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

**Umara Arain, a Mai J. Dars, Aziz A. Ujjan, b Hadi B. Bozdar, b Abdul Q. Rajput, b Saleem Shahzad**

a Institute of Plant Sciences, University of Sindh, Allama I.I.Kazi Campus, Indus Highway, Jamshoro-76080, Pakistan.

b Department of Agriculture & Agribusiness Management, University of Karachi, University Road, Karachi-75270, Pakistan.

**A B S T R A C T**

*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, Metalexyl M 4 %) with EC₅₀ 469.1 ppm, Folio Gold (Metalexyl M 4 %, Chlorothalonil 40%) at EC₅₀ 227.8 ppm, it also tolerated to Kocide (Copper hydroxide) EC₅₀ 176.1 ppm, Tilt (Propiconazole) at EC₅₀ 47.9 ppm, *T. harzianum* was highly susceptible to Score (Difenconazole) at EC₅₀ 13.2 ppm and highly Shincar (Carbendazim) at 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 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. graminiearum* 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 vide 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-trichderma (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

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Fungicides Trade name and (manufacturer)</th>
<th>Active ingredient</th>
<th>Fungicide group</th>
<th>Formulation (g Kg(^{-1})) or (ml L(^{-1})) ppm</th>
<th>Recommended dose (g) per Acre a.i. (g) per Acre</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Folio Gold (Syngenta)</td>
<td>Metalexyl M 4%, Chlorothalonil 40%g</td>
<td>Phenylamine and Chloronitrile</td>
<td>440</td>
<td>1000</td>
</tr>
<tr>
<td>2.</td>
<td>Kocide (FMC)</td>
<td>Copper Hydroxide 25%</td>
<td>Coppers</td>
<td>520</td>
<td>500</td>
</tr>
<tr>
<td>3.</td>
<td>Ridomil Gold (Syngenta)</td>
<td>Mancozeb 64, Metalex M 4%</td>
<td>Ethylenebisdithio carbamates (EBDC) and Phenylamine</td>
<td>680</td>
<td>250</td>
</tr>
<tr>
<td>4.</td>
<td>Shincar (FMC)</td>
<td>Carbendazim 5%</td>
<td>Benzinimidazoles</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>5.</td>
<td>Score (Syngenta)</td>
<td>Difenoconazole 25%</td>
<td>Triozole</td>
<td>250</td>
<td>120</td>
</tr>
<tr>
<td>6.</td>
<td>Thiovit Jet (Syngenta)</td>
<td>Sulfur 80%</td>
<td>Sulfur</td>
<td>800</td>
<td>1000</td>
</tr>
<tr>
<td>7.</td>
<td>Tilt (Syngenta)</td>
<td>Propiconazole 25%</td>
<td>Triozole</td>
<td>250</td>
<td>250</td>
</tr>
</tbody>
</table>

**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 (EC\(_{50}\)) 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 poised 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 an 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 EC\(_{50}\) value 2.5 ppm followed by Score (Difenoconazole) with EC\(_{50}\) 13.1 ppm,Tilt (Propiconazole) EC\(_{50}\) 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 EC\(_{50}\) 227.8 ppm followed by Ridomil Gold (Mecozeb 64%+Metalax 4%) with EC\(_{50}\) values 469.1 ppm > Thiovit Jet (Sulfur) as it was highly compatible with the fungi on poisoned PDA (EC\(_{50}\) 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 (EC<sub>10</sub>, EC<sub>50</sub> and EC<sub>90</sub>) to the *T. harzianum*

<table>
<thead>
<tr>
<th></th>
<th>Shinkar</th>
<th>Score</th>
<th>Tilt</th>
<th>Kocide</th>
<th>Folio Gold</th>
<th>Ridomil Gold</th>
<th>Thiovit Jet</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0.8 (0.4-1.2)</td>
<td>4.3 (2.5-6.3)</td>
<td>15.8 (10.8-21.2)</td>
<td>58.2 (40.3-81.5)</td>
<td>75.3 (49.1-116.6)</td>
<td>155.1 (92.7-269.1)</td>
<td>248.9 (127.2-521.1)</td>
</tr>
<tr>
<td>50</td>
<td>2.5 (1.7-3.3)</td>
<td>13.1 (9.4-17.6)</td>
<td>47.8 (53.3-40.0)</td>
<td>176.1 (123.4-276.5)</td>
<td>227.8 (144.7-410.6)</td>
<td>469.1 (270.2-842.5)</td>
<td>753.0 (370.5-1856.9)</td>
</tr>
<tr>
<td>90</td>
<td>7.5 (5.5-11.3)</td>
<td>39.7 (98.4-252.9)</td>
<td>144.7 (98.4-252.9)</td>
<td>532.7 (329.8-1074.6)</td>
<td>689.1 (386.1-1597.5)</td>
<td>1418.1 (725.1-3707.2)</td>
<td>2277.6 (1008.5-7080.7)</td>
</tr>
</tbody>
</table>

Pearson Goodness-of-Fit Test

<table>
<thead>
<tr>
<th>Chi-Square</th>
<th>PROBIT</th>
</tr>
</thead>
<tbody>
<tr>
<td>239.412</td>
<td></td>
</tr>
</tbody>
</table>

| dF | 34 |

Sig. 0.000

The values in parenthesis are defining the 95% confidence limit of the above values. * 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](image_url)

**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) Follio Gold, d) Thiovit Jet, while e) refers the antagonism on empoisoned PDA media as control.
The Synergism observed between low doses of fungicide with TH to fungal pathogen FO.


Rosado, I. V., M. Rey, A. C. Codón, J. Govantes, M. A. Moreno-Mateos and T. Benítez. 2007. QID74 Cell wall protein of Trichoderma harzianum is involved in cell protection and adherence to hydrophobic surfaces. Fungal Genetics and Biology, 44: 950–964.


**Contribution of Authors:**

<table>
<thead>
<tr>
<th>Author</th>
<th>Task</th>
</tr>
</thead>
<tbody>
<tr>
<td>Umara Arain</td>
<td>Conducted research</td>
</tr>
<tr>
<td>Mai J. Dars</td>
<td>Wrote manuscript</td>
</tr>
<tr>
<td>Aziz A. Ujjian</td>
<td>Supervised the study</td>
</tr>
<tr>
<td>Hadi B. Bozdar</td>
<td>Make Statistical analysis</td>
</tr>
<tr>
<td>Abdul Q. Rajput</td>
<td>Helped in experiments</td>
</tr>
<tr>
<td>Saleem Shahzad</td>
<td>Make graph and tables</td>
</tr>
</tbody>
</table>

**References:**