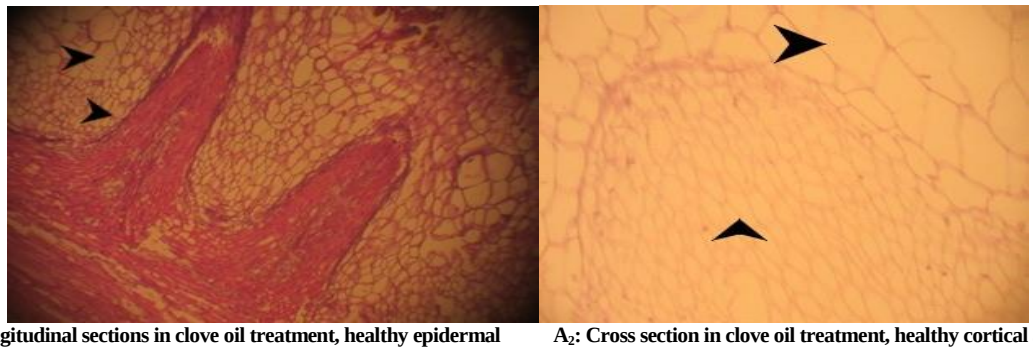
Fig. 14. Effect of different concentrations of some plant oils, *Trichoderma* spp. and urea concentrations on total phenol contents.

Histopathological features:

Effect of oil treatments on root cells anatomy:

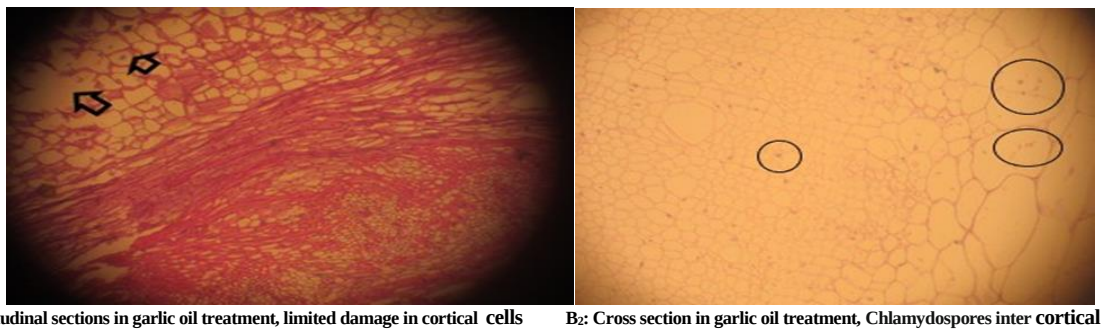
Results shown in figure (15) (A_1 and A_2) clear that clove oil treatment was the best one for controlling (FOL) fungus where there were no mycelial growth and / or fructification units within the root tissues and all tissues of secondary roots seemed completely healthy . In addition to all tissues of epidermal layer, the parenchyma of the cortex and vessels of the vascular cylinder were in intact that both in longitudinal and cross sections. Garlic oil came in the second rank where it could be noticed the tissue was ruptured and had little mycelial growth and chlamydospores within it, Fig15 (B_1 and B_2).

However, thyme oil Fig15 (C_1 and C_2) was the least effective one in suppression FOL growth within tomato root tissues, where the fungal mycelium was spread through various tissues and caused great ruptures within them.



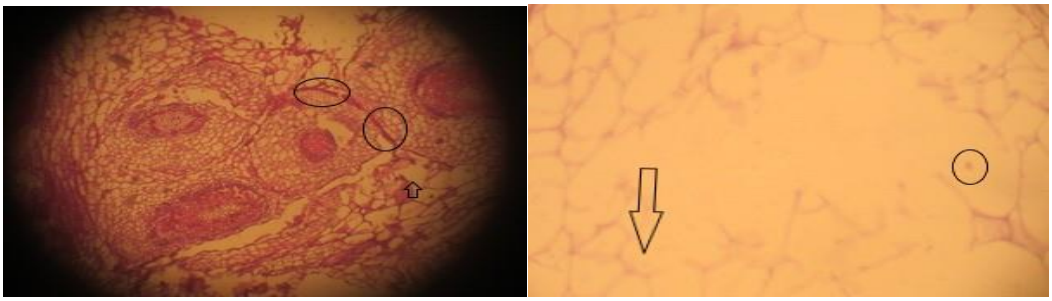
A_1: Longitudinal sections in clove oil treatment, healthy epidermal and secondary root tissues (arrowheads).

A_2: Cross section in clove oil treatment, healthy cortical and vascular tissues (arrowheads)



B_1:Longitudinal sections in garlic oil treatment, limited damage in cortical cells (big arrow) and Chlamydospore into the cell (small arrow)

B_2: Cross section in garlic oil treatment, Chlamydospores inter cortical and vascular tissues (circle).



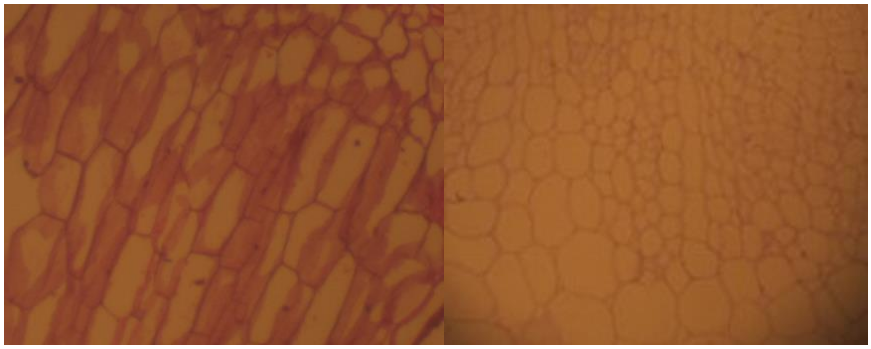
C_1: Longitudinal sections in thyme oil treatment, stained sections showing spread of pathogenic hyphae within root tissues (circles).

C_2: Cross section in thyme oil treatment, Damage cortical cell (arrow) and Chlamydospores in destroyed area (circle)

Fig 15- A_1 ,A_2, B_1, B_2, C_1, C_2. Effect of different plant oils on old tomato roots infected with (FOL): Longitudinal sections, left and cross sections right.

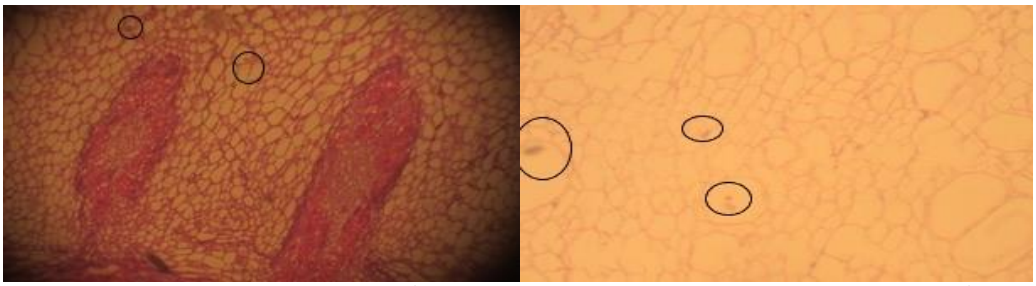
Effect of *Trichoderma* spp. isolates on root cells anatomy:

Histopathological observation of infected plants treated with *Trichoderma harzianum* , were typical of classic descriptions for non-infected plants in all tissues (epidermis , cortex and vascular bundles) .As there weren't plant cell deterioration ,fungal hyphae and chlamydospores. Figure 16 (D_1 and D_2) of *Trichoderma harzianum* showed superior effect on controlling FOL fungus where both longitudinal and cross sections were free of the mycelial growth and / or other the pathogen structures. *Trichoderma viride* was also good effective in controlling FOL fungus where a few scattered fungal chlamydospores were evident intercellular sites in root cells figure 16 (E_1 and E_2). *Trichoderma hamatum* showed less histological efficiency, where cortex tissue was ruptured and full with the pathogen mycelium and chlamydospores, figure 16 (F_1 and F_2).



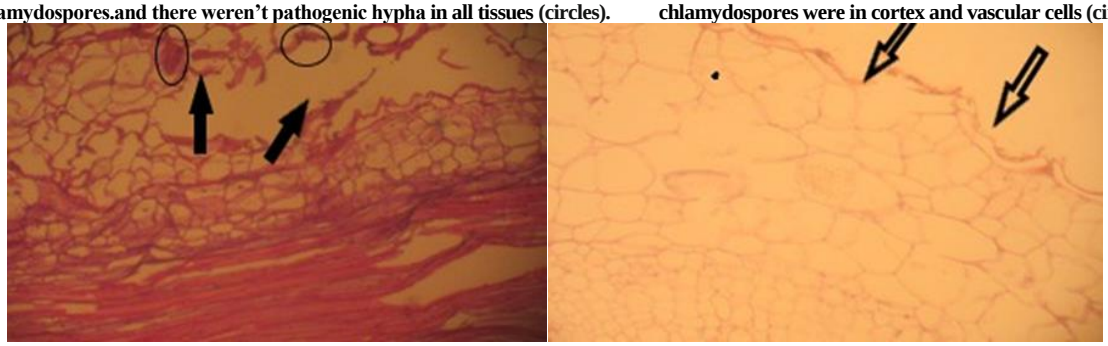
D_1: Longitudinal sections in *T. harzianum* treatment, all section appear healthy shape.

D_2: Cross section in *T. harzianum* treatment, Cortical and vascular tissues were completely empty from pathogenic hyphal or chlamydospores.



E_1: Longitudinal sections in *T. viride treatment,* little no. of chlamydospores.and there weren't pathogenic hypha in all tissues (circles).

E_2: Cross section in *T. viride treatment,* a few scattered fungal chlamydospores were in cortex and vascular cells (circles)



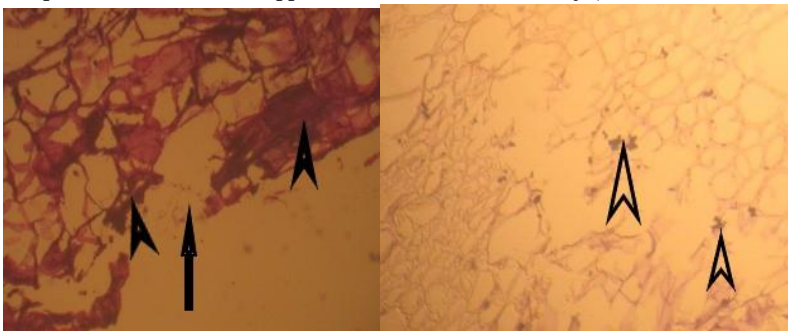
F_1: Longitudinal sections in *T. hamatum treatment,* more rupture in Epidermal and cortical tissues (arrows), spread of pathogenic hypha and chlamydospores (circles).

F_2: Cross section in *T. hamatum* treatment, more rupture in epidermal tissues (arrows).

Figure (16- D_1 ,D_2, E_1, E_2, F_1, F_2. Effect of *Trichoderma* spp. isolates on old tomato roots infected with (FOL): Longitudinal sections, left and cross sections right.

Effect of urea fertilization on root cells anatomy:

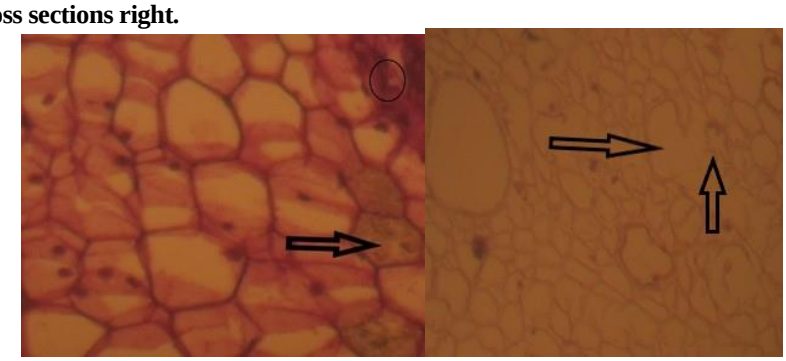
Application of urea at concentration 1.5 % to tomato plants grown in the infested soil with FOL caused great histological damage to the plant tissues comparison with all controlling treatments Fig.17 (G1 and G2). Mycelial growth are shown intracellularly Fig. 17 (G1) and the tissues were ruptured Fig.17 (G1 and G2).However, this could be due to the less thickness of cell walls in response to urea fertilizer application. On the other hand, the untreated plants (pathogen only, Fig.18, H1 and H2) were full with the mycelial growth, pathogenic, spores were present in the damage area of both cortical and vascular regions of the root, there were chlamydospores of FOL pathogen intercellular, there was condensed cytoplasm as it gain more stain and more phenolic compounds were accumulation inter root cells. These results are in harmony with those obtained by (Muhammad *et al.*, 2018*).*



G_1 : Longitudinal sections in urea 1.5 % treatment, great damage In all tissues (arrow) and more mycelial growth Inter and intercellular (arrow heads)

G_2: Cross section in urea 1.5 % treatment, stained areas showing more spread of remains pathogenic hyphae within root cells and tissues. (arrow heads).

Figure 17- G_1, G_2. Effect of urea fertilization on old tomato roots infected with (FOL): Longitudinal sections, left and cross sections right.



H_1: Longitudinal sections in pathogenic treatment, more phenolic compounds inter cortical cells (arrow), more mycelial growth intercellular (circles).

H_2: Cross section in urea 1.5 % treatment, damage of vascular tissues appear in the section (arrow).

Figure 18- H_1, H_2. Effect of pathogen only on old tomato roots: Longitudinal sections, left and cross sections right.

Histopathological studies of Fusarium wilt indicated that control treatments positively affect the anatomical structure of the plant as well as both the morphological and physiological structure. From our histopathological observation, The hyphae penetrated directly into the epidermal cells and cortex (Jiménez-Fernández *et al*.,2013), both of them were colonized by intra- and intercellular hyphal growth, then the pathogen invade vascular core (Zvirin *et al*., 2010), some changes into cells were observed such as accumulation of hyphae in the host epidermal cells and the neighbouring cortical cells ,formation of phenolic compounds which constitutes a defence response of infected plants and formation of chlamydospores (Muhammad *et al.*, 2018*).* Histopathological studies have suggested that application of clove oil, garlic oil, *Trichoderma harzianum, Trichoderma viride* and urea fertilization at 0.5% concentration can control tomato wilt disease caused by *Fusarium oxysporum* f.sp. *lycopersici* without harmful effects on plant cells.

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دراسات بيولوجيه وتشريحية ومرضية علي مرض ذبول الطماطم المتسبب عن الفطر *Fusarium oxysporum* f. sp *lycopersici*

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يعتبر مرض الذبول الفيوزاريومي في الطماطم والمتسبب عن الفطر *Fusarium oxysporum* f.sp.*lycppersici* من الأمراض الخطيره في مصرحيث يسبب خسائر كبيرة في المحصول . وقد أجريت هذة الدراسة لتقدير فعاليات بعض الزيوت (القرنفل و الثوم والزعتر) بتركيزاتها المختلفة (١٠ و ١٥و ٢٠%) وكذلك عزلات من أنواع الفطر Trichoderma (*Trichoderma harzianum* و *Trichoderma hamatum* و *Trichoderma viride*) ، بالإضافة إلي التركيزات المختلفة من سماد اليوريا (٠,٥ و ١ و ١,٥ %) ضد الفطر الممرض (FOL) . وأظهرت النتائج أن جميع الزيوت المختبرة أدت الي النقص المعنوي في نمو الفطر وإنتاج الجراثيم . وأدي استخدام زيت القرنفل إلي الحصول علي أفضل النتائج ويليه زيت الثوم وذلك تحت ظروف المعمل أو الصوبة . وعلي العكس من ذلك ، ثبت أن إضافة اليوريا بالتركيزات العالية (١ و١,٥ %) أدت الي زيادة نمو الفطر وكذلك تجرثمه مقارنة بالغير معاملة . وتحت ظروف المعمل كان الفطر *Trichoderma harzianum* هو الأفضل لتثبيط نمو الفطر الممرض (٩٤,٢%) يليه الفطر *Trichoderma viride* (٩٠,٨%) وتم الحصول علي نتائج مماثلة تحت ظروف الصوبة . ومن ناحية أخري أظهرت المعاملة بالزيوت ، عزلات Trichoderma والتسميد باليوريا تحسنا ملحوظا في النمو الخضري للنباتات . وأثبتت الدراسات التشريحية أن معاملتي *Trichoderma harzianum* وزيت القرنفل هما الأفضل في مكافحة الفطر الممرض حيث ظهرت خلايا البشرة والقشرة والإسطوانة الوعائية خالية من نمو الفطر الممرض.