



Official publication of Pakistan Phytopathological Society
Pakistan Journal of Phytopathology

ISSN: 1019-763X (Print), 2305-0284 (Online)

<http://www.pakps.com>



COMPATIBILITY OF MYCO-FUNGICIDE ISOLATE (*TRICHODERMA HARZIANUM* RIFAI) WITH FUNGICIDES AND THEIR *IN-VITRO* SYNERGISM ASSESSMENT

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ABSTRACT

Trichoderma harzianum is one of the most famous biocontrol agent in plant protection. Present study investigated the *T. harzianum*, either, to investigate there overall compatibility with commercial fungicide. The compatibility tests were conducted using the food poisoned technique adopted with dual culture methods. Among the seven commercial formulations of the fungicides *T. harzianum* strain was highly tolerable with the fungicide Thiovit Jet (Sulfur) with EC₅₀ 753.1 ppm followed by Ridomil Gold (Mancozeb 64, Metalex M 4 %) with EC₅₀ 469.1 ppm, Folio Gold (Metalexyl M 4% , Chlorothalonil 40%) @ EC₅₀ 227.8 ppm, it also tolerated to Kocide (Copper hydroxide) EC₅₀ 176.1 ppm, Tilt (Propiconazole) @ EC₅₀ 47.9 ppm, *T. harzianum* was highly susceptible to Score (Difenoconazole) @ EC₅₀ 13.2 ppm and highly Shincar (Carbendazim) @ EC₅₀ 2.5 ppm. Poisoned food media tests suggested that Kocide fungicide @ 50 ppm < EC₁₀ synergized the antagonist potential of *T. harzianum* up to 03% against a pathogenic fungal isolate of *F. oxysporum*; Ridomil Gold and Folio Gold @ 150 and 70 ppm < EC₁₀ synergized the biocontrol fungus against the pathogen in very slight values 1.0 to 0.5%, respectively; Thiovit jet showed negative effects on the biocontrol fungus; Shincar, Score and Tilt fungicides checked the *T. harzianum* growth. The *in-vivo* tritrophic tests are proposed to be examined using experimental designs for the combine utilizations of the treatments i.e. Soil + *F. oxysporum* + Kocide recommended doses + the strain of *T. harzianum*.

Keywords: *T. harzianum*, fungicide synergism, fungicide compatibility, *F. oxysporum*.

INTRODUCTION

Modernized Agricultural advancements have potential threats to environment and human health. Pesticide misuse was also a concern, as was fertilizer excess, which contaminated the drinking water reservoirs and resulted in blooms. Enhanced irrigation water use resulted in over-pumping of aquifers, while agro machines utilize a lot of fossil fuel. Perhaps, rising costs of current agriculture triggers a massive phenomenon in food security. The cereal crop production has reportedly faced 50% losses due to various disease and pest infestations, among these Fungal borne diseases of plants are also the constraints in crop

Submitted: May 08, 2022

Revised: November 11, 2022

Accepted for Publication: December 10, 2022

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production that caused 4 to 14% of world cereal production. It is essential to treat and protect the crops for quality and abundance of food, feed and fiber produced around the world (Oerke, 2006). The world food supply shorts due to exponential growth in human population. It was projected that world human population would increase from 7.4 billion (2017) to 9.7 billion (2050), it has great concern for world food supply (El-Hassan *et al.*, 2013; Fukase and Martin, 2020). The environmentalists are looking forward for friendly plant protection measures other than hazardous pesticides. The pathotypes of *F. oxysporum* were identified as resistant to fungicides i.e. Benomyl and Thiabendazole which suggested that the fungicides are losing effects on the target organisms, it resulted heavy input doses in the field (Chung *et al.*, 2009). During a resistance assessment studies Liu *et al.* (2021) reported that there were 30 moderately resistant strains of *F. graminearum* causing head

blight in wheat. The agrisoil situation in Europe can be compared with other areas of the world. This situation is hindering the natural microbial competitions among friendly and harmful microbes for plant growth. Since bio-control attributed fungi in soil are used to control the soil borne fungal pathogens of the crops, any inundation of beneficial microbes, for example *Trichoderma* spp. would not efficiently survive under the conditions where the fungicides are present as residue. Meanwhile the soil borne pathogenic fungi have had enough resistance to live with residual fungicides in soil. This situation could fearfully develop an economical loss to the farmers. Among soil borne fungal disease control strategies, the biocontrol agents are getting fame. *T. harzianum* is the most famous instrument for the mycoparasitism and recognized as mycofungicide. It has been proved with controlling attributes to the plant parasitic fungi. *T. harzianum* strains were recorded for the control of *S. rolfsii*, *R. solani*, *Botrytis cinerea*, *Fusarium* spp., *F. oxysporum*, *Pythium ultimum*, *Alternaria thaliana*. It has also been proved that some of the strains of *T. harzianum* are generalistic fungicide and easily kill the wide variety of plant pathogenic fungi (Cardoza *et al.*, 2006; Wenjun, 2006; Vizcaíno *et al.*, 2006; Li and Yang, 2007; Rosado *et al.*, 2007; Liu *et al.*, 2009; Rubio *et al.*, 2009; Ruocco *et al.*, 2009; Saiprasad *et al.*, 2009; Montero-Barrientos *et al.*, 2010; Hermosa *et al.*, 2011).

Although the *Trichoderma* spp. have been confirmed for innate or induced fungicide resistance and developing a chance to cope with traces of fungicides in agri soils (Chaparro *et al.*, 2011; Idrees *et al.*, 2019). The combined uses of microbial antagonists with reduced doses of chemical fungicides were reported as promising in results. The combination of biocontrol and commonly used fungicides were suggested as a positive association in reducing seed infection as compared with fungicide and individual fungal antagonists. The mutually applied chemical pesticides and biocontrols have combined or additional effects in controlling pathogens in the soil (Kredics *et al.*, 2003). Present study is based on the assessment of the *Trichoderma harzianum* strain for their tolerance to different group of fungicides; the study also finds the compatible doses of suitable fungicides, their effects on biocontrol attributes of *T. harzianum* against the phyto pathogenic *F. oxysporum* strain at dual

culture experiments and pathogen-plant-trichoderma (tritrophic) interactions.

MATERIALS AND METHODS

A comprehensive experiment was observed for the characterization of the strain *T. harzianum* (TH) in terms of its sensitivity to Copper Hydroxide fungicide and antagonism to plant pathogenic strain of *F. oxysporum*. The experiments were carried out at the Mycology and Plant Pathology Laboratory, Institute of Plant Sciences, University of Sindh.

Fungal cultures: The cultures of *F. oxysporum* and *T. harzianum* were acquired from the culture collection facility at Department of Agriculture and Agribusiness Management, University of Karachi. The fungal cultures were regularly maintained on Sabraud Dextrose Agar (SDA) medium poured slants, coded with accession numbers tags and incubated at 14 °C for 30 days and again revived for fresh cultures with intervals of 30 days. There were different mycological media utilized during the study. The Potato Dextrose Agar (PDA) and Sabraud Dextrose Agar (SDA) were utilized during the study. The media was prepared according to suggested additions of ingredients in sterile water. Each of the formulations of the proper media was added with 200 ml hot water in a beaker to mix the ingredients homogenously and made up to the 1000 ml by addition of distilled water. The homogenized media solution was poured into the Erlenmeyer flask, plugged with cotton swab and covered with Aluminum foil. The media filled flasks were placed in autoclave for sterilization at 121 °C (15 lbs) for 15 minutes. The media was kept to cool by 45 °C. The cooled molten medium was added with antibiotics i.e. Penicillin 40 mg and Streptomycin 20 mg in a Liter of media at the time of pouring, when it is required to use against bacterial contaminations. The commercial formulations of agricultural fungicides were utilized during the study. The active ingredients of the fungicide are detailed in Table 1. The PDA media was amended with fungicide for the food poisoned media preparation with fungicide formulation. The fungicide concentrations were made through the dilution methods as suggested by (Secor and Rivera, 2012). Each one Liter of PDA media was added with required amount of the fungicide formulation before pouring in the Petri plates. The antagonistic activities of biocontrol agents against the test pathogens were evaluated by dual culture technique (Royse, 1978; Chand and Logan, 1984; VI, 2018).

Table 1. The information of fungicides and their recommended dose for field applications

S.No.	Fungicides Trade name and (manufacturer)	Active ingredient	Fungicide group	Formulation (g Kg ⁻¹) or (ml L ⁻¹) ppm	Recommended dose (g) per Acre	a.i. (g) per Acre
1.	Folio Gold (Syngenta)	Metalexyl M 4% , Chlorothalonil 40%g	Phenylamine and Chloronitrile	440	1000	440
2.	Kocide (FMC)	Copper Hydroxide 25%	Coppers	520	500	260
3.	Ridomil Gold (Syngenta)	Mancozeb 64, Metalexl M 4 %	Ethylenebisdithio carbamates (EBDC) and Phenylamine	680	250	170
4.	Shincar (FMC)	Carbendazim 5%	Benzimidazoles	50	50	2.5
5.	Score (Syngenta)	Difenoconazole 25%	Triazole	250	120	30
6.	Thiovit Jet (Syngenta)	Sulfur 80%	Sulfur	800	1000	800
7.	Tilt (Syngenta)	Propiconazole 25%	Triazole	250	250	62.5

Food poisoned dual culture techniques: The fungicide tolerance was observed using food poisoned media technique, the fungal strains were grown on the food poisoned dual culture methods as suggested by Shah *et al.* (2018); Schmitz (1930). The radial growths of the fungi on dual culture and controls were compared and the level of growth inhibition (%) antagonism was observed with the formula as described: $A = C - T / C \times 100$, where the A stand for antagonism, C for radial growth of control fungi, T for treated fungi. Probit regression analysis was performed for the assessment of median lethal dose (EC₅₀) of fungicides to the fungus on dual culture on poised media.

Synergism calculations: The synergism between the fungicide and biocontrol agent was also calculated by a formula: $S = APM - AEM$, where the S stands for synergism, APM stands for antagonism on poisoned media and AEM stands for Antagonism on empoisoned media.

STATISTICAL ANALYSIS

All the experiments were replicated three times and repeated twice for clarity in statistical inferences, the results were analyzed for Probit regression model for dose time inhibition where the time was constant and concentrations of the fungicides were variable for calculations of Effective Concentrations (EC_{1 to 100}). The normal mean values were analyzed for variance at Analysis of variance (ANOVA) using SPSS-19 software.

RESULTS AND DISCUSSION

Compatibility of *T. harzianum* with fungicides: *T. harzianum* was highly susceptible to the Shincar

(Carbendazim) with EC₅₀ value 2.5 ppm followed by Score (Difenoconazole) with EC₅₀ 13.1 ppm, Tilt (Propiconazole) EC₅₀ value 47.8 ppm. Kocide (Copper Hydroxide) was moderately tolerable to *T. harzianum* mycelia (176.2 ppm) followed by Folio Gold (Chlorothalonil 40% + Metalax 4%) with EC₅₀ 227.8 ppm followed by Ridomil Gold (Mecozeb 64%+Metalax 4%) with EC₅₀ values 469.1 ppm > Thiovit Jet (Sulfur) as it was highly compatible with the fungi on poisoned PDA (EC₅₀ 753 ppm) (Table 2). At the lowest concentration, Shincar i.e. 1.5 ppm caused 17.5% mycelia inhibition of *T. harzianum* strain, while the Shincar restricted the highest mycelia growth 99% at 6.8 ppm, Score was 16.3% inhibitory at 7.5 ppm and it caused the highest inhibition (99%) at 33.8 ppm. The fungicide Tilt inhibited 5.2% at 7.5 ppm, while the highest inhibition 51.7 caused by 56.3 ppm of the fungicide; Kocide inhibited 2.3% mycelia growth at lower concentration 15 ppm, while the highest inhibition (23.3%) observed at 117.0 ppm, which suggested a tolerability of the fungal mycelia (Figure 1 and 2). Thiovit Jet fungicide was highly tolerable to the mycelia of *T. harzianum* isolate, it showed very low inhibition from 0.3 to 2.5% at 24 and 180 ppm, respectively. It was highly compatible to the fungal strain, it was followed by Ridomil Gold fungicide, it showed 0.6% inhibitory effects to the fungi at 20.4 ppm and increased its inhibition 5.8% at 153 ppm (Figure 1 and 2). The statistical analysis suggested that data results were highly significant at Probit analysis and chi-square tests $p < 0.05$ (Table 2).

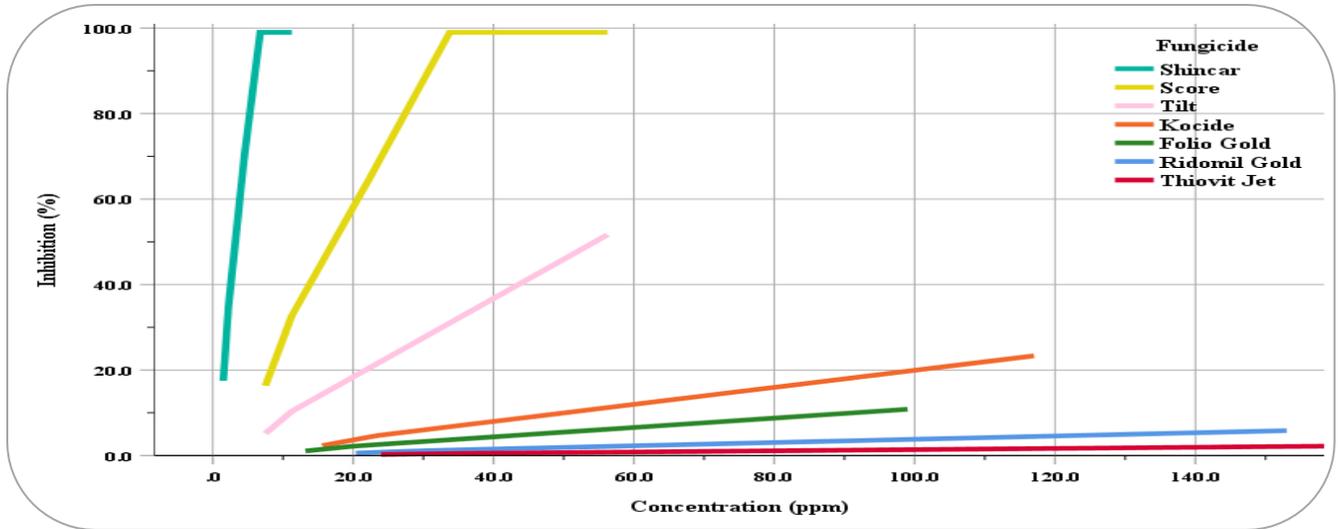


Figure 1. The caused by fungicides (% mycelia inhibition) to the *T. harzianum* at various concentrations.

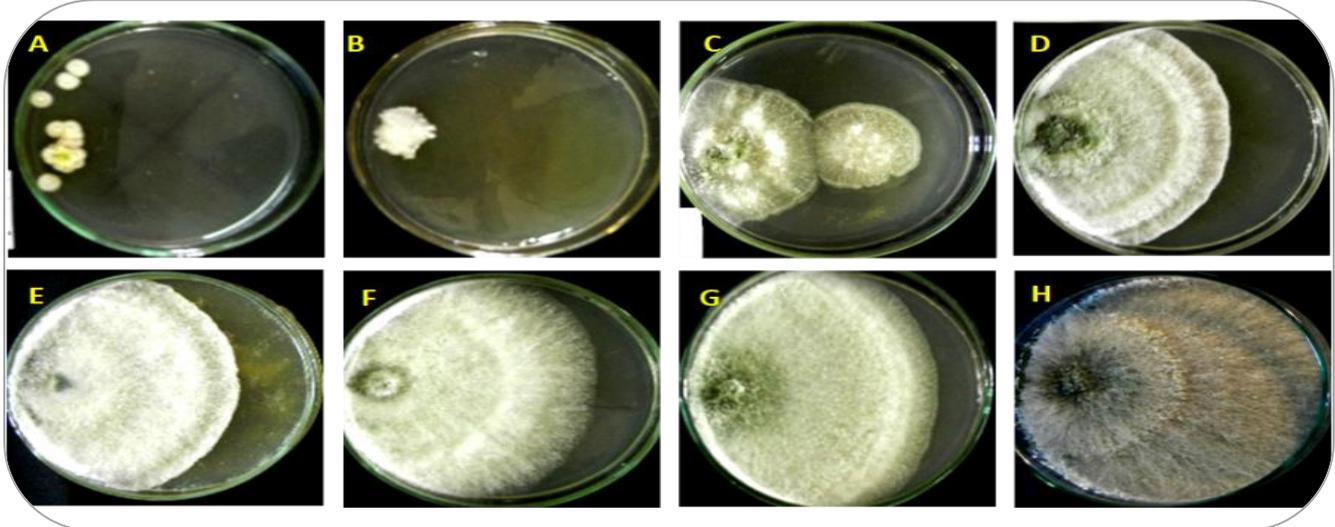


Figure 2. The growth of *T. harzianum* strain on food poisoned media with Shincar at 09 ppm (A), Score at 45 ppm (B), Tilt 50 ppm (C), Kocide at 100 ppm (D), Folio Gold at 80 ppm (E), Ridomil Gold at 120 ppm (F), Thiovit Jet at 144 ppm (G) and Control at 0.0 ppm.

Table 2. The fungicides' toxicity to the test strain, the fungicides doses/concentrations that caused 10, 50 and 90% mortality (EC₁₀, EC₅₀ and EC₉₀) to the *T. harzianum*

EC _x	Shincar	Score	Tilt	Kocide	Folio Gold	Ridomil Gold	Thiovit Jet
10	0.8 (0.4-1.2)	4.3 (2.5-6.3)	15.8 (10.8-21.2)	58.2 (40.3-81.5)	75.3 (49.1-116.6)	155.1 (92.7-269.1)	248.9 (127.2-521.1)
50	2.5 (1.7-3.3)	13.1 (9.4-17.6)	47.8 (53.3-40.0)	176.1 (123.4-276.5)	227.8 (144.7-410.6)	469.1 (270.2-842.5)	753.0 (370.5-1856.9)
90	7.5 (5.5-11.3)	39.7 (98.4-252.9)	144.7 (98.4-252.9)	532.7 (329.8-1074.6)	689.1 (386.1-1597.5)	1418.1 (725.1-3707.2)	2277.6 (1008.5-7080.7)
Pearson Goodness-of-Fit Test						PROBIT	
Chi-Square						239.412	
df ^b						34	
Sig.				0.000			

The values in parenthesis are defining the 95% confidence limit of the above values. ^b Statistics based on individual cases differ from statistics based on aggregated cases.

Most of the researchers confirmed that the Carbendazim was highly toxic to *T. harzianum* at very low doses, (Gowdar *et al.*, 2006; Aswathi *et al.*, 2019). It was also observed that a very low concentration 0.05% of Carbendazim caused 92.5% *T. harzianum* mycelia growth inhibition (Shashikumar *et al.*, 2019). Some researchers reported high doses of Carbendazim, i.e. 200 ppm caused 60.5% mycelia inhibition (Sharma *et al.*, 2016), which is very uncommon to the generalized, reported values, which could be caused by fungal adaptation to the fungicides. Some workers reported the strains of *T. harzianum* were insensitive to the best known effective fungicide i.e. Carbendazim (Kumawat *et al.*, 2019), therefore, there is a dearth of study to unearth the fungicide tolerant strains and their diverse resistance to fungicides, natural and farming habitats. It was also reported that Difenconazole inhibited 50% mycelia growth of *T. harzianum* isolate at 08 ppm of the fungicide concentration (Khalko and Pan, 2009). Very rare literature is available on the compatibility and toxicity of Difenconazole on *T. harzianum* was available. Since present results showed higher toxicity of the fungicide, therefore, it couldn't be assessed for further synergistic effects. Sarkar *et al.* (2010) reported that the mycelia growth was 100% inhibited at 25 ppm of Propiconazole which was suggesting higher toxicity of the fungicide than present findings. It was also reported that *T. asperillum* growth was inhibited 100% at 100 ppm. This refers the previous reports are supporting present study.

In earlier reports, the Copper Hydroxide fungicide was slightly tolerable to *T. harzianum* at 500 ppm concentrations, which is in support of the present finding accordingly (Erayya *et al.*, 2020), and it was highly compatible with *T. viride* (Valarmathi, 2013). The reports also suggested the fungicide could be used in combined applications; therefore, the fungicide was emphatically analyzed for synergistic studies.

Sulfur was highly compatible with the *T. harzianum*, showed maximum tolerance and grown well with the heavy doses of the fungicide. In field conditions, it was reported that the Sulfur, in combination with *Trichoderma* spp. and *T. harzianum*, increased the crop yield and reduced the disease incidence of *Alternaria porri* (Bayoumi, 2008; Bayoumi *et al.*, 2019). This was already reported that the *T. harzianum* was highly compatible with Sulfur @ 1250 and 2500 ppm, which reflects our suggestions about the fungal compatibility

with Sulfur, furthermore the same report suggested that it is also compatible with Metalax fungicide, it is supporting our findings as Folio Gold and Ridomil Gold fungicides were tolerated by the *T. harzianum* strain in present study (Ranganathaswamy *et al.*, 2012; Ranganathaswamy *et al.*, 2012; Sharma *et al.*, 2016; Vipul *et al.*, 2016; Sood *et al.*, 2020).

In-vitro synergism: Present study showed antagonism of *T. harzianum* 24% inhibition of *F. oxysporum* at dual culture plate, while it was not merely lower for antagonist reactions; but the groups of researchers suggested the varied antagonism potentials of the fungi, Carvalho *et al.* (2014) suggested 51% inhibition *F. oxysporum* by *T. harzianum*, which is higher to present study; other reported the antagonism % of the *T. harzianum* isolates from 24 to 70% to *F. oxysporum* (Altinok and Erdoğan, 2015; VI, 2018).

There is a big question; either *T. harzianum* would enhance the biocontrol attribute in presence of the highly tolerable fungicides. In next to the discussions, it would be informed the results regarding the performance of *T. harzianum* strain on poisoned dual culture technique. Hypothesis suggested that lower doses of compatible fungicides would allow the *Trichoderma* spp. to enhance their biocidal activities towards plant pathogenic fungi. Looking into the fungicide tolerance of the test fungi, the four fungicides viz. Ridomil Gold, Folio Gold, Thiovit Jet and Kocide were selected for further experimental studies. It was hypothesized that the sub-lethal doses of (concentration < EC₁₀) fungicides enhance the antagonism of the biocontrol agents i.e. synergism (Figure 3 and 4).

The calculated results of synergism experiments are as follow: Thiovit Jet (Sulfur) was highly tolerated by the biocontrol agents, EC₅₀ values were high for the mycelia inhibition of *T. harzianum* (753.2 ppm) and even it was tolerable to the pathogenic fungi i.e. *F. oxysporum* (601.2 ppm) which suggested that the fungicide was highly tolerated by the both groups of fungi (Table 2). The results were significantly evident that trace amount of the fungicide Sulfur was reducing the biocontrol potential of the *T. harzianum* strain as it reduced antagonistic potential -0.5% but supported the growth of the pathogen as compare to control (empoisoned media dual culture assay) (Figure 3 and 4). previous research work has mainly focused on understanding the general effects of sulfur on fungal specie however, (O'Neill *et al.*, 1996; Bayoumi *et al.*, 2019) no synergistic

study has been reported, the result of this study exhibited that the efficiency of the *T. harzianum* was reduced in antagonism than their antagonism on empoisoned media dual culture tests. Simply the presence of Sulfur, even in small doses, also provides support to the pathogens other than biocontrol agents in our study (Figure 3 and 4). During fungal biocidal interactions, even the highly tolerable doses were not supporting to the *Trichoderma* spp. isolates and ironically, it was resisted by fungal pathogens. Therefore, it is an important issue of fungicide resistance in fungal pathogens of the plants. This may

be predicted that the fungal pathogens of agro-ecology origins might had countered with fungicides and developed synergistic resistance in terms of their fight from two directions i.e. one from microbial competitions/parasitism and second from chemical synthetic fungicides applied by farmers.

It was observed that the Folio Gold @ 70 ppm slightly increased the potential of the *T. harzianum* strain, significantly (0.5%) and supported the biocontrol fungi with increased antagonism tendencies as compare to control against *F. oxysporum* (Figure 3 and 4).

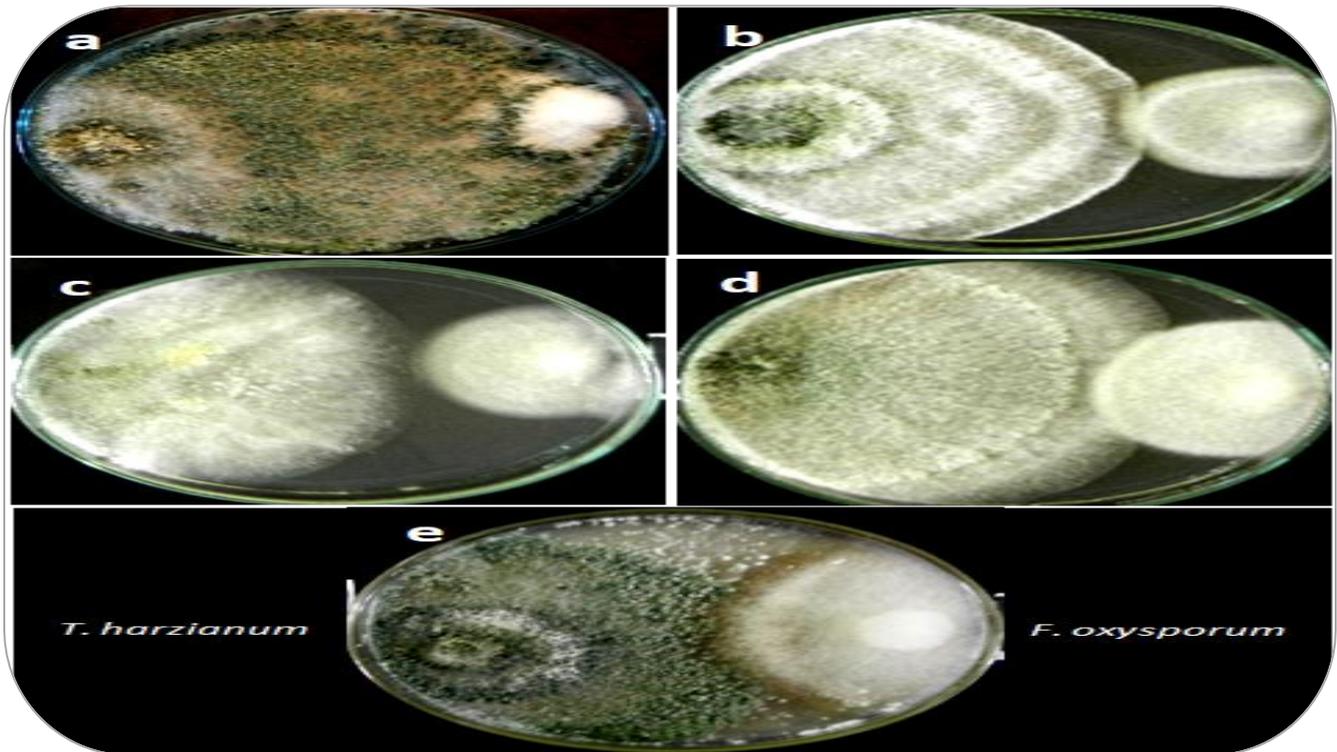


Figure 3. Visual presentation is showing *T. harzianum* (TH) on left hand side and *F. oxysporum* (FO) on right hand side under *in-vitro* antagonism test. The dual culture plates (a-d) are showing the antagonism of TH to FO on poisoned PDA media with a) Kocide, b) Ridomil Gold, c) Folio Gold, d) Thiovit Jet, while e) refers the antagonism on empoisoned PDA media as control.

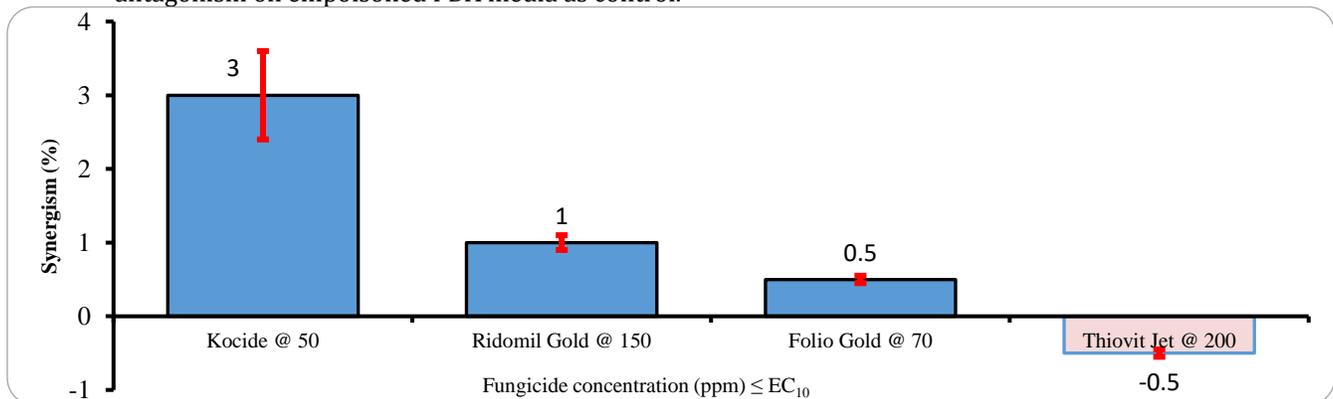


Figure 4. The Synergism observed between low doses of fungicide with TH to fungal pathogen FO.

Ridomil Gold was highly tolerable to the fungi *T. harzianum* (EC₅₀ 469.1 ppm), it was observed that the *T. harzianum* strain did synergize its effects at *in-vitro* experiments to curtail the growth of the pathogenic fungi. *T. harzianum* showed 01 % synergism and increased its efficiency from 24 to 25% antagonism to *F. oxysporum* on poison food media (Figure 3 and 4). Present study provides the information about the *in-vitro* effects of fungicides, when these were applied in combination with *T. harzianum* at sub lethal doses i.e. EC₁₀. Thus there is no such information about sublethal dose of the fungus and its *in-vitro* combined application as present study submits the findings.

Kocide (Copper Hydroxide) was tolerable to the biocontrol fungicides at *in-vitro* compatibility test. Kocide fungicide was tolerable to *T. harzianum* (EC₅₀ 176.1 ppm) (Figure 3 and 4). There was a good synergism (03%) between fungicide Kocide and biocontrol agents, *T. harzianum*, it was a positive synergism between fungicide and the biocontrol agent with increased antagonistic attributes. The Copper, Chitosan and *Trichoderma* was amended in the soil for the control of late blight of potato, it was reported that the fungus and copper amended solutions were effective to reduce disease severity from 40 to 2.3% after 90 days, which supports our findings for the combined applications (Rautela *et al.*, 2018; Erayya *et al.*, 2020). As such records were not traced for the synergistic *in-vitro* reports between Copper Hydroxide and *T. harzianum*. Present study demanded a comprehensive *in-vivo* experimental design strategy to examine the synergy between Copper Hydroxide and *T. harzianum* on *F. oxysporum* root soils of the test plant.

CONCLUSION

The fungicides have potential to support fungal biocontrol agents against antagonistic competition with plant pathogenic fungi. Present study recorded evidences that the fungicide Copper Hydroxide had attributes to support *T. harzianum* growth at lower compatible doses EC_{<10} (50 ppm) against the phytopathogen strain of *F. oxysporum*.

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Aziz A. Ujjan	: Supervised the study
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Abdul Q. Rajput	: Helped in experiments
Saleem Shahzad	: Make graph and tables