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In Vitro Integration of *Trichoderma harzianum* with Chemical Pesticides Pertain to different Classes

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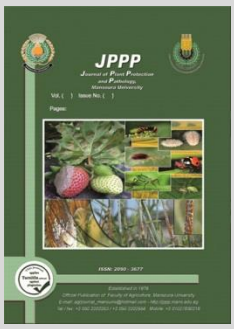

ABSTRACT

Mycoparasitic play a vital role in biological control and IPM. *Trichoderma harzianum* has the potential to control a large number of plant pathogenic fungi. Application *Trichoderma harzianum* in IPM require knowing the potential effect resulted from combination with chemical pesticides, so, the study test the mixabilities of common chemical pesticides with *T. harzianum* radial growth, sporulation and biomass production of the fungus at 0.1, 0.5 and 1 of recommended dose (RD) concentrations. Based on comparison between the tested insecticides on mycelial growth, *T. harzianum* showed profuse mycelial growth with etoxazole followed by teflubenzuron, while, profenofos caused complete inhibition. On the other hand, the tested fungicides showed that penconazole was the most toxic fungicide inhibited completely the mycelial growth with all tested concentrations, while, copper oxychloride+metalaxyl cause the same effect on the two higher concentrations. Finally, the tested herbicides showed that glyphosate isopropylammonium and bentazone inhibited completely the mycelial growth of *T. harzianum* at 1 RD, while, lower two concentrations caused a middle inhibition effect. The best sporulation results were obtained from etoxazole, bentazone, teflubenzuron and diniconazole, to surpass the control, while, the rest of the pesticides either significantly reduced or prevent sporulation completely. Based on mycelial dry weight, bentazone surpassed the control. The study recommends mixing *T. harzianum* with diniconazole, etoxazole, teflubenzuron and bentazone, respectively. Whereas, penconazole and profenofos are completely in compatible with *T. harzianum* but the rest of tested pesticides has antisporulant effect and mycelial growth inhibition was high and depend on exposure concentration.

Keywords: *Trichoderma harzianum*, compatibility, mycelial growth, sporulation, pesticides, insecticide, fungicides, herbicides.

INTRODUCTION

The pest control methods are considering indispensable processes in agricultural production due to multiple pests e.g. insects, mites, fungi and weeds which cause economic damage in field production quality as well as storage requires immediate and rapid intervention using chemical pesticides according to the damage caused. These pesticides may cause side effects on the environment and mammals due to inconsistent with sustainable environment management that enhanced by preserving the natural enemies of pests and application biocontrol agents to reduce pesticide consumption. Among the promising biocontrol agents, *Trichoderma* strains are commercially authorized by Egyptian Agricultural Pesticides Committee for controlling root-rot and late blight. *Trichoderma* strains control plant pathogenic fungi either indirectly, by nutrients and space competition, the environmental conditions modification, or promoting plant growth and plant defensive mechanisms and antibiosis, or directly, by mycoparasitism(Benítez *et al.*, 2005). *Trichoderma harzianum* registered and formulated as a commercial in Egyptian markets because of the ease of mass production (Lal *et al.*, 2014)and application methods besides the efficiency. Entering *T. harzianum* in the agricultural production system as biocontrol agent depend on chemical pesticides requires, more knowledge on compatibility to tank mix or successive application and potential side effects on *T. harzianum* survival and sporulation. Hence, the importance of this study is to determine the extent of compatibility or mixability in the tank or successive application or incompatibility in all cases between *T. harzianum* and tested chemical pesticides

MATERIALS AND METHODS

Trichoderma harzianum was isolated from commercial formulation obtained from Central Agricultural Pesticide Laboratory and market Plant Guard (*Trichoderma harzianum*, 30×10^6 cfu/ml) by serial dilution technique. Therefore, one milliliter of formulation was dissolved in 10 ml of sterile distilled water and mixed well to get 1:10 dilution. From this dilution, serial dilution was used to reach 1:1000 dilutions. One milliliter (10^3) was used to inoculate fresh PDA medium amended with streptomycin and incubated at 28±2°C for 7 days. After the incubation period, the growth of *T. harzianum* was observed and grown in pure cultures. Nine commercial pesticides which commonly used in Egypt, were used in this study, As shown in Table 1

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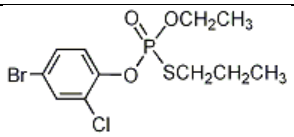
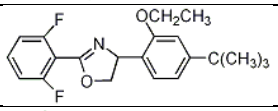
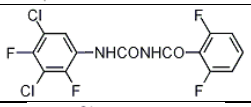
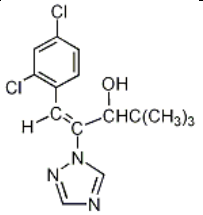
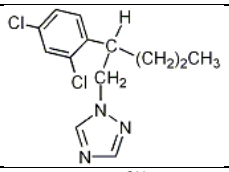
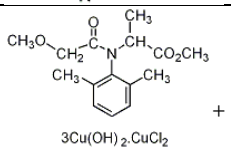
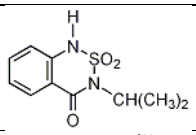
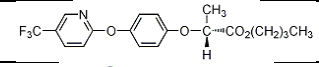
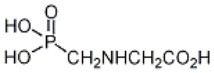
Table 1. Chemical pesticides used in the study.

Pesticide Class	Trade Name	Common Name	Chemical structure	Chemical Group	Application Rate
Insecticides	Selecron 72% EC	Profenofos		organophosphate	750 ml/100 L
	Baroque 10% SC	Etoxazole		mite growth inhibitor	25 ml/100 L
	Nomolt 15% SC	Teflubenzuron		benzoylurea	50 ml/100 L
Fungicides	Somi 8 5% EC	Diniconazole		DMI: triazole	35 ml/100 L
	Topas 10% EC	Penconazole		DMI: triazole	25 ml/100 L
	Cure-plus 50% WP	Copper Oxychloride+ Metalaxyl		phenylamide: acylalanine + multi-site: inorganic	150 g/100 L
Herbicides	Basagran 48% AS	Bentazone		benzothiadiazinone	375 ml/100 L
	Fusilade super 12.5 % EC	Fluazifop-P-Butyl		aryloxyphenoxypropionate	500 ml/100 L
	Hebrazed *48*% WSG	Glyphosate Isopropylammonium		glycine derivative	625 ml/100 L

Effect of tested chemical pesticides on radial growth and sporulation capacity of *Trichoderma harzianum*

Assessment of Mycelial Radial Growth

Different concentrations of chemical pesticides were tested 0.1, 0.5 and 1 of the recommended dose (RD) for each formulation to assess their direct inhibitory effect on tested *Trichoderma harzianum*. The inhibitory activity of the pesticides on mycelial radial growth of the bioagent was determined by growing *T. harzianum* on PDA medium containing different concentrations of the tested pesticides in Petri dishes (9 cm diameter). The pesticides were prepared directly from commercial formulation. A disc 4 mm diameters of 7 days old bioagent mycelial culture was aseptically transferred to the center of the Petri dish so that the mycelium face down on solidified PDA medium amended with different concentrations of pesticides in Petri dishes contacted with poisonous media. Pesticide free medium was used as control Petri dishes treatment. Inoculated Petri dishes were incubated at 27±2 °C with four daily observation. Mycelial growth of the bioagent was measured and the growth in PDA medium containing pesticide was compared with the control. Each treatment replicated four times. Percentage inhibition of radial growth (PIRG) was determined to estimate the bioagent growth inhibition by tested pesticide using the formula suggested by Vincent, (1947) and Pandey *et al.* (1982):

$$PIRG = \frac{Dc - Dt}{Dc} \times 100$$

Where:

PIRG: Percentage inhibition of radial growth

D_c: The average diameter of the fungal colony with control

D_t: The average diameter of the fungal colony in treatment

Assessment of sporulation capacity

Sporulation was assessed using amended growth media as described above. For tested bioagent in control, mycelial growth was permitted to extend to the edge of the control plate. Once sporulation was observed, plates were flooded with sterile distilled water (0.01% Tween 80) and spores were gently dislodged from the mycelium to release the conidiospores from mycelium using sterilized curved glass rode on the surface of media. The resulting spore suspension was filtered through two layers of cheesecloth

to get rid of mycelia fragments then the spore density of the suspension was determined using a hemacytometer (Mills *et al*., 2004). Sporulation density was reported as a percent value using the formula described above for mycelial growth measurements. The experiment was performed with three replicates per each treatment.

Effect of chemical pesticides on mycelial dry weight of *Trichoderma harzianum*

After studying the above-mentioned pesticides, most compatible one in each class were selected to know the relationship between the toxicity of etoxazole, diniconazole and bentazone on PD broth. Liquid medium samples (100 ml) of potato dextrose broth containing the following pesticides concentration 0.1 RD. Hundred ml of medium were poured into bottles and sterilized at 121°C for 15 min in autoclave. After autoclaving pesticides weights were added to the bottles containing media to reach previously the mentioned concentration. Each bottle was then inoculated with one agar mycelium disks (4 mm diameter) of 7 days old culture and incubated in the dark at 28°C for 14 days.

The mycelium was later harvested weekly through pre-weighed in Whatman No.1 filter paper and washed with several changes of double distilled water (20 ml). The filter paper, together with the washed mycelium, was dried at 60°C for 48 h. Dry weight of mycelium was recorded as grams. The experimental endpoint was after 28 days

RESULTS AND DISCUSSION

Data in Table (2) review the effects of some pesticides pertaining to different classes. With insecticide lass represented by profenofos (Selecron 72% EC), etoxazole (Baroque 10% SC) and teflubenzuron (Nomolt 15% SC). Each insecticide has a different chemical structure, target and mode of action. Profenofos showed complete mycelial growth inhibition for *T. harzianum* until the 3rd day after treatment with all tested concentration except lowest concentration (0.1 RD) started a slight growth to record 91.67% inhibition. In 4th day after treatment, 0.1 and 0.5 RD concentrations resulted in a slight mycelial growth recorded mycelial inhibition 91.09% and 92.25 %, respectively. On the other hand, 1 RD resulted in complete mycelial growth inhibition until the experimental endpoint. Etoxazole with 1 RD concentration caused complete mycelial inhibition in 1st day after treatment, while, lower concentrations (0.1 and 0.5 RD) showed a slight mycelial inhibition were 2.78 and 11.11 %. In the 2nd day showed a profuse mycelial growth recorded -25.59 and -23.24% inhibition, respectively in the 2nd day after exposure and the profuse mycelial growth continued to experimental endpoint with reduction recorded -4.64 % in both concentrations. While, 0.1 RD concentration caused a fluctuated response to record 69.38% after 4 days exposure. Teflubenzuron resulted in varied response during mycelial growth to start with a slight inhibition followed by increase mycelial growth rate to end with small inhibition with 0.1 and 0.5 RD concentrations. Although the absence growth in 1 RD concentration during 2 days at the beginning of exposure but all tested concentrations recorded percent inhibition ranged from 4.26 % to 12.79%.

Based on comparison between the tested insecticides on mycelial growth in Fig. 1, *T. harzianum* showed profuse mycelial growth with etoxazole followed by teflubenzuron that cause tolerance inhibition effect but, profenofos was very toxic to *T. harzianum* causing complete inhibition. Although the toxic effect of profenofos on mycelial growth depended on the concentration but its antisporulant effect was powerful independent on tested concentration to stop sporulation in nongrowing or growing treatments (0.1 and 0.5 RD). Using profenofos 500 g/L EC as a 59.29% reduction according to Thiruchchelvan *et al*. (2013). Sporulation capacity for *T. harzianum* after exposure to etoxazole raised with lowering concentration where 0.1 RD activate spore formation to increase with 68.53%, While, 1and 0.5 RD caused reduction of sporulation 75.06% and 44.11%, respectively. High sporulation capacity noticed with 1 RD concentration causing increasing 58.56% comparing control treatment, while, 0.5 and 1 RD concentrations reduced sporulation and recorded 24.16% and 59.33 %, respectively, as shown Fig. 4.

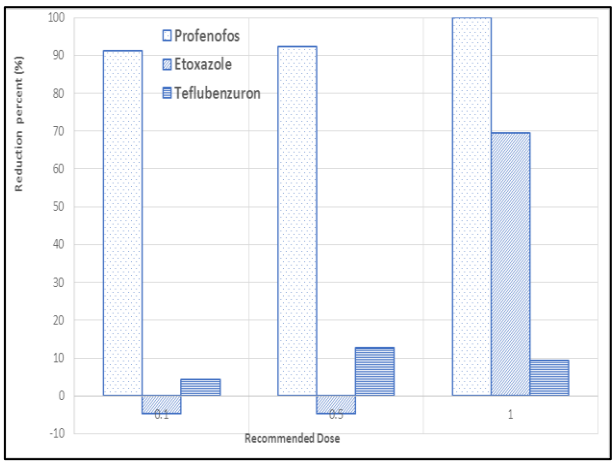


Figure 1. The inhibition effect of tested insecticides on *T. harzianum* mycelium.

With fungicide class represented by diniconazole (Somi 8 5% EC), penconazole (Topas 10% EC) and copper oxychloride+ metalaxyl (Cure-plus 50% WP). Each insecticide has a different chemical structure, target and mode of action as shown in Table (2).

Trichoderma harzianum exhibit fluctuated response after diniconazole exposure varied between inhibition and activation mycelial growth to end with equal mycelial growth activation with all tested concentrations. Increasing sporulation was exhibited with 25.54% in lowest concentrations, whereas, 0.5 and 1 RD concentrations caused a considerable reduction recorded 39.81% and 29.49%, respectively. Copper oxychloride + Metalaxyl mixture a complete caused inhibition for *T. harzianum* mycelial growth at 1RD concentration. while, 0.5 RD concentration cause the same effect until the 3rd day to grow in 4th day causing 88.95% inhibition, but, 0.1 RD start growing scarcely after incubation to result in inhibition 78.68% with equal sporulation capacity with control. In higher concentrations, 0.5 RD and 1 RD causing stopped mycelial growth in addition to spore formation as represented in Fig. 4.

Table 2. Effect of different concentrations of tested pesticides on growth and sporulation of *Trichoderma harzianum*.

Class	Pesticides	Application Rate	Colony Diameter (cm) and Inhibition (%) after Exposure period (days)				Sporulation density (spores/ml) *
			1	2	3	4	
Insecticides	Profenofos (Selecron 72% EC)	0.1	0 (100)	0 (100)	0.47±0.03 (91.67)	0.77±0.03 (91.09)	0 (100)
		0.5	0 (100)	0 (100)	0 (100)	0.67±0.07 (92.25)	0 (100)
		1	0 (100)	0 (100)	0 (100)	0 (100)	- (100)
	Etoxazole (Baroque 10% SC)	0.1	2.03±0.09 (-69.42)	3.57±0.22 (-25.59)	6.83±0.19 (-22.02)	9±0 (-4.65)	6.533×10^{6} (-68.53)
		0.5	1.33±0.17 (-10.83)	3.50±0.06 (-23.24)	6.40±0.21 (-14.29)	9±0 (-4.65)	2.167×10^{6} (44.11)
		1	0 (100)	1.20±0.26 (57.75)	1.60±0.06 (71.43)	2.63±0.15 (69.38)	9.667×10^{5} (75.06)
	Teflubenzuron (Nomolt 15% SC)	0.1	1.16±0.12 (3.33)	3.10±0.10 (-9.15)	5.77±0.15 (-2.98)	8.23±0.15 (4.26)	6.147×10^{6} (-58.56)
		0.5	1.07±0.12 (11.11)	2.90±0.06 (-2.11)	4.87±0.09 (13.10)	7.50±0.76 (12.79)	2.940×10^{6} (24.16)
		1	0 (100)	0 (100)	3.97±0.03 (29.17)	7.80±0.61 (9.30)	1.577×10^{6} (59.33)
Fungicides	Diniconazole (Somi 8 5% EC)	0.1	0.77±0.09 (36.11)	3.97±0.03 (-39.67)	7.00±0 (-25.00)	9.00±0 (-4.65)	4.867×10^{6} (-25.54)
		0.5	1.60±0.20 (-33.33)	2.77±0.17 (2.58)	2.77±0.17 (50.60)	9.00±0 (-4.65)	2.333×10^{6} (39.81)
		1	2.80±0.12 (-133.33)	3.95±0.05 (-39.08)	3.95±0.03 (29.46)	9.00±0 (-4.65)	2.733×10^{6} (29.49)
	Penconazole (Topas 10% EC)	0.1	0 (100)	0 (100)	0 (100)	0 (100)	- (100)
		0.5	0 (100)	0 (100)	0 (100)	0 (100)	- (100)
		1	0 (100)	0 (100)	0 (100)	0 (100)	- (100)
	Copper Oxychloride+ Metalaxyl (Cure-plus 50% WP)	0.1	0.47±0.07 (61.11)	0.57±0.12 (80.05)	0.97±0.17 (82.74)	1.83±0.23 (78.68)	3.877×10^{6} (0)
		0.5	0 (100)	0 ()100	0 (100)	0.95±0.03 (88.95)	0 (100)
		1	0 (100)	0 (100)	0 ()100	0 (100)	- (100)
Herbicides	Bentazone (Basagran 48% AS)	0.1	0.83±0.03 (30.56)	3.20±0.06 (-12.68)	5.43±0.03 (2.98)	7.53±0.13 (12.40)	6.247×10^{6} (-61.13)
		0.5	0.77±0.07 (36.11)	2.10±0.06 (26.06)	3.37±0.19 (39.88)	4.93±0.12 (42.64)	2.267×10^{6} (41.53)
		1	0 (100)	0 (100.00)	0 (100)	0 (100)	- (100)
	Fluazifop-P-Butyl (Fusilade super 12.5 % EC)	0.1	1.60±0.17 (-33.33)	3.17±0.32 (-11.50)	5.77±0.43 (-2.98)	8.10±0.35 (5.81)	0 (100)
		0.5	0.90±0.06 (25.00)	1.73±0.39 (38.97)	3.90±0.56 (30.36)	6.50±0.75 (24.42)	0 (100)
		1	0 (100)	0.50±0.06 (82.39)	2.47±0.03 (55.95)	4.9±0.12 (43.02)	0 (100)
	Glyphosate Isopropylammonium (Hebrazed 48% WSG)	0.1	0.97±0.07 (19.44)	2.50±0 (11.97)	3.60±0 (35.71)	4.57±0.03 (46.90)	0 (100)
		0.5	0.77±0.03 (36.11)	2.00±0.12 (29.58)	2.97±0.15 (47.02)	3.97±0.03 (53.88)	0 (100)
		1	0 (100)	0 (100)	0 (100)	0 (100)	- (100)
	Control	0	1.2±0.03 (0)	2.84±0.11 (0)	5.6± (0)	8.6±0.66 (0)	3.877×10^{6} (0)

Data expressed as mean ±SE; Values in brackets are percentage of mycelial inhibition; * enumeration of spores expressed as "0" mean that the fungus grow but no spores formed on colony; "-"mean that the fungus did not grow

Penconazole considered the most toxic fungicide inhibited completely the mycelial growth and subsequently spore formation with all tested concentrations. Penconazole was previously proved as highly toxic for mycelial growth of *T. harzianum* with ED_{50} value 11 µg ml^{-1}(Sushir, 2015). While, copper oxychloride was exhibited toxic effect to the growth of *T. harzianum* (Parab *et al*., 2009). The tested bioagent was more tolerant to copper compound than other species (Ali *et al*., 2012) while, metalaxyl recorded higher ED_{50} (1050 µg/ml) and ED_{90} (2392 micro g/ml) for radial growth(Sharma *et al*., 2001). On the other hand, diniconazole enhanced mycelial growth percent of *T. harzianum* as shown in in Fig. 2.

In the end, herbicides class represented by bentazone (Basagran 48% AS), fluazifop-P-butyl (Fusilade super 12.5 % EC) and glyphosate isopropylammonium (Hebrazed 48% WSG). Bentazone caused completely stopping of mycelial growth with 1 RD concentration during the experimental period, while, lower concentrations 0.1 RD and 0.5 RD reduced mycelial growth to 42.64 % and 12.40%, respectively at the end of experiment. On the other hand, the lowest concentration 0.1 RD activate sporulation to increase with 61.13% comparing with 0.5 RD concentration that reduced sporulation to 41.53%. Dwivedi and Vishunavat (2018) mentioned that *Trichoderma harzianum* fully compatible glyphosate. Fluazifop-P-Butyl caused a moderate reduction for mycelial growth ranged between 5.81 and 43.02% but the compound has a powerful antisporulant effect with all tested concentrations. On the other hand, glyphosate isopropylammonium causing completely stopped mycelial growth completely with 1 RD concentration, whereas, lower concentrations 0.1 RD and 0.5 RD concentrations caused reduction of mycelial 46.90% and 53.88%, respectively and stopped spore formation completely with all tested concentrations.

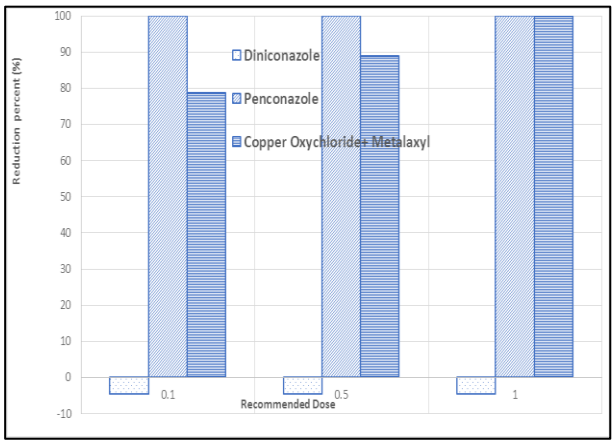


Figure 2. The inhibition effect of tested fungicides on *T. harzianum* mycelium.

Based on comparison between the tested herbicides on mycelial growth in Fig. 3, *T. harzianum* showed inhibition effect with glyphosate isopropylammonium followed by bentazone then fluazifop-P-butyl.

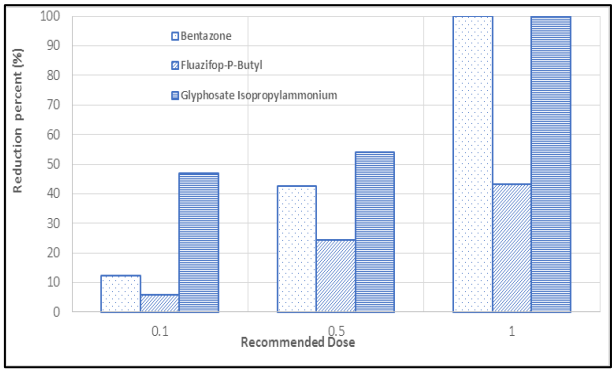


Figure 3. The inhibition effect of tested herbicides on *T. harzianum* mycelium.

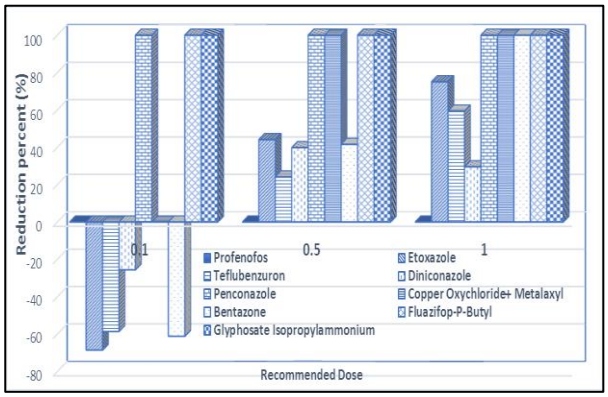


Figure 4. Effect of tested pesticides on *T. harzianum* sporulation.

Pesticides behavior (solubility) in aqueous media stimulating natural environment, so dry weight of mycelial mat of *Trichoderma harzianum* cultured on poisonous PD broth amended with 0.1 RD concentration was studied to permit growth and determined the ability *Trichoderma harzianum* to grow and utilized the pesticides by metabolization and mineralization.

All tested pesticides showed a vigor growth comparing control. The tested pesticides increased mycelial growth dry weight after 1st week then equaled approximately in 2nd weeks, with continuing incubation bentazone showed a vigor biomass dry weight followed by diniconazole then etoxazole with a big difference in the 3rd weeks. After 4 weeks incubation bentazone recorded the highest mycelial dry weight with a big difference comparing to other treatments as shown in Fig. 5.

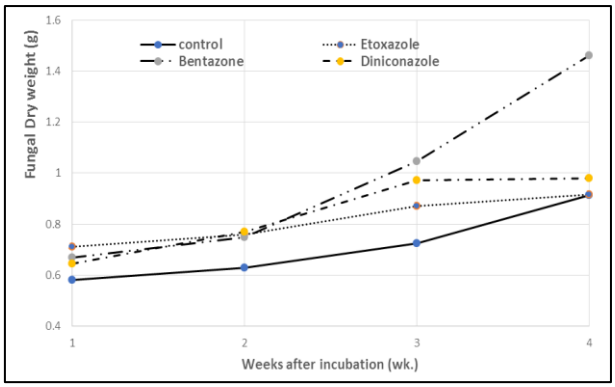


Figure 5. Biomass of *T. harzianum* in presence of chemical pesticides.

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تكامل فطر الترايكودرما هارزيانم معملياً مع مبيدات آفات كيماوية منتمية لفئات مختلفة

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تلعب الطفيليات الفطرية دوراً حيوياً في المكافحة البيولوجية والإدارة المتكاملة للآفات، ومن بين هذه الكائنات الحيوية فطر الترايكودرما هارزيانم المستخدم في مكافحة عدد كبير من الأمراض الفطرية الممرضة للنبات. إشراك فطر الترايكودرما هارزيانم في المكافحة المتكاملة للآفات يتطلب معرفة التأثير المحتمل الناجم عن خلطه مع المبيدات الكيميائية، لذلك تهدف الدراسة لاختبار توافق فطر الترايكودرما هارزيانم مع المبيدات الكيميائية الشائعة وتأثيرها على النمو الميسليومي والتجرثم وأيضاً الوزن الجاف للفطر عند تركيزات ٠.١ و٠.٥ و١ من الجرعة الموصي بها حقليا للمبيدات تحت الدراسة. بناء على المقارنة بين المبيدات الحشرية المختبرة على النمو الميسليومي، أظهر فطر الترايكودرما هارزيانم نمواً بصورة أفضل مع إيتوكسازول يليه بتفلوبنزورون والذي سبب تثبيطًا محدودًا، لكن مبيد البروفينوفوس كان سامًا جدًا لفطر الترايكودرما هارزيانم مسببا التثبيط التام للنمو الميسليومي. من ناحية أخرى عند مقارنة المبيدات الفطرية المختبرة، نجد البنكونازول أكثرها سمية مؤديا للتوقف التام للنمو وبالتالي منع تكون جراثيم مع جميع التركيزات المختبرة، أما أوكسي كلوريد النحاس+ ميتالاكسيل فسبب نفس التأثير عند استخدام التركيزات العالية مع محدودية النمو الفطري مع التركيز الأقل. وأخيرًا، أظهرت المقارنة بين مبيدات الحشائش المختبرة على نمو فطر الترايكودرما هارزيانم التوقف الكامل للنمو الميسليومي مع مبيدات الجليفوسات أيزوبروبيل أمونيوم والبنتازون عند التركيز الموصي به حقليا، بينما تسببت التركيزات المنخفضة في تثبيط النمو الميسليومي بدرجة متوسطة. تم الحصول علي أفضل توافق للتجرثم عند المعاملة بمبيدات إيتوكسازول وبنتازون وتيفلوبنزورون ودينيكونازول على التوالي، أما باقي المبيدات فإما خفضت التجرثم بدرجة كبيرة أو منعته كليا. وجد أن أفضل توافق بناءً على الوزن الجاف للميسليوم عند تركيز ٠.١ من المعدل الموصي به حقلياً، تنتج عن البنتازون يليها دينيكونازول وأخيراً الإيتوكسازول والكنترول على السواء مما يشير لاحتمالية مقدرة الفطر على تحطيم المبيدات والاستفادة منها شرط التعرض بتركيزات غير مثبطة كليا. لذا توصي الدراسة بخلط فطر الترايكودرما هارزيانم مع دينيكونازول وإيتوكسازول وتفلوبنزورون وبنتازون على التوالي. أما بينكونازول والبروفينوفوس فينصح بعدم الخلط أو التطبيق منفرداً الرش بهذة المبيدات، أما بقية مبيدات الآفات المختبرة لها تأثير مضاد للتجرثم وتثبيط النمو الفطري لها كان مرتفع ويعتمد على التركيز المتعرض له.