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Inhibition Activity of Mycorrhizal Fungi *Glomus mosseae* and *G. intradicas* with *Trichderma harizanum* Against *Rhizoctonia solani* in Okra Plant *Abelmoschus esculentus* (L.)

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Abstract: The agricultural production processes currently targeted reducing chemical fungicides usage and increasing bio-agent application through controlling diseases alone or integrating it with other factors. The study aimed to investigate the induction of systemic resistance by multi bio-agents represented by mycorrhizal fungi *Glomus mosseae*, *G. intradicas* and *Trichoderma harizanum* against pathogenic fungus *Rhizoctonia solani* which caused wilt disease and growth defoliation to Okra seedling. Three isolate of *R. solani* were recorded on root of Okra seedling, named (local - Batra). Isolate no. (3) was more virulence than other isolates in damping off disease in the pre and post emergence. Results also showed that *G. mosseae* and *G. intradicas* with *T. harizanum* had a positive influence in reducing detrimental effect of *R. solani* in all growth parameters (e.g. fresh and dry weight of root) on disease severity on Okra plant caused by *R. solani*. Bio-agents (*G. mosseae*,*G. intradicas* and *T. harizanum*) increased resistance in Okra plants by raising production of enzymescatalase and Peroxidase.this experiment was revealed that using a complex of bio-agent's factors were greatly increase the efficiency of biological control than using each of them individually. We conclude that the broad diversity of rhizosphere micro-organisms as well as the confronting between the bio-chemical and physical changes could be reflected the variations in the metabolic secondary products that could inhibit pathogens.

Keywords: Mycorrhiza, Peroxidase, *Trichoderma,* Xylanase.

Introduction

Crops protection from diseases still depending on the chemical control, that could causes a serious effects on both environment and human health compared to other agricultural methods (Aly *et al*., 2007). Currently, the main challenge is to minimize chemical pesticide usage by replacing them with safe the techniques based on Biocontrol Agents (BCAs) and bio-elicitors compound which led to induced systemic resistance

(ISR) in plants (Aly *et al*., 2007; Takur, & Sohal, 2013).

Enzymes can be described as one of bio-elicitors compound can be induced systemic resistance (ISR) and could be the most important plant's defense means, include polyphenoloxidase, peroxidase, chitinase and phenyl alanine ammoniase. ISR led to the accumulation of callus, phenols, lignin and systemic mechanisms in plant after infection or during treatment by elicitors (Sirin, 2011).

R. solani is one of the important pathogens to plants, it's characterized by producing several enzymes and toxins and play a role in the pathogenesis susceptibility and responsible for the appearance of symptoms which related to pathogen. Dawar *et al*. (2008) was found that there is a group of enzymes responsible for dismantling and degradation of cell walls such as pectinase and pectinmethylhydrase which related to *R. solani* in producing toxic substances (toxins),these substances have phenolic properties such phenylacetic acid.

Trichoderma sp. succeed as bio-pesticide because it's rapid growth, high ability to be productive, controlling plant diseases and ability to compete with other microorganisms in rhizosphere and/or resistance to fungicides. Moreover, it has the ability to live and grow under harsh conditions and has good efficiency in exploitation soil nutrients. It is highly virulent to parasitic pathogen, significantly improving plant growth (Vinale *et al*., 2006) and has ability to inducting systemic resistance (Harman, 2006).

Duffy *et al*. (1996) reported that most biological control agents are used as individual environmental agents and as biologically for one. This may in part be caused in the contradictory response to the formulation of biocidesis individual bioreactor may not be effective in soil environment conditions. This vital agent will be used against different types of pathogens that attack the plant, as well as a combination of biotic factors that mostly mimic the actual reality of the soil environment. This may expand the scope of biological enemies' activity against a wide range of pathogens as well as increase the efficiency of biological control.

Using mycorrhizal fungi to protect plant from fungal infection and increase plant tolerance to ecological stress conditions (Finlay, 2008), and improve plant growth by increasing the availability of nutritional elements as a nitrogen, phosphorus and potassium. In addition to that increase plant chlorophyll content (Sheriff, 2012).

Okra (*Hibscus esculentus* L.) is one of important vegetable crop in Iraq. It has a high nutritional value due to the availability of some elements such as calcium, magnesium, phosphorus and many vitamins. Also, its flowers were used for medicinal purposes (Hafez, 1992). As well as the importance of this crop, the planted areas have increased significantly in Iraq. This increment was accompanied by distribution of fungal infection especially damping off and root disease (Fakir, 2000).

The study was aimed to induce systemic resistance using multi bio-agents. Mycorrhizal fungi *Glomus mosseae* and *G. intradicas* with *T. harizanum* against the causal agent of damping off and root rot disease (*R. solani*) on growth parameters of okra plant.

Material and Methods

Isolation and diagnosis of *Rhizoctonia solani*

Okra seedlings infected with damping-off were collected from different fields in Basrah Province and transfer to the laboratory of Plant Pathology, College of Agriculture, Basrah University. Infected seedling washed with water, cut into small pieces (0.5-1cm). Sterilized with 10% of NaOCl for 3-5 min, then washed with sterilized distilled water, dried on filter paper and planted in a Petri dish diameter 12 mm containing sterilized P.D.A. 250 mg /L of Chloramphenicol was added to the growth medium, then medium was cooled to 40° C and poured into the plates according to the taxonomic characteristics mentioned by Barnett and Hunter (2006). The isolates were activated and inoculated in slant contain solid media

and stored in the refrigerator at 4°C for subsequent experiments.

Pathogenicity of *R. solani* on okra plants:

Plastic pots with of 5 kg capacity were used in this experiment containing soil and peat moss mixed in 1: 2 ratio that were sterilized by formalin/water 1:50 (Tawajin, 1979). The pots were inoculated with millet seed fully colonized with *R. solani* 0.5% weight/weight. Control treatment contains sterilized millet seeds only. Each Pot was planted after three days with five seed of okra (Local variety), irrigated whenever needed. The percentage of seedling death (damping off) before emergence and after three weeks of germination was calculated according to the following equation:

% seeding death= (of dead seedlings/of germinated seedlings) *100

Antagonistic ability of *T. harizanum* against *R. solani:*

In order to identify the antagonistic ability of bio-agent fungus *T. harizanum*, against isolate of *R. solani*, the Dual-culture technique were used in a sterile Petri dishes with 9 cm diameter contains PDA media. Plates of PDA were inoculated with a 5 mm disc from five-day-old cultures of the *R. solani* 10 mm from the edge of the plate, a 5 mm disc of the *T. harzianum* was placed 55 mm from the *R. solani* disc. Paired cultures were incubated in the incubator at 28±2°C for five days. The growth of the fungus was recorded by measuring the radial growth of the *R. solani*. The degree of antagonism for each isolates measuring according to Bell *et al.* (1982), as follows:

Degree of growth (bio-agent fungi)

(1) The bio agent fungi fill entire the Petri dish

(2) The bio agent fungi fill two-thirds Petri dish

(3) The bio agent and the pathogenic fungus both fill half of the Petri dish

(4) Pathogenic fungus fills two-thirds of the Petri dish

(5) Pathogenic fungus fills entire the Petri dish

Bio-effect of *Glomus mosseae* , *G. intradicas* and *T. harizanum* on *R. solani* in plastic pots *:*

Plastic pots capacity 5 kg used in this experiment were filled with a mixture of soil and peat-moss in 1:2 ratio and commercialized as in the previous paragraph (3-7) in a 5 kg plastic bag. The soil was contaminated with *S. solani*, loaded with millet seeds, then water-fed. Three days later, the soil was contaminated with fungus (1) *G. mosseae* and *G. intradicas*, which were obtained from (fungus + rhizosphere + rhizosphere), were added by 30 g of each seed from the fungus *G. mosseae* and G. intradicas. The Agriculture Research Centre affiliated to the Ministry of Science and Technology is a comparative treatment of soil Contaminated with mushrooms *R. solani* and other non-polluted only sterile millet seeds, and then planted with the seeds of the plant of the amphibian petrified and watered as needed to become transactions as follows.

The germination percentage of okra seed was calculated after 10 days of planting and after three weeks later the severity of an injury and fresh and dry weight of roots was calculated in addition to the percentage of a severity of classified following Wheeler (1970) scale which consists of four grades as follows:

(0) plant life health

(1) The seedling life but its root infected with rot

(2) The seedling death before emergence

(3) The seedling death after emergence

the percentage of a severity of injury was calculated according to the following equation:

$$\% \text{ severity of injury} = \frac{\text{total \# of seedling} \times \text{degree}}{\text{total \# of seedlings} \times \text{higher degree}})$$

Effect of *R. solani* on enzymatic activity in root of okra plant treated with *G. mosseae*, *G. intradicas* and *T. harizanum*:

Catalase and Peroxidase activity

Roots of okra plants planted in treatments were collected and placed in Polyethylene bags and placed in cooling box. Then they transported to the laboratory. 300 mg fresh weight of roots was taken and washed with distilled water free of ions and added to it 6 ml of 0.05 M potassium phosphate buffer solution (K_2PO_4 31g,K_2HPO_4 0.006, EDTA 0.1g, poly vinyl pyrolidon (pvp) 5g, ascorbic acid 0.2g, and adjust pH to 6). After that, centrifuged at 12000r/min for 20 min for catalase activity the effectiveness of enzymatic in UV spectrophotometer was estimated at 240 nm (Aebi, 1984).

For peroxidase apply to the potassium phosphate buffer solution 250 µl from both 0.5% of Gauiacol pigment and hydrogen peroxide 0.3% v/v. Finally, the effectiveness of enzymatic in UV spectrophotometer was estimated at 470 nm (Kim et al., 1988).

The enzymatic activity of both enzymes estimated using the following equation:

$$\text{Enzymatic activity} = \frac{\text{Device Reading}}{\frac{\text{Example weight}}{\text{Extraction size}} \times \text{solution reading Size}}$$

Statistical analysis:

The study results analyzed using Complete Random Design (CRD) and Least Significant Difference (L.S.D) (Al-Rawei and Khalaf Allah, 1980). GenStat statistical software and Microsoft Excel were used to analyze the data and the means ($P< 0.05$) were compared between treatments of plants.

Results and Discussion

Identification and pathogenicity of *R. solani*

The results revealed the presence of three isolates of *R. solani* in roots of okra seedlings (Fig. 1). Based on pathogenicity most virulence isolate was isolate no. (3) (Table 1). The results showed that no. (3) isolate of *R. solani* was observed to be more pathogenic because it reached 31.1 % of Okra seedling death pre-emergence percentage while isolate no (1) and (2) reached 22.4 % and 17.9% respectively. In a post-emergence the percentage of seedling death was observed to be high in isolate no. (3) reached 60.0% more than isolate no (1) and (2) reached 53.0% and 46.6% respectively.

The pathogen factor is depending on variation of *R. solani* isolates and its ability to infection Okra seedling beside the subsequent disease development. These results were significant and consistent in repeated experiments. One possible reason for this is ability to produce a lot of toxins and enzymes that effect seeds germination because *R. solani* is one of major fungi that causing rotting seeds (Dewan *et al*., 1985). Based on a previous results, isolate (3) of *R. solani* were selected to complete subsequent studies.

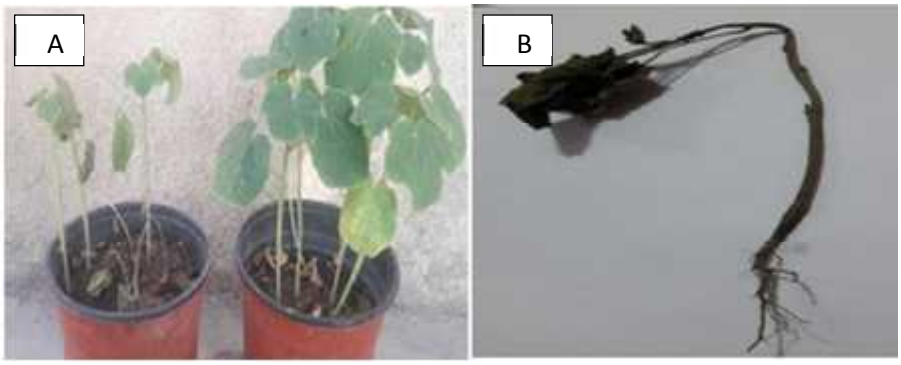

Fig. (1): Pathogenic effect of *R. solani* on okra plants in plastic pots,

A: Pathology B: Dead seedling.

Table (1): Pathogenicity of *R. solani* isolates on Okra seedling in plastic pots.

R. solani Isolates	% seedling damping off	
	pre- emergence	post- emergence
Isolate-1	22.4	53.0
Isolate- 2	17.9	46.6
Isolate-3	31.1	60.0
Control	0	0
L.S.D 0.05	**11.3**	**6.87**

Antagonistic ability of *T. harizanum* against *R. solani*

Antagonist activity (Dual culture assays) provided evidence that *T. harzianum* substantially reduced the growth of the pathogens *R. solani* (Fig. 2). *T. harzianum* were able to inhibit the growth of three isolates of pathogens *R. solani* and gave scale no. (1) of degree of antagonism according to the scale mentioned by Bell *et al*. (1982) (Table 2). The bio agent *T. harzianum* over grew the host resulting into complete degradation of the latter and sporulation of the former over the entire plate (Shafique *et al*., 2015).

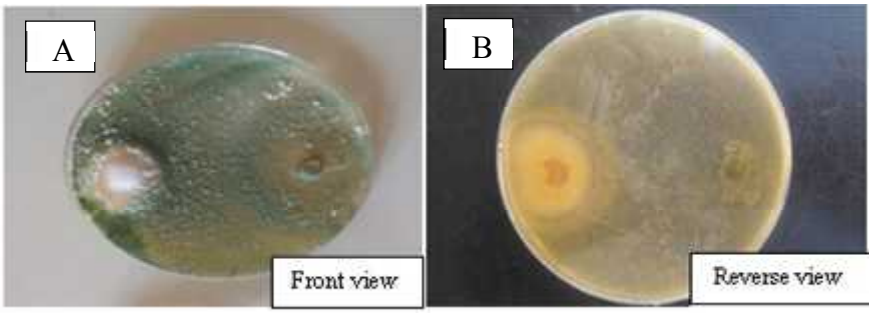

Fig. (2): Antagonistic activity of *T. harizanum* against *R. solani* grown on PDA after five days of inoculation at 28±2°C, A: Front view, B: Reverse view.

Table (2): Antagonistic activity of bio-agent *T. harizanum* against isolates of *R. solani*

Bio-agent fungi	*T. harizanum*
R. solani isolates	Antagonism scale
Isolate-1	1
Isolate- 2	1
Isolate-3	1

Okra seeds could be penetrated by *T. harzianum* and induced systemic resistance (Howell *et al.,* 2000). The bio-pesticide has a significant role in inhibiting pathogenic fungal growth. These results are consistent with many researchers such as Vinale *et al.* (2006), they mentioned that species of *Trichoderma* produce many antibiotics and these will be synergistic when they associate with cell wall analytic enzymes. So, this would work as an "inhabitation" effect for many pathogens.

ISR by *Glomus mosseae, G. intradicas* and *T. harizanum* on Okra plants infected by *R. solani* in plastic pots:

The results of this experiment showed that the fungus had a significant biological effect against *R. solani* isolate (3). The results proved the efficiency of the fungus of *G. mosseae*, *G. intradicas* and *T. harizanum* in reducing an effect of *R. solani* isolate (3) in growth parameters (Table 3) in growth parameters. The treatment *G. mosseae* + *R. solani* were characterized by highest percentage of seedling death 24.1%, while the lowest percentage with 11.9% recorded in *T. harizanum* + *R. solani*. The interaction between fungi and the isolate of pathogen was clearly varied in their effect in the percentage of seedling death. However, all the interactions were less effective compared with control treatment (21.7%). The fresh weight of seedling roots of Okra plant, revealed that *R. solani* + *G. intradicas*, *G. mosseae* + *G. intradicas* + *R. solani* and *G. mosseae* + *T. harizanum* + *G. intradicas* + *R. solani* reached the highest average 14.66g, 13.61g and 12.29 g respectively, comparing to the control treatment (10.33 g). The dry weight of seedling roots showed a significant increase in dry weight of *G.mosseae* + *T.harizanum*+*R. solani* reached 1.99 g, but exhibited lowest dry weight of the roots of *T. harizanum* + *G. intradicas* + *R. solani* reached 0.98 g comparing to control treatments; pathogenic fungi and millet seeds, where dry weight of seedling roots were 1.44 g and 2.17 g respectively.

The results showed a significant bio-effect on the percentage of infection severity of Okra plants infected by *R. solani* beside interaction with other fungi which used in this study (Table 3). Disease severity was decreased for treatments *T. harizanum*+ *G. intradicas* + *R. solani*, *T. harizanum* + *R. solani* and *G. mosseae* + *T. harizanum* + *R. solani* reached 31.25, 32.50 and 38.30%, respectively comparing to the control treatment with an *R. solani* reached 55.00 %. but they were generally less than the control.

The bio-agents interaction effects was increased against the pathogenic fungus due to its compatibility, in addition to, the toxic and enzymatic capacity which enhance the ability of plant resistance against pathogen beside, increment phytochemicals production which is toxic to plant pathogens (Eman *et al*., 2012).

Mixture of compatible bio-agents inhibited the growth of the target organisms through its ability to grow much faster than the pathogenic fungi thus competing efficiently for space and nutrients. Starvation is the most common cause of death for microorganisms, so that competition for limiting nutrients results in biological control of fungal phytopathogens (Siameto *et al*., 2010). Thus, high inhibitory effect would happen against a wide range of plant pathogens (Vinale *et al*., 2006). This also reported by El-Fiki *et al.* (2004) when the soil of sesame plant by *T. harzianum* and *Glomus* sp. used alone or together were treated by *Macrophomina phaseolina* which leads to increased host defences by increasing the oxidation of enzymes and increases the total phenol in the plant. Metcalf and Wilson (2001) and Sharon *et al.* (2001) demonstrated possible role of chitinolytic and/or glucanases enzymes in bio-control by *Trichoderma* , these enzymes function by breaking down the polysaccharides, chitin, and glucans that are responsible for the rigidity of fungal cell walls, thereby destroying cell wall integrity limiting the growth of the pathogen.

Table (3): Biological effect of *Glomus mosseae*, *G. intradicas* and *T. harizanum* on Okra seedling plants infected by *R. solani* in plastic pots.

Treatments	% Seedling death	Fresh weigh of roots (gm)	Dry weigh of roots (gm)	% Infection severity
G.mosseae+R. solani	24.1	9.54	1.08	42.50
T.harizanum+R. solani	11.9	11.78	1.62	32.50
R. Solani + G. intradicas	19.8	14.66	1.67	40.0
G.mosseae+T.harizanum+R. solani	14.5	11.05	1.99	38.30
G.mosseae+G. intradicas+R. solani	17.5	13.61	1.15	43.75
T.harizanum+G. intradicas+R. solani	16.8	10.93	0.98	31.25
G.mosseae+T.harizanum+G. intradicas+R. solani	13.5	12.29	1.59	40.00
(Control-1) *R. solani* only	21.7	10.33	1.44	55.00
(Control-2) millet seeds only	0	14.01	2.17	0
L.S.D (0.05)	**5.91**	**3.11**	**0.37**	**9.42**

Mixture of compatible bio-agents inhibited the growth of the target organisms through its ability to grow much faster than the pathogenic fungi thus competing efficiently for space and nutrients. Starvation is the most common cause of death for microorganisms, so that competition for limiting nutrients results in biological control of fungal phytopathogens (Siameto *et al*., 2010). Thus, high inhibitory effect would happen against a wide range of plant pathogens (Vinale *et al*., 2006). This also reported by El-Fiki *et al.* (2004) when the soil of sesame plant by *T. harzianum* and *Glomus* sp. used alone or together were treated by *Macrophomina phaseolina* which leads to increased host defences by increasing the oxidation of enzymes and increases the total phenol in the plant. Metcalf and Wilson (2001) and Sharon *et al.* (2001) demonstrated possible role of chitinolytic and/or glucanases enzymes in bio-control by *Trichoderma* , these enzymes function by breaking down the polysaccharides, chitin, and glucans that are responsible for the rigidity of fungal cell walls, thereby destroying cell wall integrity limiting the growth of the pathogen.

The positive roles of the bio-agent fungi can be summarizing in promoting roots growth, increasing nutrients availability in addition to encouraging growth which indicated by increasing both fresh and dry weight (Howell *et al.*, 2000; Alwan, 2005; Al-Obaidi, 2007). Vinale*et al.* (2006) reported that *Trichoderma* produces many antibiotics; include Gliotoxin, Alamethicins, Viridol, Trichoviridine and Harzianic acid; these antibiotics are synergistic when they were associated with different cell wall enzymes. The bio-agent produce analytic enzymes that could have the ability to inhabit many pathogens and analyze them would be considered an important tool for effective control of plant diseases (Viterbo*et al.*, 2007).

The enzymatic activity of Okra plant treated by *G. mosseae, G. intradicas, T. harizanum* and infected by *R. Solani*

Enzymatic activity of catalase and peroxidase showed ability of *G. mosseae*, *G. intradicas* and *T.harizanum* to induce resistance in Okra plants (table-4). The enzymatic activity of catalase was significant by *G.mosseae + G. intradicas + R. solani*; *T. harizanum+ G. intradicas + R. solani* and *G .mosseae + R. solani* treatment, reached 3.129, 2.655 and 2.430 unit/gm, respectively compared with control treatment reached 0.896 unit/gm fresh weight. For Peroxidase activity, the best enzymatic activity reached 4.760, 4.654 and 4.541 unit/gm fresh weight in treatments *G. mosseae + G. intradicas + R. solani*; *G. mosseae + R. solani* and *R. solani + G.*

intradicas, respectively, compared with control treatment.

The interaction between bio-agents led to significant production of catalase and peroxidase enzymes; this would indicate an increasing effectiveness and inhibition against pathogenic fungi. Sirin (2011) reported that treating sunflower plants with *Glomus* sp. and *T. harzianum* fungi were leaded to increase the effectiveness of enzymes in defending against pathogens, and they found that inoculating plants with these bio-agents leaded to an increase in effectiveness of peroxidase and increase resistance against *R. solani*. Peroxidase associated in a production of reactive oxygen will be toxic to the pathogens directly or indirectly, have its role in reducing the spread of pathogen through increased lignin in the cell wall (Hammond-Kosack and Jones, 1996), and A mixture of several enzymes might be necessary for efficient cell wall lysis (Siameto *et al*., 2010).

The catalase is considered as one of defense enzymes which produced by the plant because of exposure to the invasion of pathogens. This would also cause degradation to the cell walls of fungus and may enhance the antagonist activity of *T. harzianum* (El-Katatny *et al*., 2000).

*Trichoderm*a sp. play an important role in inducing mechanism of plant defenses. Jayalakshmi *et al*. (2009) studied the metabolisms toxic substances, volatile or non-volatile which produced by *Trichoderm*a and that could have inhibited the settlement of micro-organisms leading to a synthesis of phytoalexins and proteins and other compounds in the plant against plant pathogens. Gailite *et al.* (2005) found that treating bean plants with *T. viride* will increase phenolic substances level. The cotton seeds treated by isolates of *Trichoderma* had a clear effect in increasing Peroxidase level (Hamid, 2002). Studies have reported that high efficacy enzymes will conjugate with a high level of plant resistance. The peroxidase acts with hydrogen peroxide in breaking down of pathogenic enzymes, inducting phytoalexins and building a structural defence to strengthen the cell walls, such construction of lignin also interacts with cell wall proteins forming transverse bands and multiple compounds which increase the hardness of cell wall (Hibar *et al.*, 2007).

Table (4): Effect of *G. mosseae, G. intradicas* and *T. harizanum* treatment on enzymatic activity (catalase and Peroxidase) of Okra seedling infected by *R. solani*

Treatments	Catalase activity	Peroxidase activity
	(absorption unit/g fresh root weight)	
G. mosseae+R. solani	2.430	4.654
T.harizanum+R. solani	1.013	3.229
R. solani + G. intradicas	1.772	4.541
G. mosseae+T. harizanum+R. solani	1.994	4.189
G. mosseae+G. intradicas+R. solani	3.129	4.760
T. harizanum+G. intradicas+R. solani	2.655	4.012
G. mosseae+T. harizanum+G. intradicas+R. solani	2.067	1.985
R. solani only	0.896	1.656
L.S.D. (0.05)	**0.218**	**0.782**

The inhibition of pathogenic fungi will increase by using a complex of compatible bio-agents, due to producing many antibiotics, that synergistic with different enzymes to degrade cell wall of a wide range of pathogenic fungi. Singh *et al*. (2013) reported that phenolic compounds are major factors in disease resistance of many plant families. Peroxidase and polyphenoloxidase are associated in phenols oxidation to

Quinones which is more toxic to pathogens. These two enzymes are more effective in sunflower plants which used *T. harizianum* to control *R. solani*. This study indicates the high efficiency of these enzymes are associated with high levels of resistance in addition to Peroxidase is associated with hydrogen peroxide in breaking pathogenic enzymes such a pectinase that strengthens the cell wall. The induction of phytoalxins and the strength and structural defense of walls such as lignin building and interaction with cell wall proteins (Hibar *et al.*, 2007)

This work also showed that *Trichoderma* sp. strains were compatible with other bio-agents factors. Wahid (2006) and Al-Taie *et al*. (2016) reported when using combination of bio-agents was gave better results than it used alone. *T. harzianum* and *T. viride* were compatible with *P. fluorescens* causes significant reduction in tomato seedling (Rini and Sulochana, 2007).

Conclusion

Based on this study, it can be concluded that use of a combination of bio-agents was more efficient in biological control than in individual treatment, because of the wide diversity of rhizosphere organisms and the biological, chemical and physical changes that occurred leading into a variety of secondary metabolic products that contribute to inhibiting the plant pathogens.

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