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Effect of Gibberellic Acid, Indole Acetic Acid and Ethylene Gas on The *In vitro* Biocontrol Activity of *Trichoderma harzianum* Refai Against *Alternaria solani* and *Botrytis cinerea.*

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ALTERNARIA solani and *Botrytis cinerea* were isolated from molds infecting tomato plant in Egypt. The biocontrol activity of *Trichoderma harzianum* Refai against these two pathogens were investigated in presence and in absence of growth regulators . The antagonistic activity of *T. harzianum* against these two pathogens were affected by the presence of growth regulators. IAA and GA_3 alone or in combination was stimulator for *Alternaria solani;* however this stimulation was suppressed in dual culture with *T. harzianum.* Ethylene gas was stimulator for the growth of *Botrytis cinerea*, although this stimulation was hampered when the ethylene gas and *T. harzianum* were existing together. Although , IAA and GA_3 alone or in combinations stimulated *Alternaria solani* especially at low concentrations , it couldn't affect the growth of *T.harzianum* .The hormones mycoparasitism relationship were monitored by endo-polygalacturonase , β-1, 3- glucanase, and Endo-chitinases enzymes . The secretion of endo- PG from *A. solani* was reduced when it was grown in the presence of IAA and GA_3 when they were used separate or in mixture .The secretion of endo-PG from *T. harzianum* was increased as the hormones were also increased, but in the presence of the phytopathogens the secretion was reduced.The activity of enzymes were increased at concentrations less than 40 ppm for both IAA and GA_3 and at concentrations less than 75 ppm for ethylene .The hormones separatley or in mixture couldn't affect the secretion of Endo-chitinase (endo-CH) and β-1, 3- glucanase from *T. harzianum* directly but it act together with the phytopathogen. Ethylene gas was stimulator for *Botrytis cinerea* but it couldn't affect the growth of *T.harzianum* .

Keywords: Growth hormones, *Alternaria solani*, *Trichoderma harzianum*, *Botrytis cinerea*, Enzymes.

Phytopathogens are responsible for considerable losses ($30–$50 billion annually) in crops (Baker *et al*., 1997). No other single fungal genus has received as much attention as the *Trichoderma* spp for biocontrol of plant pathogens. *Trichoderma harzianum* p1 was used *in vitro* and *in vivo* to suppress many phytopathogens (Latorre *et al*., 1997). Some commercial products of *Trichoderma* spp. were used such as (Belgium) against *Rhizoctonia solani*,

Pythium spp. and *Fusarium verticillium*; Tricodex and Trichopulvin 25 PU against *Botrytis* of vegetables and grapevines (Monte, 2001). The development of combinatory approaches that involve two or more biocontrol components, each occupying a different environmental niche and / or expressing a different mode of action, will improve the consistency and efficacy of the biocontrol activity (Schmitt *et al*., 2002). The presence of growth regulators such as auxins, gibberellins and ethylene in the soil come from applications done to the plants or even produced by some fungal pathogens could affect the phytopathogencity biocontrol relationship or to mediate fungal-plant interaction (Cristescu *et al*., 2002). High concentrations of IAA can inhibit the hypersensitive response (Robinette & Matthysse, 1990 and Jouanneau *et al*., 1991) and may suppress expression of plant defense genes or give similar disease symptoms (Tudzynski & Sharon, 2002).

Chitinolytic enzymes and β-1, 3-Glucanases which produced extracellular by *Trichoderma* spp. are well-known pathogenesis-related proteins (Whipps, 1992 and Zimand *et al*., 1996). Furthermore, they can also work indirectly by releasing oligosaccharides that can act as elicitors to activate other plant defense responses (Shibuya & Minami, 2001). It has been claimed that these two enzymes are a battery of antibiotic substances produced by *Trichoderma* spp. (Ghisalberti & Sivassithamparam, 1991). The presence of growth regulators in the soil could affect the activity of these enzymes (Aub *et al*., 1993).

Alternaria solani and *Botrytis cinerea* are plant necrotrophic pathogen that colonizes senescent or dead plant tissues and causes softening of fruits. *Alternaria solani* is the causal agent of early blight (EB) disease, infects aerial parts of tomato at both seedling and adult plant stages (Chaerani *et al*., 2007). On the other hand, *B. cinerea* attacks different plant tissues and has a broad host range. Furthermore , *B. cinerea* can synthesize ethylene themselves to assess in attacking the plant tissues and rapid maturity of the infected fruits (Cristescu *et al*., 2002). The role of ethylene in plant–pathogen interactions is complex (Díaz *et al*., 2002) . The availability of the new tools for studying *B. cinerea* biology will make the characterization of fungus-produced ethylene in pathogenesis possible, and it will provide more answers on the physiology of the event of *B. cinerea* infection in fruits with respect to biocontrol . Recently, it has been reported that ethylene is a primary marker for fruit pathogenesis and several other infection-related plant products have also been studied as early markers of pathogenesis (Polevaya *et al*., 2002)**.** Knowledge of the factors that could affect biocontrol activity would allow improving the biocontrol conditions. Limited information is available concerning the role of the plant growth regulators on the relationship between *Trichoderma* spp. and phytopathogens (Aub *et al*., 1993 and Badri *et al*., 2007). It is necessary to provide the information of how growth regulators secreted by the pathogens or pollute the soil could benefit or decrease the biocontrol effect of biocontrol organism. So,the aim of the present work was to investigate the role of IAA , GA3 and ethylene gas on the *in vitro* biocontrol activity of *Trichoderma harzianum* Refai strain against the most two tomato pathogens , *Alternaria solani* and *Botrytis cinerea* .

Materials and Methods

Chemicals

All chemicals and plant hormones were purchased from Sigma Aldrich Company. Ethylene gas and medical air were purchased from the Egyptian Company for Artificial Gases, Cairo, Egypt.

Microorganisms

Alternaria solani was isolated from mold infecting tomato fruits and were maintained on potato dextrose agar according to the method described by Kennedy *et al*. (2000) and Strobel & Daisy (2003). Briefly, infected tissues are thoroughly surface treated with 70% ethanol. With a sterile knife blade, outer tissues are removed from the samples and the inner tissues are carefully excised and placed on water agar plates. After seven days of incubation, at 25°C, hyphal tips of the fungi are removed and transferred to potato dextrose agar. *Botrytis cinerea* was isolated from the infected tissues according to the method of Kritzman & Netzer (1978). The infected tissues rinsed in deionized water for 60 min, then dried in a laminar flow hood on paper towels sterilized with 70% ethyl alcohol, and plated onto Kritzman's agar. The seed plates were incubated at 24°C with 12 h of day and 12 h of night (cool white fluorescent light by day) for 14 days, and examined microscopically (up to ×100 magnification) for development of the fungus. The cultures were then transferred to acidified potato dextrose agar for the following studies. Identification of isolates based on colony morphology, rate of colony growth, sporulation, and sclerotium characteristics (Gilman, 1957; Ellis, 1971 and Barnett & Hunter, 1998). *Trichoderma harzianum* Refai EMCC 583 was obtained from Egypt Microbial Culture Collection (Cairo Mircen), Faculty of Agriculture, Ain Shams University.

Preparation of inocula of A.solani and B. cinerea.

Isolates of *A.solani* and *B. cinerea* were maintained on potato dextrose agar (PDA) plates for 14 days at 25°C before inocula were isolated. To prepare a spore suspension, the fungus was removed from the cultured plates by gentle brushing of the plate surfaces with a sterile platinum loop and was suspended in distilled water. The fungal suspension was filtered through two layers of gauze under sterile conditions to separate the spores. The concentration of spores was determined by using a haemocytometer. Inocula of each fungus have 10^5 spores ml^{-1} was prepared in sterilized water supplemented with 0.03% Tween 20 to ensure uniform distribution of the spores.

Biocontrol activity of Trichoderma harzianum against Alternaria solani in presence of growth hormones

Disks (0.5 cm diameter) from pure cultures of *A. solani* were seeded with *T. harzianum* in a 9 cm Petri dishes containing autoclaved PDA separately or in dual cultures. Gibberellic acid (GA_3) or indole acetic acid (IAA) was added at 0, 10, 20, 40, 60, and 80, 160 ppm before the inoculation of the fungi and after cooling the medium. Fungi were grown up to ten days at 28°C $\pm$ 2°C. Colony

diameter was observed every two days and finally recorded at the tenth day. Dual cultures of *A. solani* and *T. harzianum* were seeded in the same dish at opposite sides and their growth was evaluated with radial growth of both. For each treatment, triplicate sets were run with six Petri dishes of the same procedure and repeated for three independent experiments. Control experiments were performed with seeding each fungus against itself.

In vitro growth of B. cinerea and ethylene production

The isolate of *B. cinerea* obtained from infected tomato fruits was investigated to produce ethylene *in vitro* by the method of Simona *et al*. (2002) with slight modification. Briefly, the isolate was grown on PDA supplemented with 35 mM of L-methionine (an ethylene precursor). The precursor was added to PDA, and the pH of the medium was then adjusted to 3.8 before autoclaving. Portions (160 µl) of a suspension containing a 10^5 of conidia were uniformly plated by placing on 9-cm-diameter Petri dishes containing solidified PDA. For each treatment, Petri dishes with the same previous procedure were placed into Desiccator jars in five groups ,each one has triplicate samples .The Desiccator jars are incubated for 120 h at 28°C $\pm$ 2°C . The ethylene emission was detected at 24 h intervals up to 120 h. To accurate detection of ethylene gas emission, the experimental groups were removed from the incubator one by one at the end of each incubation time and the gas collected was estimated per volume of Desiccator jar as ppm.

Analysis of gas concentrations

Ethylene / air mixtures were prepared by mixing medical air with ethylene, to achieve the appropriate final ethylene concentration for use in the flow through system. Ethylene concentrations were monitored by Gas Chromatography (GC) using (Carle AGC series 400, HACH Carle Chromatography. Detector tube was model / type 8014-2805, its limits from 20-2000 ppm. Standard was analyzed prior to analysis of each set of samples. Sample concentrations were calculated based on the concentration of standards. In all gas experiments the design system was adjusted at flow rate 50 nl h^{-1} to remain the concentration of gas constant under the selected concentrations.

Effect of ethylene on the biocontrol activity of Trichoderma harzianum against Botrytis cinerea

Disks (0.5 cm diameter) from *Botrytis cinerea* was seeded with *T. harzianum* in a Petri dish containing autoclaved PDA separately or in dual cultures then ethylene gas was supplied at concentrations 0,25,50,75,100,150 and 200 ppm . Fungi were grown up to ten days at 28°C $\pm$ 2°C. Colony diameter was observed every two days and finally recorded at the tenth day. In case of dual cultures, controls were performed seeding each fungus against itself. Ethylene treatment was conducted in Desiccator jars using a novel flow through system described by Roze *et al*. (2004). Where no ethylene was added ethylene scrubbers (5.5 g $KMNO_4$) were placed in the Desiccator jars to minimize effects due to production of the gas by the fungus. Air/gas mixtures were water saturated by passing medical air and ethylene through a sparger containing sterilized distilled

water. For each treatment, triplicates sets were run with six Petri dishes of the same procedure and repeated for three independent experiments. Finally at the end of incubation the data were analyzed statistically.

Spore germination in presence of growth regulators

In vitro spore germination was tested in 96-well micro titer plates essentially as previously described with some modifications (Lorito *et al*., 1994). Briefly, a suspension of 3 x 10^3 spores of the experimental fungi and 50 µl of broth culture (PDB) with 5 mM potassium phosphate buffer (pH 6.7) were placed in a well of a micro titer plate, and 10 µl of the tested hormone concentration (IAA,GA3 or mixture of (IAA + GA3 , 1:1) was supplemented under the same previous conditions . Addition of 10 µl H_2O instead of the hormone supply was used as a control. The ethylene gas was supplied under the same concentrations and conditions that were described previously. The germination tube length and the number of germinated spores were measured after 8 h of incubation.

Effect of growth regulators (IAA, GA3 and ethylene) on the enzymes activity for T. harzianum , Alternaria solani and Botrytis cinerea

Endo-polygalacturonase (endo-PG) activity

The secretion of Endo-polygalacturonase was evaluated in submerged broth culture (PDB) supplemented with hormones as described previously. For each treatement , 250 ml Erlenmeyer flasks, containing 50 ml of PDB supplemented with 10 g l^{-1} citrus pectin were inoculated with 10^5 spores of either two pathogens or *T. harzianum* in absence or the presence of different concentrations of growth hormones . To investigate the effect of *T. harzianum* on the ability of *A. solani* or *Botrytis cinerea* to secrete endo-PG, 10^5 conidia of each fungus were inoculated under the same conditions to give a final concentration of 2 x 10^5 conidia per flask then incubated at 28 $^oC \pm 2\ ^oC$ for 7 days. After the end of incubation ,the whole medium was then centrifuged at 9,000 x *g* for 10 min to remove mycelia, and the supernatant was used to test endo-PG. Endo-polygalacturonase (endo-PG) activity for the two pathogens was tested by a modified Nelson-Somogyi assay (Nelson, 1944). The reaction mixture contained 0.5% (w/v) polygalacturonic acid in 100-mM sodium acetate pH 5.2 (Pérez *et al*., 1991). One unit was defined as the amount of enzyme that released 1 mM of reducing sugars per minute. Controls of the enzyme activity were performed with boiled enzyme (20 min). The results correspond to the mean of six different experiments run in triplicates. To test the effect of growth hormones on the biosynthesis of endo-PG by *T. harzianum*, it grows alone under the same conditions of the dual culture then the enzyme was assayed.

Endo-chitinase (endo-CH) activity of Trichoderma harzianum

The secretion of Endo- chitinase enzyme was evaluated in submerged (PDB) broth supplemented with hormones as described previously. For each treatment, 250 ml Erlenmeyer flasks, containing 50 ml of PDB broth supplemented with 1% glycol chitin were inoculated with 10^5 spores of either two pathogens or *T. harzianum* in absence or the presence of different concentrations of growth

hormones. Endochitinase activity of *T. harzianum* strain was tested by the method of Rojas-Avelipaz *et al*. (1999). To test the effect of *A. solani* or *Botrytis cinerea* on the ability of *T. harzianum* to secrete endo-CH, 10^5 conidia of each fungus were inoculated in the same media to give a final concentration of 2 x 10^5 conidia per flask. Flasks were incubated at 28°C $\pm$ 2°C up to 10 days with shaking at 150 rpm. The whole medium was then centrifuged at 9,000 x *g* for 10 min to remove mycelia, and the supernatant was used to test endo-CH activity. One unit of the enzyme activity was defined as the amount of enzyme that releases 1 µmol of N-acetyl glucosamine per hour. The results correspond to the mean of six different experiments run in triplicates.

β-1, 3-Glucanase activity

In the previous described experiment, *β* -1, 3-Glucanase of *T. harzianum* was also assayed based on the release of reducing sugar from laminarin as described by Santos *et al*. (1977). One unit of enzyme activity (U) was defined as the amount of enzyme that catalyzes the equivalent release of one µmol of glucose per minute under the described assay conditions.

Statistical analysis

Data were analyzed through least significant differences using the statistical analysis system SPSS soft ware version 14 (Robert & Jane Gradwohl, 2005). Standard error bars of means for each treatment were expressed in the graphs.

Results and Discussion

Biocontrol activity of Trichoderma harzianum in presence of plant growth hormones

To study the mycoparasitism relationship between the biocontrol organism and the two pathogens in presence of hormones, the plan of investigations was designed to study such two parameters in separate and in dual cultures for both pathogens and *T. harzianum*. The results in Table 1 show that *T.harzianum* Refai has antagonistic activity against *A.solani*. When the hormones were added alone or in combination they have no effect on the growth of *T. harzianum* in absence of *A.solani*. On the other hand, the growth of *A. solani* was suppressed in the presence of concentration less than 40 ppm while, an inhibitory activity increased when the concentrations of hormones increased than 40 ppm alone or in dual cultures. The results are also indicated that the effect of IAA alone or in combination with GA_3 was more inhibitory effect than GA_3 alone. The growth of *T. harzianum* in dual cultures treated with the growth regulators increased significantly than in case of its use alone .These results proved that the hormones have no effect on the growth of *T. harzianum*. Therefore, the stimulation of *T. harzianum* is due to the presence of the phytopathogen not due to the treatment of hormones. Finally, the presence of *T. harzianum* and *A. solani* in combination with IAA, GA3 alone or in the mixture could increase the mycoparastism *in vitro* by *T. harzianum*. In this manner, IAA inhibited the growth of *Agrobacterium* and many other plant-associated bacteria but not the growth of bacteria that occupy other ecological niches (Pu Liu & Nester, 2006). In the same respect, the growth of red pepper plants was enhanced by treatment

with *Rhizobacterium* producing giberillic acid. (Joo *et al*., 2005). Although, no one could explain the effect of auxins and gibberellins on the fungi, Lu *et al*. (2000) discussed the effect of IAA on the basis of stimulation of the pathogen. The results of the present work are nearly in line with the results of Sharaf & Farrag (2004) who found that 40 ppm of IAA inhibited the growth of *Fusarium solani*.

TABLE 1. Effect of different concentrations of IAA and GA_3 on the growth of *T. harzianum* and *A. solani* in separate and in dual cultures.

Growth hormone	Concentration (ppm)	Colony diameter (mm) of			
		T. harzianum **in separate cultures**	***T. harzianum*** **in dual cultures**	***A. solani*** **in separate cultures**	***A. solani*** **in dual cultures**
IAA	0.0(control)	81.2 ±0.31	81.2 ±0.31	52.1 ±0.24	52.1 ±0.24
	20	81.4 +0.24	83.2 +0.27	53.3**±0.22	50.4**±0.22
	40	82.1±0.33	83.2 ±0.29	56.8**±0.19	39.2**±0.18
	60	81.9±0.28	90.1**±0.24	42.7**±0.12	38.3**±0.29
	80	82.1±0.17	92.3**±0.20	40.3**±0.18	34.5**±0.23
	160	81.7±0.18	88.6**±0.12	39.1**±0.14	20.2**±0.22
GA_3	0.0(control)	81.2 ±0.31	81.2 ±0.31	52.1 ±0.24	52.1 ±0.24
	20	81.2±0.20	85.5 **±0.29	52.7**±0.21	51.6**±0.20
	40	81.4±0.26	87.3 ±0.25	53.8**±0.19	50.8**±0.17
	60	81.9±0.16	89.2 ±0.34	47.9**±0.22	49.2**±0.25
	80	81.1±0.28	90.1**±0.20	46.4**±0.17	47.2**±0.23
	160	81.7±0.21	89.8 **±0.21	40.6**±0.10	40.2**±0.20
Mixture of IAA and GA_3 (1:1)	0.0(control)	81.2 ±0.31	81.2 ±0.30	52.1 ±0.24	52.1 ±0.24
	20	81.5+0.11	85.3 **±0.25	44.2**±0.17	40.4**±0.20
	40	81.7±0.16	87.4*±0.26	38.9**±0.15	35.7**±0.11
	60	81.6±0.19	88.2 **±0.24	31.1**±0.20	26.7* ±0.24
	80	81.9±0.12	91.1**±0.22	24.6**±0.15	21.3**±0.19
	160	81.7±0.12	87.2**±0.20	20.2**±0.18	18.8**±0.20

** means highly significant L.S.D at 5 %.

Spore germination of Trichoderma harzianum and Alternaria solani in presence of growth regulators

The results in Table 2 show that the rate of spore germination of *T. harzianum* stay without change than control samples when they treated with IAA or GA_3 separate or in combination. However, the germination tube length increased significantly at 40 and 60 ppm if the hormones were used separate or in combination. The germination of *A.solani* spores was reduced; however the tube elongation was stimulated. These findings are in agreement with the results of Roco *et al*. (2001) who found that neither spore germination nor growth of T. *harzianum* could be change in the presence of growth hormones except at 40 ppm which was stimulator

for *T. harzianum* growth in dual culture. In the same manner, Gryndler *et al.* (1998) stated that 30 µM IAA completely suppressed the growth of *Glomus mossae* and *G. fistilosum.* Recently, Badri *et al*. (2007) found that the spore germination of both *Fusarium oxysporum* and *T. harzianum* were reduced in the presence of the growth hormones. They also found that 40 ppm of GA_3 didn't affect the germination of *Fusarium oxysporum* spores. All these findings showed a variation in hormonal response for both biocontrol organism and pathogen, the thing that indicated the effect of IAA and GA3 may be a strain dependent and complex.

TABLE 2. Percentage of *in vitro* spore germination and germ tube elongation of both *T. harzianum* and *A. solani* in presence of different concentrations of IAA- and GA3.

Growth hormone	Concentration (ppm)	Percentage of Spore germination of *T.harzianum*	Germ tube length of *T. harizianum* (µm)	Percentage of spore germination of *A. solani*	Germ tube length of *A.solani* (µm)
IAA	0.0	84.2 ±0.24	124.2 ±1.0	56.4 ±0.22	88.3±0.9
	20	84.6 ±0.21	124.7 ±2.3	55.6**±0.11	92.4**±1.4
	40	84.9 ±0.18	133.2** ±1.0	53.9** ±0.23	97.9**±1.3
	60	84.1±0.20	127.2** ±2.0	50.1**±0.12	98.3**±0.8
	80	80.1**±0.16	107.3** ±1.5	50.1±0.20	90.3**±0.6
	160	80.9±0.22	85.9 **±1.2	56.9**±0.22	89.4**±1.1
GA_3	0.0	84.2 ±0.23	124.2 ±1.0	56.4 ±0.25	88.3 ±0.9
	20	84.3±0.20	128.9** ±1.3	55.4**±0.32	90.3**±1.4
	40	84.2 ±0.19	125.4 **±1.0	53.5 **±0.13	92.6**±0.7
	60	84.1±0.15	124.5 ±1.2	50.1**±0.15	95.4**±1.2
	80	84. 1±0.23	85.4** ±1.6	59.1**±0.21	90.4**±0.6
	160	84.9±0.24	64.3** ±1.1	55.9**±0.22	91.1**±1.2
Mixture of IAA + GA_3 (1:1)	0.0	84.2 ±0.23	124.2 ±1.0	56.4 ±0.26	88.3± 0.9
	20	84.6±0.18	137.6** ±1.5	52.4**±0.30	94.1**±1.4
	40	84.3 ±0.20	131.8** ±1.3	51.5 **±0.18	99.7**± 2.0
	60	84.4±0.14	126.4** ± 2.1	50.1**±0.14	97.2**±1.8
	80	84.4±0.11	121.2** ± 1.0	49.1**±0.21	95.4**±1.5
	160	84.3±0.12	121.2 ± 1.0	45.9**±0.20	93.9**±1.2

** means highly significant L.S.D at 5 %.

Effect of IAA and GA3 on the enzymes activity for both T. harzianum and Alternaria solani.

Endo-polygalacturonase (endo-PG) activity

Endo-polygalacturonase (endo-PG) are enzymes secreted by phytopathogens to dissolve the cell wall of the host and they also secreted by *T. harzianum* (Pérez *et al*., 1991 and Sicilia *et al*., 2005). The results (Fig. 1-3) showed that the secretion of endo- PG from *A. solani* was reduced when it was grown in the presence of these two growth regulators regardless they were used separate or in mixture. In this regard, the secretion of endo-PG from *A. alternata* (Pérez *et al*., 1991) into submerged cultures was reduced to 20 - 25% when this phytopathogen was grown in the presence of 40 ppm of either IAA or GA_3; these results suggested that the hormones interrupt the rate of the enzyme secretions because the rate of secretion decreased than control. A stronger inhibitory effect was observed in the presence of active *T. harzianum*, which may be explained as a consequence of the growth inhibitory effect of the biocontrol agent on *A. solani.* Due to the fact that *T. harzianum* also secretes endo-PG, and to avoid misleading in the results as a result of endopolygacturonase activity by *T. harzianum*, it was grown separately in presence and in absence of the growth hormones. The results in Fig. (1-3) show that the enzyme from *T. harzianum* was secreted increasingly as the hormone secretions were also increased than control, but in the presence of the phytopathogens the secretion was decreased. These results indicated that the effect of the hormones on the total endoploygalacturonase in a dual culture *in vitro* may introduce the expected inhibition of the *A.solani* in the presence of the biocontrol organism in a combination with hormones .In this respect , Pérez *et al*. (1991) stated that its level is much smaller than those secreted by *A. alternata* but it was necessary to determine if endo-PG secretion by *A. solani* was altered by the presence of the *T. harzianum* . These findings may be suggested that during antagonism *T. harzianum* affects cellular mechanisms in *A. solani* that result in a slow down in its development and in a decrease of its infectious ability based on the levels of secreted endo-PG.Similar results were obtained by Roco *et al*. (2001) . They mentioned that the addition of 10^5 M IAA or GA_3 enhanced cellulase production by *T. reesei* whereas higher concentrations were inhibitory.

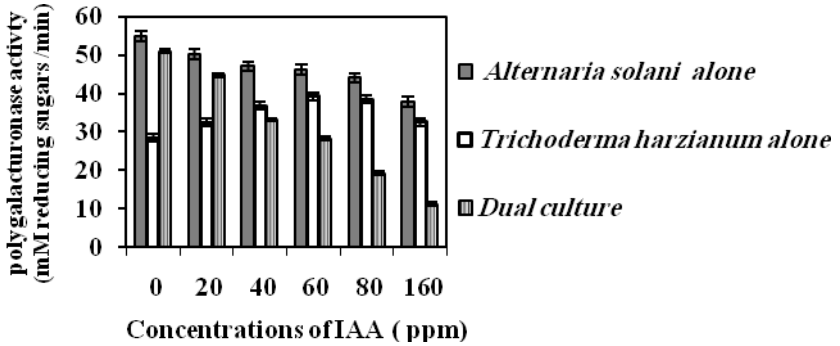

Fig. 1. Effect of IAA on Endo -polygalacturonase activity secreted by *Alternaria solani* and *Trichoderma harzianum* in both separate and in dual culture .

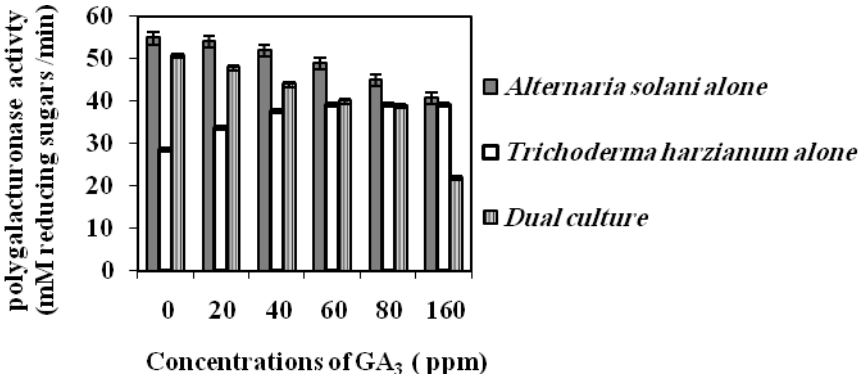

Fig. 2. Effect of GA_3 on polygalacturonase activity secreted by *Alternaria solani* and *Trichoderma harzianum* in both separate and in dual culture.

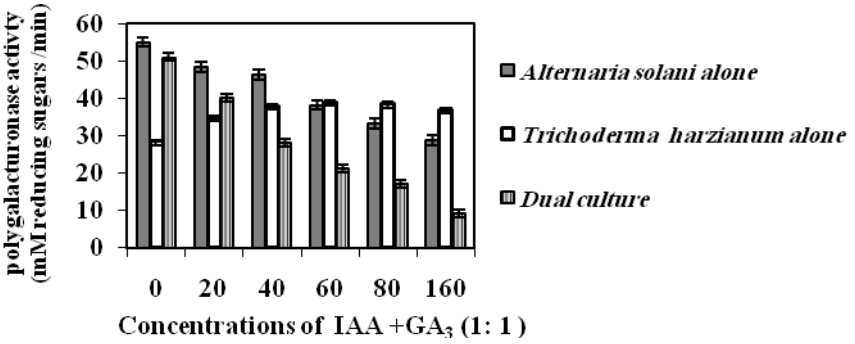

Fig. 3. Effect of mixture of IAA + GA_3 on polygalacturonase activity secreted by *Alternaria solani* and *Trichoderma harzianum* in both separate and in dual culture.

Endo-chitinase (endo-CH) and β-1, 3-Glucanase activity

The role of IAA and GA_3 on Endo-chitinase (endo-CH) and β-1,3- glucanase activity secreted from *T.harzianum* were represented in Fig. 4-9. The results show that the presence of 20,40,60, ppm of either IAA or GA_3 could affect the ability of *T. harzianum* to secrete endo-CH , however the secretion was reduced at 80 and 160 ppm. The results are also showed that the secretion is only increased in dual cultures while in separate cultures it still near to the control.This indicated that the two hormones separately or in mixture couldn't affect the secretion of enzymes directly but it act together with the phytopathogen. These results are in accordance of Haram *et al*. (1996). They suggested that the presence of the pathogen could serve as an additional inducer of this fungal cell wall degrading enzymes. Then, the presence of this pathogen would stimulate one of the mechanisms by *T. harzianum* for its biocontroller activity. The importance of Endo-chitinase (endo-CH) and β-1, 3-glucanase activity in the inhibitory activity is due to the degradation of cell walls of

the pathogens. They can hydrolyze cell walls and inhibits spore germination and germ tube elongation of various fungi (De la Cruz *et al*., 1992 and Lorito *et al*., 1994). The results indicated that the stimulation of the two enzymes in *T. harzianum* is not due to the direct effect of the growth regulators on *T. harzianum* itself but due to the stimulation of *A. solani* that stimulate the mycoparasitism of *T. harzianum* by the enhancement of its antagonistic enzyme secretions.

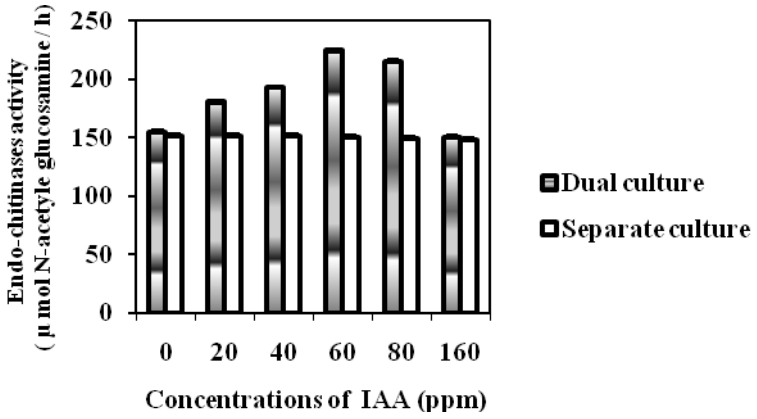

Fig. 4. Effect of different concentrations of IAA on Endo-chitinases activity of *Trichoderma harzianum* in separate and dual culture with *Alternaria solani*

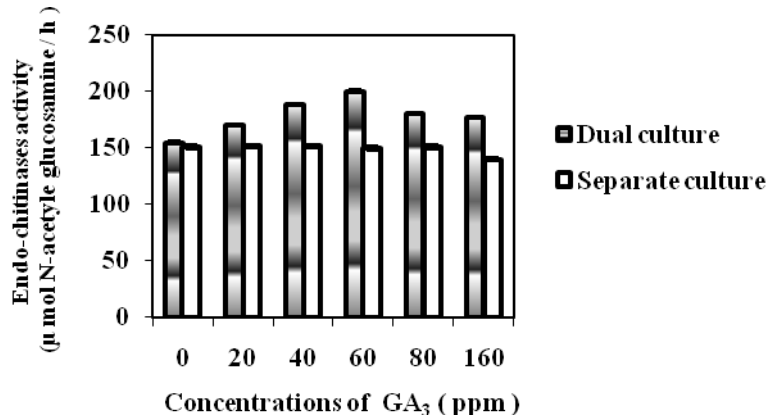

Fig. 5. Effect of different concentrations of GA_3 on Endo-chitinase activity of *Trichoderma harzianum* in separate and in dual culture with *Alternaria solani.*

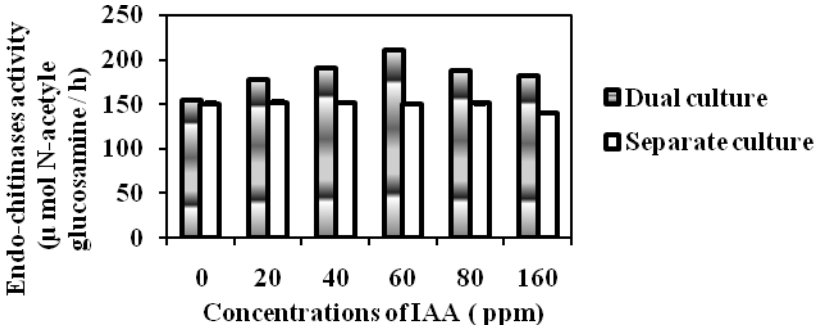

Fig. 6. Effect of IAA + GA_3 on Endo-chitinase activity secreted by *Trichoderma harzianum* in both separate and in dual culture with *Alternaria solani*.

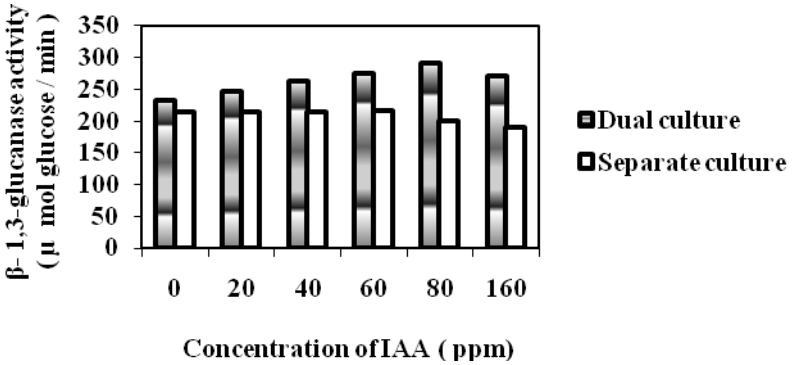

Fig. 7. Effect of IAA on β- 1,3 - glucanase activity of *Trichoderma harzianum* in separate culture and in dual culture with *Alternaria solani*.

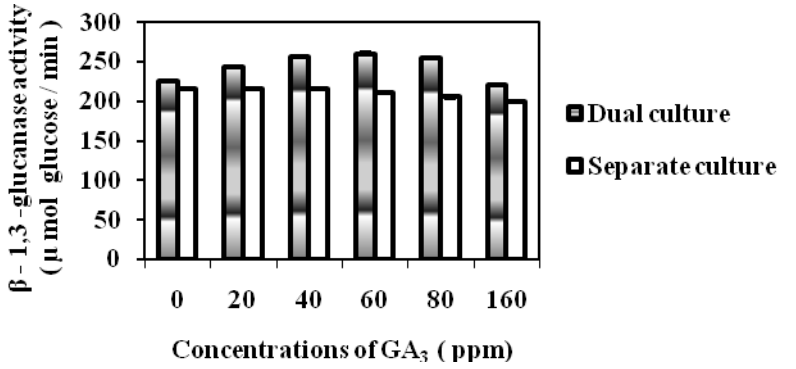

Fig. 8. Effect of GA3 on β- 1,3 - glucanase activity of *Trichoderma harzianum* in separate culture and dual culture with *Alternaria solani*.

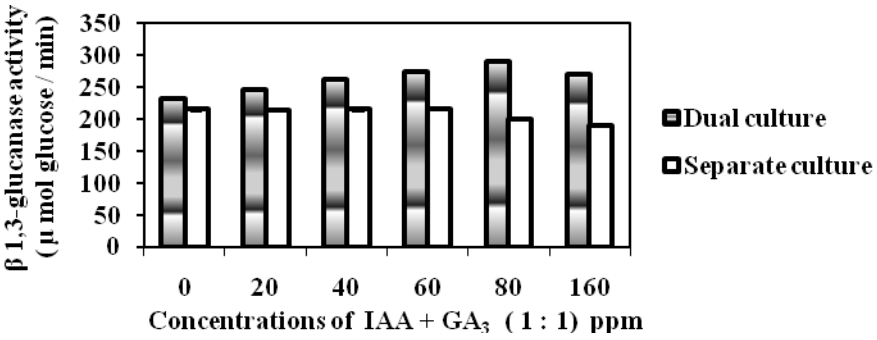

Fig. 9. Effect of IAA + GA3 on β- 1,3 - glucanase activity of *Trichoderma harzianum* in separate culture and in dual culture with *Alternaria solani*.

In vitro growth of B. cinerea and ethylene production

An increase of ethylene formation in pathogen-challenged plants has been related to defense responses leading to resistance as well as to symptoms development during pathogenesis. The role of ethylene in incompatible interactions is mostly contradictory and depends on the pathogen (Thomma *et al*., 1999). In addition, recent findings indicated the role of ethylene in systemic acquired resistance and in induced systemic resistance (Knoester *et al*., 1998). Literatures have been proved that some strains of *Botrytis cinerea* enable to produce ethylene gas to assess the pathogen in its attack the host. So, in the present study, the isolate of *Botrytis cinerea* isolated from infected tomatoes fruits was subjected to test its *in vitro* biosynthetic abilities to produce ethylene gas.The results (Fig. 10) show that the isolate of *Botrytis cinerea* enabled to produce ethylene in presence or absence of its precursor (methionine), although the production was more in presence of methionine. In this regard, Qadir *et al*. (1997) found that the maximum amount of ethylene was produced by grey mold isolated from tomatoes grown on PDA supplemented with 35 mM L-methionine after 7 days. In the same line, Chagué *et al*. (2002) stated that *Botrytis cinerea* during the first 48 h of culture the fungus uses methionine to produce ethylene *in vitro*.

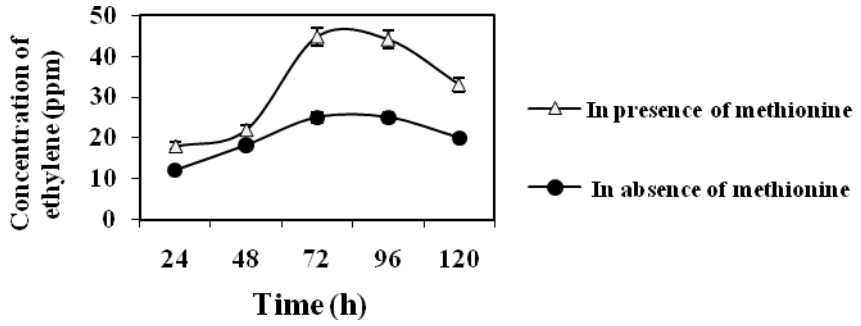

Fig. 10. Time course of ethylene production by *Botrytis cinerea* on PDA in the presence and absence of methionine for 120 hr at 24 hr intervals.

TABLE 3. Effect of Different concentrations of ethylene on the growth of *T. harzianum* and *Botrytis cinerea* in separate and in dual cultures after incubation at 28° C for one week.

Concentrations of ethylene (ppm)	Colony diameter (mm) of			
	***Trichoderma harzianum* in separate culture**	***Trichoderma harzianum* in dual culture**	***Botrytis cinerea* in separate culture**	***Botrytis cinerea* in dual culture**
0.0(control)	83.2±0.31	85.23±0.22	69.5±0.33	67.3±0.14
25	84.7**±0.21	94.11**±0.15	71.22**±0.15	69.25**±0.22
50	87.4**±0.22	96.4 **±0.18	74.33**±0.40	70.13**±0.19
75	89.2**±0.18	99.2**±0.11	75.2**±0.25	69.18**±0.14
100	83.1**±0.27	92.2**±0.20	70.11**±0.16	56.15**±0.13
200	83.6±0.10	87.2**±0.12**	68.22**±0.10	45.14**±0.50

** means highly significant L.S.D at 5 %.

Effect of ethylene on the biocontrol activity of Trichoderma harzianum against Botrytis cinerea

Limited studies have been focused concerning the role of ethylene on microorganisms but the exposure to exogenous ethylene has been demonstrated to escalate the biosynthesis of endogenous ethylene and to enhance the rate of membrane lipid breakdown (Kim & Wills, 1995) Mechanisms of *B. cinerea* suppression by different *Trichoderma* spp. are diverse (Rey *et al*., 2001 and Hanson & Howell, 2004). The results (Table 3) show that ethylene was a stimulator for both *T. harzianum* and *Botrytis cinerea* at the first three concentrations when they grown alone. On the other hand, in case of dual cultures the growth of *T. harzianum* was increased significantly while growth of *Botrytis cinerea* decreased significantly than that of separate culture. This may be due to the mycoparasitism of *T. harzianum* against *Botrytis cinerea* occurred following the suppression of ethylene production. This hypothesis was confirmed in results shown in Fig 11.The results showed that the biosynthesis of ethylene in dual culture was decreased than separate cultures which is consequently suppress the growth of *Botrytis cinerea* .In this regard, Elad & Eversen (1995) stated that grey mold development is associated with an increase in ethylene production from the infected tissues, which is most often attributed to the host plant. The level of ethylene production by *B. cinerea*-infected tomatoes was significantly higher than the level in mock-infected tomatoes and started to increase before there was visible decay (Polevaya *et al*., 2002). The results (Table 4) show similar results in spore germination and germ tube elongation for both pathogen and the biocontrol organism. In this manner, Díaz *et al*. (2002) found that exogenously applied ethylene was shown to stimulate gray mold, caused by *B. cinerea*, on tomato, pepper, cucumber, bean, rose, and carnation. All these data showed that ethylene is being stimulator for *Botrytis cinerea* when it exists alone in absence of the biocontrol organism. Moreover, the presence of

T. harzianum and *Botrytis cinerea* together suppress the production of ethylene and consequently, the growth of Botrytis *cinerea*. When ethylene was supplied exogenously it leads to the stimulation of mycoparasitism and inhibition of the pathogen.

TABLE 4. Percentage of *in vitro* spore germination and germ tube elongation of both *T. harzianum* and *Botrytis cinerea* in presence of different concentrations of ethylene gas.

Concentration of ethylene (ppm)	Percentage of spore germination of *Trichoderma harzianum*	Germ tube length of *Trichoderma harzianum* (µm)	Percentage of spore germination of *Botrytis cinerea*	Germ tube length of *Botrytis cinerea* (µm)
0.0(Control)	83.6 ±0.20	124.8 ±1.3	62.5 ±0.33	92.5±1.2
25	83.8 ±0.17	124.5 ±1.4	65.4**±0.99	99.1**±1.5
50	83.1 ±0.10	124.9** ±1.8	70.1** ±0.82	102.4**±2.1
75	83.1±0.25	120.1** ±1.1	84.2**±0.76	106.6**±2.2
100	83.5**±0.22	117.2** ±1.0	76.1±0.44	94.5**±1.3
200	83.7±0.15	111.3 **±1.7	60.3**±0.41	88.2**±0.9

** means highly significant L.S.D at 5 % .

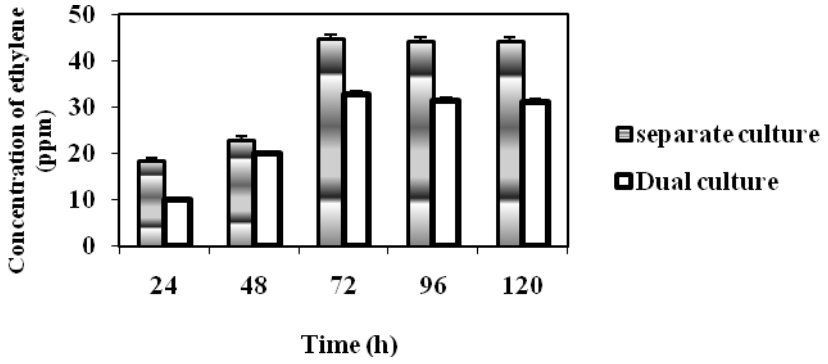

Fig. 11. Time course of ethylene production by *Botrytis cinerea* on PDA media in separate and in dual culture with *Trichoderma harzianum* for 120 h at 24 hr intervals.

Effect of ethylene on the enzymes activity for both T. harzianum and Botrytis cinerea

The results in Fig. 12 indicate that Endo-polygalacturonase enzyme was secreted from both organisms. The results were also showed that *Botrytis cinerea* was more active than *T. harzianum* when they grow separately; in addition to ethylene was stimulator for *Botrytis cinerea* only, however it couldn't affect *T.*

harzianum .Moreover, the total enzyme production was reduced in dual culture than each organism alone. The reduction of the production in such case is due to the mycoparasitism relationship. In this line, Sicilia *et al*. (2005) stated that during infection *with Botrytis cinerea*, it secretes several endopolygalacturonases (PGs) to degrade cell wall pectin, and is constitutively an important virulence factor. In the same regard, ethylene seems to be stimulator for the defense mechanism by enzymes from the hosts (Rakwal *et al*., 2004). In addition, virulence of the *B. cinerea* strain B05.10 on pear fruits was dependent on the polygalacturonase gene *Bcpg1*, but not on the pectin methyl esterase gene *Bcpme1*. (Akagi & Stotz, 2007).

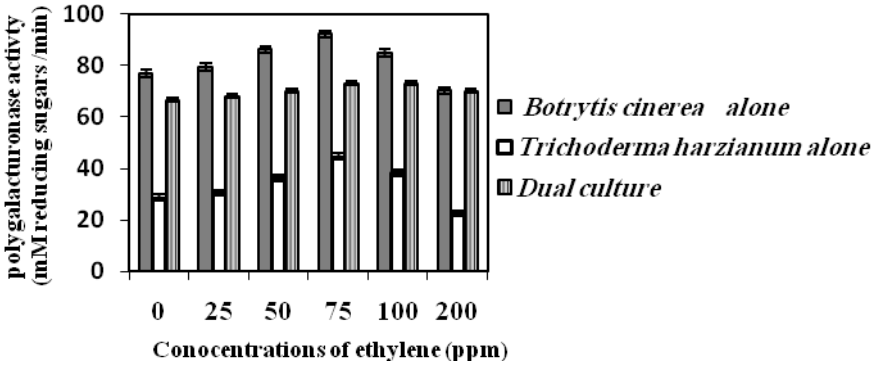

Fig. 12. Effect of different ethylene concentrations on Endo-polygalacturonase activity secreted by *Botrytis cinerea* and *Trichoderma harzianum* in both separate and in dual culture.

The results (Fig. 13) show that both Endo-chitinase and glucanases were stimulated significantly as the concentration of ethylene was increased than control where no ethylene was supplied. The results were also indicated that both enzymes were stimulated in presence of *Botrytis cinerea* than that of *T. harzianum* when cultivated alone. Therefore, the presence of the pathogen and ethylene could increase the mycoparastism by *T. harzianum* which reached maxima at 100 ppm of ethylene gas .In this respect, Some isolates interferes with *B. cinerea* enzymes (Elad & Stewart, 2004).The fact that ethylene is a known inducer for several pathogen defense-related enzymes, *e.g.* peroxidase, glucanase, and chitinase, lends support to the regulative role of ethylene in resistance responses (Ohme-Takagi *et al*., 2000). In this regard ,they can hydrolyze *in vitro Botrytis cinerea* cell walls and inhibits spore germination and germ tube elongation of various fungi (Lorito *et al*., 1994 and Schirmböck *et al*., 1994).

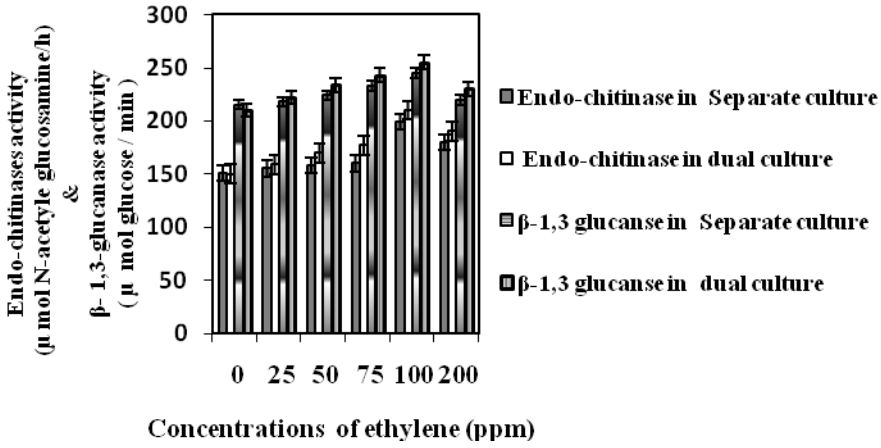

Fig. 13. Effect of different ethylene concentrations on Endo-chitinases and β-1,3 glucanases activity secreted by *Trichoderma harzianum* in both separate and in a dual culture .

Conclusion

The conclusion consists in that the presence of growth regulators affect the ability of *T. harzianum* Refai to control both *A. solani* and *Botrytis cinerea* isolated from infected tomato fruits .The stimulation of *T. harzianum* antagonisity by IAA or GA_3 alone or in combination occurred in the presence of the two organisms together although, it stimulates the phytopathogen activity. Not many studies are available regarding the role of ethylene in the establishment of mycoparasitism relationships. The results of this study indicated that ethylene gas could increase the phytopathogencity of *Botrytis cinerea in vitro* at low concentration while the increase in the concentrations inhibits its pathogencity in the presence of *T. harzianum*.

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تأثير حمض الجبريلك، اندول حمض الخليك وغاز الأثيلين معمليًا على النشاط الضد حيوي لفطرة *تريكوديرما هارزيانم* ضد فطرتي *الألترناريا سولاني* و*البوتريتس سينريا* المعزولتان من ثمار الطماطم

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تستخدم فطرة *تريكوديرما هارزيانم* على نطاق واسع فى المقاومة البيولوجية للأفات الميكروبية التي تصيب المحاصيل الزراعية. وتعتبر فطرتي *الألترناريا سولاني* و*البوتريتس سينريا* من الأفات الضارة التي تصيب نبات الطماطم. ولقد أثبتت الدراسات السابقة التوسع فى أستخدام منظمات النمو النباتية و خاصة فى الفترة الأخيرة للعديد من الأسباب والتى قد تؤثر على النشاط الفطري.

لهذا فقد استهدفت الدراسة الحالية التحقق من تأثير كل من حمض الجبريلك، اندول حمض الخليك وغاز الأثيلين معمليًا على النشاط الضد حيوي لفطرة *تريكوديرما هارزيانم* ضد فطرتي *الألترناريا سولاني* و*البوتريتس سينريا*. تم أولاً التحقق من النشاط الضد حيوي لفطرة *تريكوديرما هارزيانم رفاعي* و فطرة *الألترناريا سولاني* فى غياب حمض الجبريلك، اندول حمض الخليك و فى وجودها بتقنية المزرعة المزدوجة، بينما تم دراسة تأثير غاز الأثيلين على كل من فطرة *تريكوديرما هارزيانم رفاعي* و فطرة *البوتريتس سينريا*.

توصلت الدراسة إلى وجود علاقة معنوية بين منظمات النمو موضوع الدراسة وكل من القدرة الضد الحيوية *لفطرة تريكوديرما هارزيانم* ونموفطرتى *الألترناريا سولاني* و*البوتريتس سينريا*. تبين من الدراسة أن كل من أندول حمض الخليك وحمض الجبريلك زادا من نشاط فطرة *ألترناريا سولاني* خاصة عند التركيزات أقل من ٨٠ جزء فى المليون الا أن التنشيط قد ثبط فى وجود فطرة *تريكودرما هارزيانم رفاعي* . على الجانب الأخر نشط غاز الأثيلين فطرة *بوتريتس سينريا* سواء فى المزارع المزدوجة أو المنفردة مما أكد تأثير غاز الأثيلين على تثبيط القدرة الضد حيوية لفطرة *تريكودرما هارزيانم* ضد فطرة *بوتريتس سينريا*.

للتحقق من تأثير منظمات النمو على العلاقة بين فطرة *تريكودرما هارزيانم* والفطرتين موضوع الدراسة تم تتبع تأثر الأنزيمات ذات العلاقة بالقدرة المرضية للفطريات وهى انزيم بولى جالاكتويورينيز، و الجلوكاناز و الأندوشيتينيز. تبين أن الأنزيمات هى الأخرى قد نشطت فى المزارع المزدوجة و قد زاد نشاطها فى التركيزات أقل من ٤٠ جزء فى المليون بالنسبة لكل من أندول حمض الخليك وحمض الجبريلك و عند التركيزات أقل من ٧٥ جزء فى المليون بالنسبة لغاز الأثيلين.

استخلصت الدراسة أن تلوث التربة بكل من أندول حمض الخليك و حمض الجبريلك وغاز الأثيلين قد يؤثر على النشاط الطفيلي لفطرتي *ألترناريا سولاني* و*البوتريتس سينريا* اللتان تصيبان الطماطم و النشاط الضد حيوي لفطرة *تريكودرما هارزيانم*، ولهذا فالدراسة تحتاج الى تجارب حقلية أخرى.