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Effect of some Chemical Inducers on Antagonistic Potential of *Trichoderma harzianum* against *Rhizoctonia solani* and it's Metabolites Production

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ABSTRACT

The present study was conducted on different supplemented gliotoxin growth medium by using some chemical inducers (micronutrients mixture, potassium tartrate and thiamine) to improve antagonism, crude extracts and volatile compounds activities of *T. harzianum* against *R. solani*. *T. harzianum* grown on medium supplemented with potassium tartrate decreased the mycelial growth of *R. solani* by 89% compared with *T. harzianum* alone (63.3%). Also, exhibited the significantly higher activity of the crude extract and the volatile compounds. The micronutrients followed by the thiamine addition recorded the highest activity of the secondary metabolite. Treated Trichoderma enhanced bioactivity against the pathogen when transferred on PDA medium without addition of the tested materials. Light microscope observation revealed that malformed and damaged pathogen hyphae occurred more clearly after contact with the treated *T. harzianum*. The GC-MS analysis showed detection of antifungal compounds as 2,3-butanediol, Decane, 2,4,6-trimethyl-, phenylethyl alcohol, acetic acid, 1 H-Benzocycloheptene-7-ol,2,3,4,4a,5,6,7,8-octahydro-1,1,4a,7-tetramethyl-,cis- in the crude extract of Trichoderma grown on medium supplemented with different tested compounds. Soaking faba bean seeds Giza 429 in treated Trichoderma suspension before sowing in infested soil increased survival faba bean seedlings compared with the untreated Trichoderma. As a result, the tested chemicals improved the antagonistic activity of *T. harzianum* and can be used as biological fungicides.

Keywords: *Trichoderma harzianum*, specific activity, potassium tartrate, thiamine, micronutrients, *Rhizoctonia solani*

INTRODUCTION

R. solani causes severe damages to economic crops and once established in the field the fungus often remains forever. The control strategies such as the application of fungicides are insufficient to manage diseases caused by *R. solani* because it persists in soil by producing sclerotia which are a hard-resistant structure. Also, it's a large broad host range and unavailability of resistant plant varieties (Abbas *et al*., 2017). The consumers growing health attention and environmental pollution associated with fungicide use, the emergence of fungicide resistant strains of pathogen have motivated the search for alternative methods. Therefore, the strategies to control *R. solani* are limited. Therefore biological control is effective management for long term sustainability for such fungus (Priya and Upadhyay 2017). Its environmentally friendly alternative way to prevent the pathogen. Also, the biological control progress the soil health and increase the productivity of plant by inhibition of pathogen (Abbas *et al*., 2017). Improve the antagonism efficacy of Trichoderma is essential for the biocontrol of diseases and to develop alternative methods which are, by definition, safe in the environment. Micronutrients effect on different cellular structures and influence metabolic processes by blocking of enzymes (Badura and Piotrowska, 2000). Application of microelements has been a common practice that can affect biological control activities (Yousef *et al*., 2016). Manganese increased the production of chitinase in some *Trichoderma* spp. and can be used in the biological control application. Attempts have been made to enhance the bioefficacy of *T. viride* with sodium salts for minimizing rhizome rot of turmeric (Jagtap et *al*., 2011).

Vitamins increase the production of different metabolites and the activities of enzymes (Chen *et al*., 2004). Mycoparasitism is being considered as a mechanism of the biocontrol leads to the destruction of some of the structures of the pathogen (e.g. mycelium, spores and sclerotia). Occurs in several successive stages. Starts with the chemotrophic growth of Trichoderma to the host. After this, Trichoderma secret hydrolytic enzyme primarily chitinases, glucanases and proteases, which degrade the cell wall of the host mycoparasitism as major mechanisms of action of a fungal biocontrol agent is concerned (Bhat, 2017).The secondary metabolites of Trichoderma perform an important role in the mycoparasitism as reported by (Vinale *et al*., 2008). Seed treatment with a fungicide is insufficient method, especially if the pathogen is internal borne of the seed, therefore, alternatives must be examine (Fialho *et al*., 2011).

Therefore, this research was subjected to study the effect of some chemical inducers (micronutrients mixture, thiamine and potassium tartrate) for improving the biological control efficacy of *T. harzianum* against *R. solani* growth, sclerotia production and hypeparasitism on microscopic slides. In addition to its impact on the production of metabolites in the crude extract and identified using GC-MS.

MATERIALS AND METHODS

Microorganisms

R. solani was isolated from infected cucumber seedlings, showing root rot symptoms in previous studies by (Yousef *et al*., 2013) and isolate of *T. harzianum* (T1) was isolated from the rhizosphere soil of healthy bean plants (Yousef *et al*., 2016).

Tested materials

Potassium tartrate (0.3%), mixture of micronutrients (0.02% Zn sulfate, 0.04% Mn sulfate, 0.01% boric acid and 0.06% sodium selenite) and thiamine (0.1%) was added to GM medium separately. All tested chemical materials were obtained from Al-Gomhouria Co, Egypt.

Seeds of faba bean cv. Giza 429 were obtained from Legume Research Department, Field Crops Research Institute, Agricultural Research Center, Giza, Egypt.

Preparation of the culture medium

Gliotoxin medium (GM) composed of (g/l) 2 ammonium tartrate; 2, di potassium hydrogen orthophosphate; 25, glucose and 0.001 ferrous sulfate.

Effect of some micronutrients, K tartrate, and thiamine on *R. solani* growth

The micronutrients (Mn SO_4, Zn SO_4, boric acid, sodium seleate and their mixture), potassium tartrate and thiamine were used to study their effect on the mycelial growth of *R. solani*. Five mm mycelial discs were landed onto the center of GM medium supplemented with different chemicals. Three Petri-dishes free from any addition performed as the control. Three replicates were taken for each treatment.

Bioassay study against *R. solani*

Two bioassay experiments were conducted:

- Antagonistic activity of Trichoderma on GM medium supplemented with chemical inducer individually.
- Antagonistic activity of modified Trichoderma discs on PDA free from any addition.

Antagonistic activity on GM medium supplemented with chemical inducer

The antagonistic activity was studied by using the dual culture technique (Naeimi *et al*., 2010). In which, 5 mm diameter of discs of the pathogen and the Trichoderma were placed opposite each other in Petri dishes containing GM medium supplemented and non- supplemented with the tested materials as the control. Three replicate dishes per concentration were designed for each treatment.

Antagonistic activity of modified Trichoderma discs on PDA free from any addition.

In another experiment antagonism test carried out by cultivating modified mycelial discs 5 mm in diameter of actively Trichoderma growing colonies from GM modified plates and transferred to non- supplemented PDA medium plates with *R. solani*. The control treatment plates inoculated with Trichoderma non-modified mycelial discs with *R. solani* isolate. Three replicate dishes per treatment. The dishes were incubated in the dark at 25°C until the full growth of *R. solani* control and the mean colony diameter of the pathogen was measured. The percent of inhibition of the growth was calculated.

Induction of highly active of crude extract of *T. harzianum* against *R. solani* using chemical inducers

The methodology described by Dennis & Webster (1971a) was used to evaluate the crude extract activity of *T. harzianum* against *R. solani*. Crude extract was obtained from GM broth medium amended individually with the three tested compounds (potassium tartrate, micronutrients and thiamine). 50 ml of the medium was inoculated with one disc of the active mycelium of *T. harzianum* of 250-ml flasks. Another experiment applied to PDA without addition tested materials. The medium was inoculated with one disc of *T. harzianum* previously grown on GM medium supplemented with potassium tartrate, micronutrients and thiamine. After incubation at 25°C for 14 days in the dark, the supernatant was filtered, centrifuged at 10000 rpm for 20 min and sterilized by filtration using millipore membrane (0.25 µm). 20 ml of the filtrate were mixed with 80 ml of PDA medium before solidification. The mixture was poured in Petri dishes and each was inoculated by 5 mm disc of the active mycelium of *R. solani* at the center of each dish. Then incubated at 25°C until *R. solani* control overgrown the plate and mycelial growth was measured. Three replicates were used for each treatment.

Induction of highly active of volatile compounds produced by *T. harzianum* against *R. solani* using chemical inducers

Volatile compounds produced by *T. harzianum* were evaluated on GM medium plates containing the previously inducer in concern. *T. harzianum* was used to inoculate plates of the three days later; the lid of each plate was replaced with the bottom of other plate then inoculated with five mm mycelial disc of the pathogen. Both bottoms were closed with adhesive tape (Dennis and Webster, 1971b). The control treatment did not contain *T. harzianum*. Cultures were further incubated at 25°C until the colony of the pathogen was spread on the whole Petri plate in the control treatment and the diameter of the *R. solani* colony in each plate was measured. The inhibition percent was calculated. Three replicates were used for each treatment.

Induction of highly active of the secondary metabolites of *T. harzianum* against *R. solani* using chemical inducers

Secondary metabolites of *T. harzianum* was produced on GM plates with the addition tested materials, containing sterilized cellophane paper, placed on the surface of the medium, which was inoculated with 5 mm mycelial discs of *T. harzianum* and incubated for 2 days at 25°C, then cellophane paper containing the mycelial growth of *T. harzianum* was throw it away and a five mm disc of pathogen was placed on the same medium containing the secondary metabolites (Dennis and Webster, 1971a). The control treatment contained only pathogen disc grown without cellophane paper. The cultures were further incubated at 25°C until the colony of the pathogen was spread on the whole Petri plate in the control treatment and the diameter of the *R. solani* colony in each plate was measured. Another experiment applied on PDA without addition tested materials.

Effect of *T. harzianum*, chemical inducers and their combination on the sclerotial formation and the germination percentage.

PDA agar medium amended with potassium tartrate, micronutrients separately and their combination and thiamine (three replicate dishes per treatment). Three Petri- dishes free of chemicals performed one check. After solidification of the medium, each dish was inoculated in the centre with a mycelial disc (5-mm diameter) of *R. solani*. The plates in previous experiments were maintained for one more week and the sclerotia formation was recorded visually. Sclerotia of *R. solani* were transferred to PDA Petri plate amended with different chemical inducers. The germination rate of sclerotial was recorded after 24 h incubation at 25˚C.

Detection of mycoparasitism using slide culture technique

Thin layer of molten water agar was poured to form a thin agar film over the sterile slide. One end of the slide was kept free of the medium to facilitate handling. One end of the slide was kept free of the medium to facilitate handling (Naeimi *et al*., 2010).

Mycelial discs of Trichoderma modified with the tested chemical inducers and non-modified as the control were placed with the *R. solani* on the sterile glass slide 1 cm apart from each other. Inoculated slides were placed in sterilized Petri dishes contain two filter papers (Whatman No.1) saturated with 10 ml of sterilized distilled water just to maintain humidity around the inoculated slides. Plates

with slides were incubated at 25 ºC for 4 days. The hyphae of Trichoderma interaction with the hyphae of *R. solani* were inspected under a light microscope for wall disintegration in the hyphae of the pathogen and photographed.

Extraction of the bioactive compounds from *T. harzianum* cultures

Five mm mycelial discs from 7 days old cultures of *T. harzianum* was inoculated into 500 ml flasks containing 250 ml of 1/2 strength GM supplemented with different tested materials and then incubated in the dark at room temperature. Forteen days after inoculation, spores and mycelia of modified Trichoderma cultures were removed from broth culture by filtration. The extraction of the bioactive compounds of *T. harzianum* was done using the procedure according to Vinale *et al*., (2006) and Siddiquee *et al*., (2012) with slight modification, in which, 100 mL of ethyl acetate were added to the grown culture in the Erlen meyer flask, and the mixture was incubated at room temperature overnight under shaking (50 rpm). The mixtures were then transferred to separating funnel to separate the ethyl acetate phase from the aqueous phase. The extracted mycelia (cell debris) were thrown away, and the filtrate containing the ethyl acetate phase was collected for further processing. The ethyl acetate phase was washed with distilled water twice and filtrated on anhydrous sodium sulphate to remove the moisture. The ethyl acetate extracts were evaporated at 40 ºC on a rotary evaporator until dryness; then the dry film was dissolved in dichloromethane (chromatographic grade) and filtered through 0.22 µm PTFE filter. This sample was stored in the deep freezer at -20 ºC until use for GC–MS analysis

Detection of the bioactive compounds using GC-MS

The analysis of GC-MS was carried by a Thermo Scientific, Trace GC Ultra / ISQ Single Quadru column MS, TG-5MS capillary column of milted silica (30m, 0.251mm, 0.1 mm film thickness). An electron ionization system with ionization energy of 70 eV was used, gas of Helium was used as the carrier gas at a constant flow rate of 1ml min^{-1}. The injector and MS transfer line temperature was set at 280 ºC. The temperature of the oven was programmed at 50°C as an incipient temperature (hold 2 min) to150°C at an increasing rate of 7°C min^{-1}, posteriorly to 27°C at an increasing rate 5°C min^{-1} (hold 2min) subsequently to 310 as a temperature final at an increasing rate of 3.5°C min^{-1} (hold 10 min). The identified components quantification was carried by using a percent relative peak area. Identification tentative of the compounds was carried by based on the comparison of their time retention relative and spectra of mass with those of the NIST, WILLY the GC-MS library data according to Meena *et al*., (2017) with slight modification.

Effect of seed treatment with *T. harzianum* (grown on GM broth medium supplemented with different chemical inducers) on damping-off disease caused by *R. solani*

T. harzianum grown on different GM broth medium supplemented with different chemical inducers in dark conditions (25°C) for 1 week. Spores suspensions were prepared at the rate 1:20. Faba bean seeds of variety Giza 429 were surface sterilized (sodium hypochlorite 3% for 3 min after that water for 10 min). Then, they were soaked in treated and untreated Trichoderma suspension treatment 1 hour. Treated and untreated seeds were sown in pots infested with *R. solani* in soil at the rate of 1% of planting soil while, the control was seed soaking in water. The pots were sown with 4 seeds per pot and four pots were used for each treatment. Data for seedling damping off and survival (%) of plant were recorded after 10, 15 and 30 days, respectively.

Statistical analysis:

Data were subjected to statistical analysis by software packages CoStat (version 6.4, CoHort Software, U.S.A). The one-way completely randomized design was applied. Duncan's multiple range test was used to compare the means at probability (P) value of ≤ 0.05. Experiments were performed with three replicates. Data were subjected to statistical analysis by software packages

RESULTS AND DISCUSSION

Effect of the some micronutrients, K tartrate, and thiamine on the growth of *R. solani*.

Fig (1) showed that the different chemicals had no effect on the *R. solani* growth. This effect may due to these chemicals present the inducer resistance in plants and bioagent activities

Bioassay study against *R. solani*

Table (1) showed that the three chemical inducers supported the production of highly active antagonism, crude extract, secondary metabolites and volatile compounds of *T. harzianum* against *R. solani*. Potassium tartrate was the best in improving the antagonism, 89% inhibition compared with the untreated Trichoderma 63.3 %. Whereas enhance crude extract activity to decrease *R. solani* growth to 2.8 cm and 5.3 cm with volatile compound instead of 9 cm in the control.

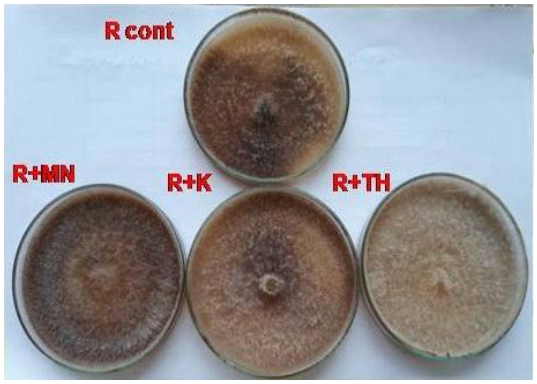

Fig. 1. Effect of different chemical inducers on the growth of *R. solani* grown on GM medium. *R. solani* colony grown GM medium supplemented with micronutrients mixture (MN), potassium tartrate (K) and thiamine (TH).

The secondary metabolites were greatly enhanced by micronutrients being 1.2 cm of *R. solani* linear growth compared with the untreated Trichoderma 9 cm.

In support of our results, Morid and Zafari (2013) reported that the highest specific activity of the chitinase enzymes were found in isolates *T. brevicompactum* and *T. koningiopsis* grown in medium containing manganese micronutrient Fig. (2, 3 and 4).

Table (2) showed the positive effect of chemical inducer on improving Trichoderma activity when transferred to PDA medium free from any addition. Trichoderma treated with potassium tartrate increased *R. solani* inhibition

from 63.3% to 78.9%. In addition to improve crude extract activity to reduce *R. solani* growth to 3 cm compared with untreated Trichoderma 8 cm. Trichoderma treated with thiamine was found to increase the biological activity of volatile compounds and secondary metabolites against *R. solani* linear growth (5.2 & 1.5 cm) respectively compared with 8 cm in untreated Trichoderma. In discussion of these results there are several lines of evidence.

Slight growth of the pathogen was observed under stress of crude extract, secondary metabolites and volatile compounds as a response of *Trichoderma* species and/or their extracellular metabolites and can be exploited as a biocontrol agents or biological fungicides (Vinale *et al*., 2006). The addition of antioxidants can lead to stimulate secondary metabolite gene expression by *Aspergillus flavus* RCP08108 Nesci *et al*., (2003). The mechanisms of action include secretion of antibiotics, competition for nutrients and secretion of enzymes (Harman *et al*., 2004) and this can improve the biocontrol potential of *T. viride* against *M. phaseolina* in micronutrients amended medium (Sundravadana and Alice, 2006), they added that application of microelements fertilizer has been a common practice that may have an impact on biological control efficacy of microorganisms.

The effect of micronutrients on growth of microorganisms can result from its binding with various biomolecules, influence on different cellular structures and metabolic processes by blocking of enzymes **(**Abbas *et al*., 2017). The positive effect of chemical inducers on the bio-agent was an expected, as most of them improve the metabolic activity and can be used by the fungus as nutrients or coenzyme. Micronutrients increased the production of chitinase in some *Trichoderma* species and can be used in biological control application. Addition of an antioxidant increased the biomass yield of *Lentinus tigrinus*, as well as *Cunninghamella japonica*. *Trichoderma* spp. prefer and grow well in acidic pH and in soil or media have high organic matter. (Ivashechkin *et al.*, 2015).

The micronutrients and thiamine in the nutritional media, induced the production of individual enzymes in fungal proteome, through increasing the expression of several proteins, However, the present results confirm that the supplementation of *T. harzianum* medium with the tested chemicals could improve and increase the efficacy of the overall biological control process (Joo *et al.*, 2009).

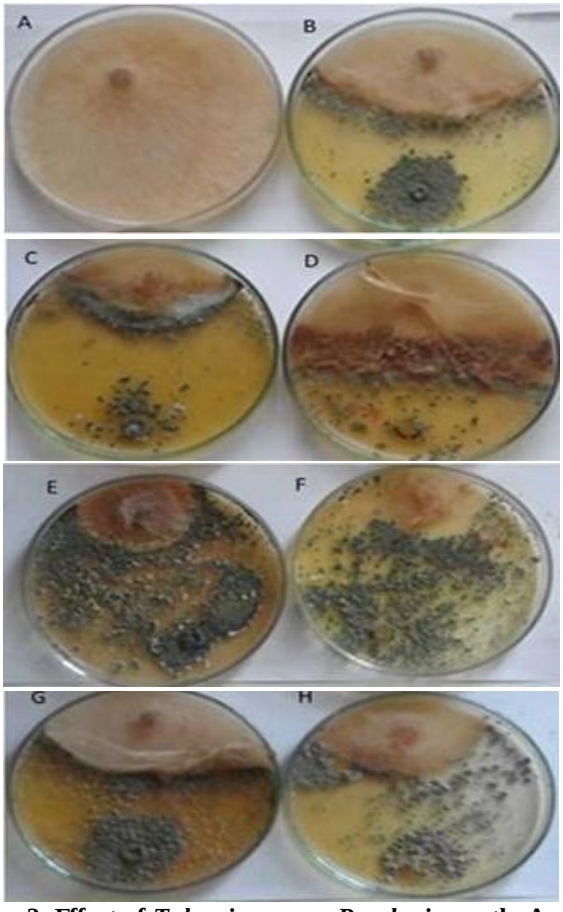

Fig. 2. Effect of *T. harzianum* on *R. solani* growth. A: *R. solani* grown on PDA medium, B: *T. harzianum* against *R. solani* on GM medium (control), D, F &H: antagonism on GM medium supplemented with micronutrients, K-tartrate and thiamine. C,E&G antagonism on PDA free from any addition and inoculated with treated Trichodema discs with previous chemical inducers.

Table 1. Antagonistic effect of *T. harzianum* against *R. solani*, crude extract, secondary metabolites and volatile compounds activities as affected by growing on different GM medium supplemented with the tested antioxidants compounds.

Treatment	Dual culture test		Crude Extract	Volatile compounds	Secondary metabolites
	Growth(cm)	Inhibition %			
R. solani	8.0a	00.0	9.0a	9.0a	9.0a
T. harzianum (T)+ *R. solani*	3.3b	63.3	9.0a	7.1c	9.0a
T + potassiumtartrate	1.0g	89.0	2.8f	5.3g	3.0d
T +micronutrients	1.7f	81.1	3.7d	5.6f	1.2f
T +thiamine	1.8ef	80.0	4.5b	6.2e	3.0d

Means followed by the same letter in each column are not different significantly according to LSD test P=0.05).

Table 2. Effect of treated *T. harzianum* with different chemical inducers on antagonism, crude extract, secondary metabolites and volatile compounds activities against *R. solani* grown on PDA medium free from any addition.

Treatment	Dual culture test		Crude Extract	Volatile compounds	Secondary metabolites
	Growth (cm)	Inhibition %			
R. solani	8.0a	00.0e	8.0a	8.0a	8.0a
T. harzianum (T)+ *R. solani*	5.3b	63.3d	8.0a	7.1b	8.0a
T* potassiumtartrate	1.9e	78.9a	3.0c	8.1b	7.4b
T* micronutrients	2.1d	76.7b	3.7bc	6.6c	3.8c
T* thiamine	2.4c	73.3c	3.9b	5.2d	1.5d

Means followed by the same letter in each column are not different significantly according to LSD test P=0.05).
T*= Trichoderma treated

The positive effect of antioxidants improved the metabolic activity and can be used by the fungus as a nutrients or coenzyme. Our studies revealed that the addition of different supplements to the growth media helped *T. harzianum* to excrete secondary metabolites over those produce on the basal medium. In previous studies found that potassium tartrate and micronutrients enhanced the antagonism leading to complete inhibition of *S. sclerotiorum* growth. In addition to all *Trichoderma* isolates supplemented with all antioxidants caused great effect in the new sclerotia formation by *S. sclerotiorum* (Yousef *et al*., 2016). Our studies documented a link between increased secondary metabolites production and the addition of different media *R. solani*.

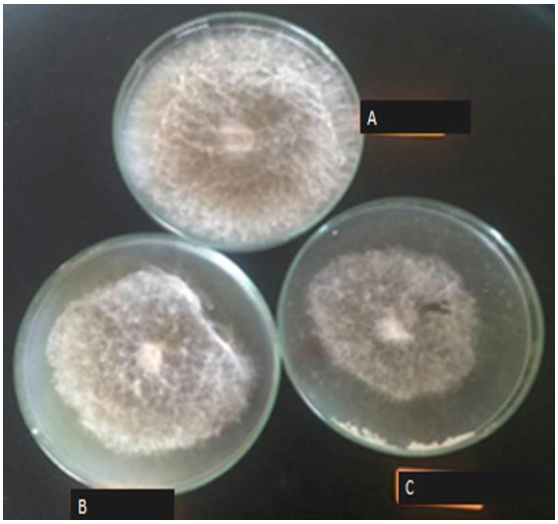

Fig. 3. The positive effect of volatile compounds of modified *T. harzianum*, A: *R. solani* control, B: untreated *T. harzianum* (control), C: slightly growth of *R. solani* by the action of volatile compounds of *T. harzianum* induced by micronutrients.

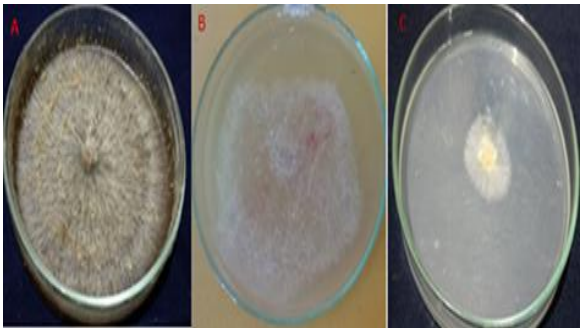

Fig. 4. Effect of crude extract of *T. harzianum* supplemented with potassium tartrate on mycelial growth of *R. solani*. A: *R. solani* (control), B: untreated *T. harzianum* control and C: filtrate supplemented with potassium tartrate from cultures of modified *T. harzianum*.

Effect of *T. harzianum*, chemical inducers and their combination on sclerotial formation and germination

Data in Table (3) indicate that growing *R. solani* in medium containing potassium tartrate, thiamine and micronutrients mixture or one of the used micronutrients have negative effect on sclerotia formed in varying degrees. *T. harzianum* recorded 52% reduction in the sclerotia germination. Micronutrients separately did not affect the germination percentage of the fungus sclerotia. The only exception was recorded with boron to give 60% reduction in the sclerotia germination. Micronutrients mixture alone gives 55% reduction in the sclerotia germination. Whereas Trichoderma supplemented with all chemical inducers completely prevent sclerotia formation and germination. It is well known that these elements are necessary for the growth and sclerotia germination of different fungi but after exceeding a certain concentration they inhibit both, and thus they are used as fungicides (Yousef *et al*., 2015a).

Table 3. Effect of *T. harzianum*, chemical inducers and their combination on sclerotial formation and germination of *R. solani* after 24 incubation.

Treatment	Sclerotia formed	Sclerotial germination%
Check	+++++	100.0a
T. harzianum	+++	52.0e
Potassiumtartrate	++	99.0b
Mn	+++++	99.0b
Zn	+++++	99.0b
Se	+++++	99.0b
B	++	60.0c
MN mixture	+++	55.0d
Thiamine	+++	100.0a
T+chemical inducers	0	00.0f

Means followed by the same letter in each column are not different significantly according to LSD test P=0.05).
+= sclerotia formation rate; 0=nil, ++=low, +++ =moderate, +++++=dense and T=*T. harzianum*

Detection of mycoparasitism using slide culture technique

Mycoparasitism by *Trichoderma* sp. is one of the important modes of action exhibited by biocontrol agents against host fungi. The hyphal contact between both fungi under the light microscope was observed more clearly in treated Trichoderma compared with untreated one as shown in Figs. (5,6,7 and 8). *T. harzianum* penetrated the hyphae of *R. solani*. Malformed hyphae of *R. solani* as affected by the toxic substances produced by *T. harzianum*. Mycelium change in color to deep dark, also increased in width of the infected mycelium. The same figures also show lysis of hyphae and heavy rally of *T. harzianum* spores, especially with potassium tartrate and thiamine treatment, around the hyphae of *R. solani*. (Harman, 2006) showed that malformation might be due to the effect of toxic substance produced by *T. harzianum*, which led to a clear lytic area started to appear in the hyphae of pathogen. The mycoparasitism of treated Trichoderma may be due to cell wall degrading enzymes such as chitinase, glucanase and proteases are related to the mycoparasitism. (Naeimi *et al*., 2010) reported that Trichoderma formed appressorium-like structures contact branches. After interaction with the host, pronounced collapse of *R. solani* and breakdown of the cell wall of *R. solani* and hyphal disintegration. Later, the hyphae showed extreme shrinkage and shriveling. Inhibitory volatile substances activity induces by antioxidants and micronutrients may also contribute to the biocontrol activity of treated Trichoderma. Micronutrients in soil may influence growth, sporulation and enzymatic Trichoderma enzymes. (Jaworska and Dluzniewska, 2007), which can cause changes in the metabolites produced in addition to biological activity against the pathogens

(Kredics *et al*., 2001 a, b). The positive effect of antioxidants and micronutrients was an expected result as most of them improved the metabolic activity and can be used by the fungus as a nutrients or coenzyme (Ivashechkin *et al*., 2015; Yousef *et al*., 2016) The present study revealed that chemical inducers enhance production of primary and secondary metabolites of *T. harzianum*. The bioagent fungi destroy sclerotia, thus reducing inoculum of the pathogen, makes them a promising strategy (Knudsen *et al*., 1991). Our studies showed that Trichoderma treatments prevented sclerotia formed of the *R. solani in vitro*, and thus reduce fungus inoculum in the soil because sclerotia are the main primary inoculum that initiates the disease in the following season.

In addition to the obvious activity of the crude extract, volatile compound and secondary metabolites of Trichoderma grown on medium containing the tested chemicals, these chemicals also increased the growth and sporulation, as well as induced Trichoderma activity against the pathogen (Almeida *et al*., 2007; Yousef *et al*., 2016). Eziashi *et al*., (2007) suggested that the antagonists had also an affinity for the host cell wall, which my involve chemical bonding between functional sites of carbohydrates present on the cell wall of Trichoderma and pathogen, which triggers the events leading to host wall penetration.

The production of antifungal compounds, also play an important role in antagonistic activity of Trichoderma. These include; antibiotics, mycotoxins and low-molecular weight secondary compounds (Schuster and Schmoll, 2010).

These results indicated that all three tested chemicals improved the secondary metabolites activity that reduced the mycelial growth of *R. solani*. Poatassium tartrate and micronutrients mixture proved to be the highest producer of these metabolites. The present data suggest that these three chemicals play a positive role in the production of primary and secondary metabolites of *T. harzianum* and could be considered as enhancers to improve bioagent activities.

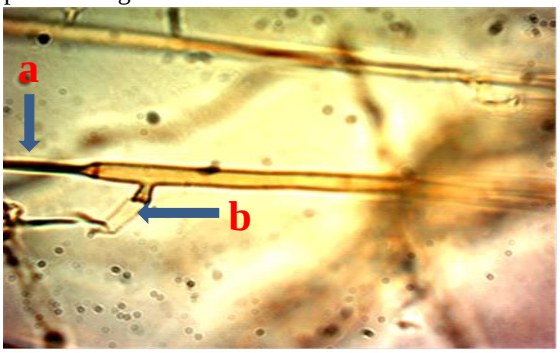

Fig. 5. Light microscope of hyphal interaction of untreated *T. harzianum* (control) and *R. solani* shows a clear damage and lysis hyphae (a) as affected by the metabolites of *T. harzianum*. b: malformed mycelium disrupted or changed in colors to deep dark (x40).

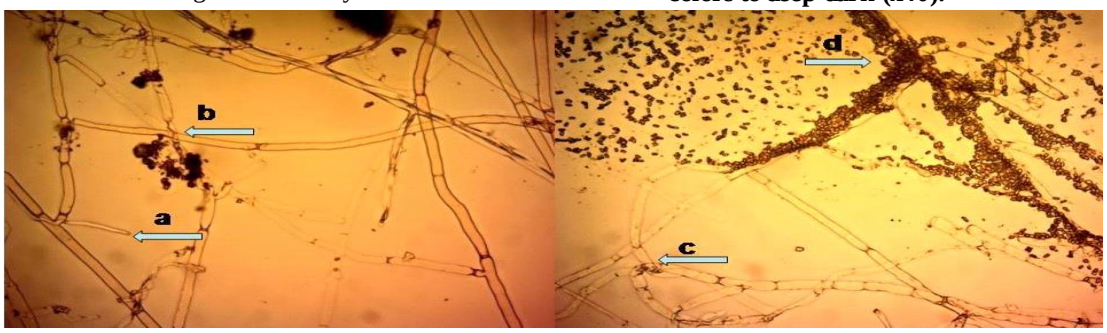

Fig. 6. K-tartrate treatment showed penetrated the hypha of Trichoderma of pathogen mycelium (a) and lysis (b), coiled (c), prolific gathering of *T. harzianum* spore around the hypha of *R. solani* (d), leading to morphological changes in the hyphal growth (x40)**.**

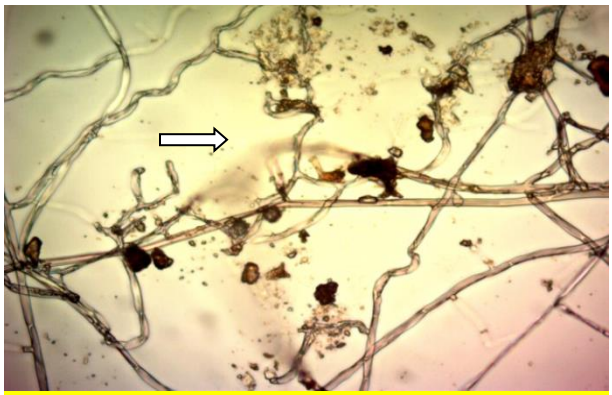

Fig. 7. The effect of *T. harzianum* grown on GM medium supplemented with micronutrients mixture on *R. solani* shows lysis (arrow), malformed mycelium, changes in colors to deep dark and increased in the area of the infected mycelium (x40).

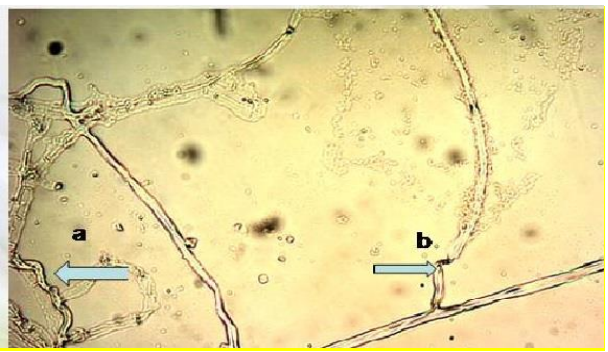

Fig. 8. Coiling (a), malformed mycelium (b) and accumulation of *T. harzianum* spores around the mycelium of *R. solani* as a result of *T. harzianum* supplemented with thiamine (x40).

Identification of chemical compounds produced by treated and untreated *T. harzianum* using GC-MS

This study on these promising antagonist treatments are identify potential compounds secreted, to examine

possible modes of action related to biocontrol and to evaluate efficacy of chemical inducer in stimulation of Trichoderma to produce substances with a high disincentive effect on the pathogen. The major identified compounds from each growth trails were summarized in Table (4). The identified compounds are belonging to various chemical classes, including hydrocarbon, alcohols, ketones, cyclohexane, esters and terpenes.

Table 4. The major chemical compounds of *T. harzianum* grown on GM broth medium amended or un-amended with different supplements.

No.	RT	Chemical Name	Molecular Formula	Abundance % of each chemical compound with the corresponding supplement			
				Cont.	potassium tartrate (A)	Micronutrients (MN)	thiamine (TH)
1	4.27	Trichloromethane	$CHCl_3$	------	0.33	0.29	--------
2	5.14	Tetrahydropyrrole-3-amino-2,5-dione	$C_4H_6N_2O_2$	------	0.20	0.14	------
3	9.13	Acetic acid	$C_2H_4O_2$	------	2.17	-----	-------
4	9.20	Cyclohexan-1,4,5-triol-3-one-1-carboxylic acid	$C_7H_{10}O_6$	------	------	0.17	1.44
5	10.18	Decane, 2,4,6-trimethyl-	$C_{13}H_{28}$	-------	0.16	--------	-------
6	11.19	4,4-Ethylenedioxy-1-pentylamine	$C_7H_{15}NO_2$	0.24	0.21	0.92	0.81
7	11.79	Ethane, 1,1,2,2-tetrachloro-	$C_2H_2Cl_4$	-------	0.18	0.16	-------
8	12.37	sec-Butyl nitrite	$C_4H_9NO_2$	------	0.50	0.64	------
9	12.94	Hexadecane	$C_{16}H_{34}$	------	0.42	0.36	------
10	13.38	Terpineol, cis-α-	$C_{10}H_{18}O$	0.78	------	------	0.98
11	13.36	2,3-Butanediol	$C_4H_{10}O_2$	------	7.41	2.77	3.14
12	14.12	2,3-Butanedioldiacetate	$C_8H_{14}O_4$	------	2.07	------	------
13	15.04	3-Cyclohexene-1-methanol, α, α,4-trimethyl-,	$C_{10}H_{18}O$	1.10	------	------	1.71
14	15.51	1,3-Butanediol, diacetate	$C_8H_{14}O_4$	------	1.83	0.65	1.02
15	16.99	1,3-Dioxolane-2-acetic acid, 2-methyl-	$C_6H_{10}O_4$	--------	1.44	0.92	--------
16	17.49	2,5-Hexanedione, 3,4-dihydroxy-3,4-dimethyl-	$C_8H_{14}O_4$	--------	0.30	0.17	--------
17	18.27	2-Acetoxytetradecane	$C_{16}H_{32}O_2$	--------	0.75	0.63	--------
18	18.51	Phenylethyl Alcohol	$C_8H_{10}O$	--------	2.00	3.07	1.59
19	19.29	Tetradecanoic acid, ethyl ester	$C_{16}H_{32}O_2$	--------	0.60	0.55	-------
20	19.70	Octadecane, 6-methyl-	$C_{19}H_{40}$	------	0.66	0.56	------
21	20.20	Phenol, 2-methyl-5-(1-methylethyl)-	$C_{10}H_{14}O$	30.87	------	0.06	22.83
22	20.37	3- Trifluoroacetoxypentadecane	$C_{17}H_{31}F_3O_2$	0.19	0.43	0.10	0.19
23	21.31	Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$	0.18	0.76	0.64	0.35
24	22.30	1H-Benzocyclohepten-7-ol, 2,3,4,4a,5,6,7,8-octahydro-1,1,4a,7-tetramethyl-, cis-	$C_{15}H_{26}O$	0.62	3.18	4.59	1.95
25	23.09	[1,1'-Bicyclopropyl]-2-octanoic acid, 2'-hexyl-, methyl ester	$C_{21}H_{38}O_2$	0.33	0.21	0.15	----------
26	23.28	Octadecanoic acid, ethyl ester	$C_{20}H_{40}O_2$	----------	0.43	0.44	0.26
27	31.17	1,2-Benzenedicarboxylic acid, butyl octyl ester	$C_{20}H_{30}O_4$	0.12	0.24	----	----
28	40.38	2-[4-methyl-6-(2,6,6-trimethylcyclohex-1-enyl)hexa-1,3,5-trienyl]cyclohex-1-en-1-carboxaldehyde	$C_{23}H_{32}O$	0.52	0.45	0.92	0.59
29	48.79	9-Octadecenamide	$C_{18}H_{35}NO$	0.30	0.32	0.63	0.73

The major chemical compounds identified were 2,3-Butanediol, Cyclohexan-1,4,5-triol-3-one-1-carboxylic acid, Hexadecanoic acid ethyl, ester, 1H-Benzocyclohepten-7-ol,2,3,4,4a,5,6,7,8-octahydro-1,1,4a,7-tetramethyl-,cis-, 9-Octadecenamide, 1,3-Butanediol diacetate , Phenylethyl Alcohol, Phenol, 2-methyl-5-(1-methylethyl) or (Carvacrol), acetic acid, 2,3-Butanedioldiacetate, 1,3-Dioxolane-2-acetic acid 2-methyl-, Octadecanoic acid, ethyl ester, 2-[4-methyl-6-(2,6,6-trimethylcyclohex-1-enyl) hexa-1,3,5-trienyl] cyclohex-1-en-1-carboxaldehyde, 3- Trifluoro acetoxy pentadecane Terpineol, cis-a- and Hexadecane, Trichloromethane, Tetrahydropyrrole-3-amino-2,5-dione,Decane,2,4,6-trimethyl-4,4-thylenedioxy-1-pentylamine,Ethane, 1,1,2,2- tetrachloro-, 3- Cyclohexene-1-methanol,à,à,4-trimethyl-,2,5-Hexanedione, 3,4-dihydroxy-3,4-dimethyl, and 2-Acetoxytetradecane. Fig. (9) shows the chemical structure of major identified compounds.

Table (4) illustrated that the identified compound abundance ratio was changed with the addition of different medium supplement. Generally, the addition of different supplements to the growth medium helped *T. harzianum* to excrete secondary metabolites over those produce on the basal medium. For example; 2, 3-butanediol was not detected in the extract of the basal medium trail, while when the basal medium supplemented with K-tartrate, micronutrients or thiamine, the recorded percent of 2,3-Butandiol was 7.41%, 2.77% and 3.14%, respectively. Also, the phenyl ethyl alcohol present was raised up from nil in the basal medium trail to be

2.0% (potassium tartrate), 3.07% (micronutrients) and 1.59% (thiamine). In the same manner, 1H-Benzocyclohepten-7-ol,2,3,4,4a,5,6,7,8-octahydro-1,1,4a,7-tetramethyl-, cis- ratio was raised up from 0.62% in the basal medium trail to be 3.18%, 4.59% and 1.95%, respectively. In contrary, phenol, 2-methyl-5-(1-methylethyl) or (carvacrol) ratio was decreased from 30.87% in the basal medium trail to be zero, 0.06% and 22.83%, respectively. Some identified compounds have a slight change in their abundance ratio, such as 3- Trifluoro aceto xy pentadecane, 9-Octadecenamide, Hexadecanoic acid ethyl ester and 2-[4-methyl-6-(2,6,6-trimethylcyclohex-1-enyl)hexa-1,3,5-trienyl]cyclohex-1-en-carboxaldehyde.

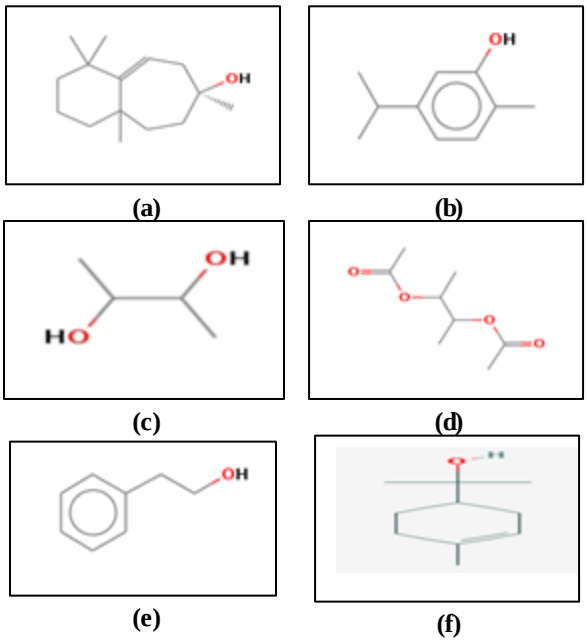

Fig. 9. (a) 1H-Benzocyclohepten-7-ol,2,3,4,4a,5,6,7,8-octahydro-1,1,4a,7-tetramethyl-, cis- (b) Phenol, 2-methyl-5-(1-methylethyl)-, (c) 2,3-Butanediol, (d) Phenylethyl Alcohol (e) 2,3-Butanediol diacetate and (f) 3-Cyclohexene-1-methanol, α, α,4-trimethyl-,.

Previous work has shown that the production of VOCs by antagonists agent with antimicrobial activity against plant pathogens, for examples, Fialho *et al.*, 2011 evaluated volatile organic compounds (VOCs), produced by *Saccharomyces cerevisiae*, to control *S. sclerotiorum in vitro* and bean seeds. The compounds 2-methyl-1-butanol and 3-methyl-1-butanol cause completely inhibition its mycelial growth of *S. sclerotiorum*, followed by the ethyl acetate. Bean seeds treated with the VOCs at 3.5 µL mL^{-1} showed a 75% inhibition in *S. sclerotiorum* incidence after four days of fumigation, they reported that VOCs have potential to manage the pathogen in stored seeds. Volatile compounds from wheat seed culture of *Streptomyces philanthi* RM-1-138 was able to control the rice sheath blight disease caused by *R. solani* (Boukaew *et al.*, 2013).

Shi *et al.*, (2017) reported that 2, 3-butanediol triggers the secretion of root exudates that modulate soil fungi and their roles as important inducers of plant defenses against fungal infection and insect damage. As well as, induced plant defense against *R. solani* for creeping bentgrass. In addition to improve plant growth. 2, 3-butanediol suppressed the expression of some regular stress-related genes in creeping bentgrass. These results suggest that 2, 3-butanediol may induce changes to the plant transcriptome in induced systemic resistance pathways.

The volatile compounds have been identified as mono- and sesquiterpenes, alcohols, ketones, lactones, esters compounds (Hynes *et al.*, 2007; Nemcovic *et al.*, 2008). *Trichoderma* spp. are able to secrete different secondary metabolites that may contribute to their mycoparasitism action. These volatile and nonvolatile toxic metabolites inhibit the colonization of pathogen (Reino *et al.*, 2008; Sharma, 2011), induce resistance and promote the plant growth (Shalini *et al.*, 2006; Siddiquee *et al.*, 2012; El-Sharkawy *et al.*, 2018).

Evaluate of treated *T. harizanum* for managing the damping off disease in faba bean

Results in Table (5) indicated that all Trichoderma treatments as seed soaking significantly increase Trichoderma efficacy to reduce the incidence of damping off comparing with the control and untreated Trichderma. *T. harizanum* supplemented with micronutrients mixture and potassium tartrate protect from damping off and give more plant survival (93.7 & 87.5%) respectively than untreated Trichoderma 62.5 % and control 25%**.** These treatments seem to stimulate a systemic affect that effect on suppress plant pathogen. In addition, it has direct effect on the pathogen. It is may be due to that modified Trichoderma stimulate the enzymes in beans which protect plant against disease. In addition the chemical inducers had a positive role in plant defence activity (Yousef *et al.*, 2015b).

Table 5. Damping-off of faba bean seeds cv. Giza 429 seedlings in pots infested with *R. solani* and resulted from seed soaking with Trichoderma supplemented with different chemical inducers.

Treatment	Pre-emergence	Post-emergence	Plant Survival %
Untreated T	18.75b	18.75b	62.5d
T+ micronutrients	0.00d	6.25d	93.75a
T+ thiamine	6.25c	18.75b	75.0c
T+ potassium tartrate	0.00d	12.5c	87.5b
R. solani (control)	50.0a	25.0a	25.0e

Means in each column followed by the same letter are not significantly different according to LSD test P=0.05).
T= *T. harzianum*

CONCLUSION

Some chemical inducers enhance the *T. harzianum* activities against *R. solani*. However modified *T. harzianum* shows different metabolic pathways, producing various metabolites based on the composition of growing medium. Suppression effects were observed on both sclerotia formation and damping off diseases. Such activity making *T. harzianum* as useful to be used eco-friendly as a biocontrol agent for developing control strategy for *R. solani* and other plant pathogens. More studies are needed for separation and purification of such bioactive fractions to be used in agriculture as well as pharmaceutical applications.

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تأثير بعض المستحثات الكيميائية على القدرة التضادية لتريكودرما هارزيانم ضد ريزوكتونيا سولانى وإنتاج أيضها

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اجريت الدراسة الحالية على بيئة النمو جليوتوكسن المدعمة ببعض المستحثات الكيميائية مثل (خليط المغذيات الدقيقة، طرطرات البوتاسيوم والثيامين) لتحسين النشاط الحيوي للتضاد والمواد المتطايرة والغير متطايرة لتريكودرما هارزيانم ضد الريزوكتونيا سولانى. سجلت التريكودرما المنماه على بيئة مدعمه بطرطرات البوتاسيوم تقليل النمو الميسليومى للريزوكتونيا سولاني بنسبة 89% مقارنة بالتريكودرما بمفردها 63,3%، إضافة إلى زيادة ملحوظة في النشاط الحيوى لمركبات التريكودرما المتطايرة والغير متطايرة. سجلت إضافة المغذيات الدقيقة أعلى نشاط للمركبات الثانوية يليها معاملة الثيامين. عند نقل التريكودرما المعاملةعلى بيئة بطاطس بدون اضافة المواد المختبرة أظهرت نشاط حيوى ضد المسبب المرضى اشار فحص الميكروسكوب الضوئى تلف وتدمير لهيفات الفطر الممرض ظهر بشكل واضح بعد التداخل مع التريكودرما المعاملة على بيئة بطاطس بدون إضافة المواد المختبرة. أظهر تحليل GC-MS اكتشاف مركبات جديدة مضادة للفطريات بمستخلص التريكودرما المنماه على بيئات الجليوتوكسن المدعمه بمواد الاستحثاث الكيماوية مثل 2,3-Butanediol، 1H-Benzocyclohepten-7-ol,2,3,4,4a,5,6,7,8-octahydro-1,1,4a,7-tetramethyl-,cis-, acetic acid ، phenylethyl alcohol و Decane, 2,4,6-trimethyl. أدى نقع بذور الفول البلدى جيزة 429 في معلق التريكودرما المعاملة بالمركبات المختبرة قبل الزراعة في التربة المعدية إلى زيادة فى نسبة البادرات المتبقية مقارنة بالتريكودرما الغير معاملة. طبقا لنتائج المواد المختبرة وجد أنها تزيد النشاط التضادى للتريكودرما ويمكن إستخدامها كمبيدات حيوية.