

Induction of defence related enzymes and biocontrol efficacy of *Trichoderma harzianum* in tomato plants infected with *Fusarium oxysporum* and *Fusarium solani*

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Induction of defence related enzymes and biocontrol efficacy of *Trichoderma harzianum* in tomato plants infected with *Fusarium oxysporum* and *Fusarium solani*

Abstract: *Fusarium* wilt of tomato plants caused by *Fusarium oxysporum* Schlecht. emend. Snyder & Hansen and *Fusarium solani* (Mart.) Sacc. are serious problem limiting tomato production worldwide. Biological control has emerged as one of the most promising alternatives to chemical fungicides. The biological control capability of a *T. harzianum* isolate against *F. solani* and *F. oxysporum* has been investigated. It inhibited colony growth of two *Fusarium* species by more than 80 % in dual culture tests. Results of greenhouse experiments revealed that disease severity in the tomato plants co-inoculated with *T. harzianum* was significantly lower than plants only infected with the *Fusarium* pathogens. Tomato plants inoculated with the antagonistic *T. harzianum* isolate, showed enhanced peroxidase and polyphenol oxidase activities in greenhouse experiments and increased resistance to *F. solani* and *F. oxysporum*. The *T. harzianum* isolate indirectly affected the *Fusarium* pathogens by enhancing plant defence.

Key words: fungal pathogens; antagonistic fungus; peroxidase; polyphenol oxidase; plant growth and biological control

Vzpodbuditev aktivnosti encimov povezanih z obrambo in učinkovitost biokontrole z glivo *Trichoderma harzianum* Rifai v paradižniku okuženem z glivama *Fusarium oxysporum* Schlecht. emend. Snyder & Hansen in *Fusarium solani* (Mart.) Sacc.

Izvleček: Fuzarijska venenja paradižnika, ki jih povzročata glivi *Fusarium oxysporum* Schlecht. emend. Snyder & Hansen in *Fusarium solani* (Mart.) Sacc. so resen problem, ki omejuje svetovno pridelavo paradižnika. Biološka kontrola se je pokazala kot najbolj obetajoča alternativa kemičnim fungicidom. V raziskavi je bila preučevana sposobnost biološkega uravnavanja gliv *F. solani* in *F. oxysporum* z izolati glive *Trichoderma harzianum*. Izolati so zavrli rast kolonij obeh vrst iz rodu *Fusarium* za več kot 80 % v preiskavih dvojnih kultur. Izsledki iz poskusov v rastlinjaku so pokazali, da je bila obolelost paradižnika značilno manjša, če je bil ta inokuliran hkrati z obema patogenima glivama iz rodu *Fusarium* in z glivo *T. harzianum*. Ratline paradižnika, ki so bile inokulirane z izolati antagonistične glive *T. harzianum* so imele v poskusih v rastlinjaku povečani aktivnosti peroksidaze in polifenol oksidaze ter povečano odpornost proti patogenima glivama *F. solani* in *F. oxysporum*. Izolati iz glive *T. harzianum* so neposredno vplivali na patogena iz rodu *Fusarium* s povečanjem obrambe rastlin.

Ključne besede: paradižnik; patogene glive; antagonistične glive; rast in biološka kontrola; peroksidaza; polifenol oksidaza

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1 INTRODUCTION

Tomato plants can be attacked by different soil-borne fungi, which cause severe diseases such as wilt and root rot (Morsy et al., 2009). *Fusarium* species are the most common pathogens, which can live in organic materials and nearly all soil types (Boyaci et al., 2010). They can attack the vascular system in infected plants, block water transport through xylem by inducing vessel plugging, leading to foliage wilt and finally plant death (Portal et al., 2018; Malandrakis et al., 2018).

The attempts to minimise the use of fungicides make it necessary to develop economical and effective methods to obtain biopesticides that are based on endogenous microorganisms (de los Santos-Villalobos et al., 2012). *Trichoderma* (Hypocreales) accommodates various soil-borne and pathogen-antagonistic species (Verma et al., 2007). *Trichoderma harzianum* is well known for its antagonistic effect on pathogenic fungi. It is the most potent biocontrol agent that inhibits the growth of *Fusarium* species. Mechanisms of biocontrol agent are based on antifungal metabolites, mycoparasitism, competition for nutrients and induced resistance (Perello et al., 2003; Silva et al., 2019).

Moreover, an increased activity of polyphenol oxidase and peroxidase in response to the infection by the pathogen is considered to play an active role in contributing to disease resistance in certain plant hosts (Vidhyasekaran, 2004). Pradeep and Jambhale (2002) and Vidhyasekaran (2004) observed that plants show increased activities of polyphenol oxidases and peroxidases in response to pathogen infections and the ability of a plant to resist disease. The present study investigated the effectiveness of *T. harzianum* to induce systemic resistance against *Fusarium solani* and *Fusarium oxysporum* when infecting tomato plants.

2 MATERIALS AND METHODS

2.1 ANTAGONISTIC ACTIVITY

Trichoderma harzianum isolate UPM40 was obtained from the plant protection department, faculty of agriculture, University Putra Malaysia and was evaluated in vitro for its antagonistic activity against two *Fusarium* isolates in dual culture method (Rahman et al., 2009). DNA barcoding strategies based on ITS sequences were used to identify one of the *Fusarium* as a member of the *F. solani* (KM039055) and the other as a member of the *F. oxysporum* species complex (KM039054) (from previous study Rashid et al., 2016). Isolate UPM40 was identified as a member of *Trichoderma harzianum* species complex

on the basis of morphological characters. Discs of 6 mm diameter of the antagonist and pathogens were cut from the edge of actively growing colonies on potato dextrose agar (PDA Difco™) and placed at opposite sides (4.5 cm from each other) on PDA plates. Each antagonist/pathogen combination was set up in triplicates. The plates were incubated at 24 ± 2 °C. The antagonistic effect of the test fungus was estimated by measuring their radial growth in comparison to control plates using the following formula: $I = [(C-T) / C] \times 100$ where: I = % of inhibition in the mycelia growth, C = the growth of pathogens in control plates and T = the growth of pathogens in the dual culture plates.

2.2 SOIL PREPARATION AND EXPERIMENTAL DESIGN

Soil comprising clay, sand and organic matter (1:1:1 v:v) from Erbil Governorate (pH 5.5-6) was air dried and sieved (5 mm), sterilised twice in an autoclave at 121 °C for one hour and allowed to cool for 24 hours before use.

The tomato ('Baccarat 322') seeds were surface disinfected by soaking them in 95 % ethanol for 10 seconds, followed by 3 % sodium hypochlorite for 1 minute and then washed 6 times with sterile distilled water. Two tomato seeds were planted per plastic cell in seedling growing trays. The seedlings were trimmed down to one seedling per cell after two weeks. The one-month-old tomato plants were then moved to clean pots (two plants/pot). On a daily basis, these plants were watered with tap water. The independently repeated experiment was conducted in a completely randomised design.

2.3 IN VIVO TEST

A Neubauer haemocytometer was used to adjust the *T. harzianum* spore concentration to 2×10^5 spore/ml (working solution). The soil with one-month-old tomato plants was drenched with 100 ml of working solution (Mahato et al., 2018). After 24 hours, the same amount of 2×10^5 spore/ml pathogen suspension was added to the soil per pot.

2.3.1 Chlorophyll content measurement

The chlorophyll content was measured in ten repetitions per leaf (5 plants per treatment) with a CL-01 Chlorophyll Content Metre after 30 days of inoculation. This device measured the relative chlorophyll content via dual wavelength optical observance (620 and 940 nm).

2.3.2 Sample collection for the biochemical analysis

The roots of the treated and untreated tomato plants were collected 10, 20 and 30 days after the treatment application; they were washed in running tap water and stored in a deep freezer (-80 °C) until they were used for the biochemical analysis.

2.3.3 Total protein content measurement

Protein extracts were generated from roots and protein concentrations were determined using the method described by Arora and Wisniewski (1994), of which the latter was based on Bradford assays.

2.3.4 Quantification of polyphenol oxidase (PPO) activity

The reaction mixture consisted of 0.5 ml of the enzyme extract and 2.3 ml of 0.1M phosphate buffer (pH = 6.1). Both were mixed in a cuvette and adjusted to the zero absorbance of a spectrophotometer (Mahadevan and Sridhar, 1982). Catechol solution (0.1 M, 0.2 ml) was added and the reactants were quickly mixed. The enzyme activity, measured as the change in absorbance per minute (ÅA/min) at 400 çm, was measured immediately after the addition of the catechol solution.

2.3.5 Quantification of peroxidase (PO) activity

Peroxidase activity was determined according to Sreedevi et al. (2011). The peroxidase enzyme activity was determined from the roots of the un-inoculated and inoculated tomato plants. About 0.5 g of freshly harvested material was ground in a pre-chilled mortar with 20 ml of 0.1 M cold ice phosphate buffer (pH 7.1) and centrifuged at 2000 rpm for 10 minutes. The supernatant

was made up to 25 ml and used for the assay. Freshly prepared pyrogallol (0.2 M) reagent (0.1 ml), 1.0 ml of the enzyme extract, and 1.4 ml of the 0.1M phosphate buffer (pH, 7.1) were mixed in a cuvette, and the mixture was immediately adjusted to the zero absorbance of a spectrophotometer. The H₂O₂ solution (0.5 ml of 0.01M) was added to it, and the content was mixed by inverting the cuvette. The enzyme activity was recorded according to the changes in absorbance per minute (ÅA /min/ä) at 430 cm.

3 RESULTS AND DISCUSSION

3.1 ANTAGONISTIC ACTIVITY

The results indicated that *Trichoderma harzianum* (T. h) reduced the radial growth of *F. solani* (F. s) colony by 82.31 % and *F. oxysporum* (F. o) by 80.25 %.

3.2 IN VIVO EXPERIMENT

Trichoderma treated plants showed increased plant height, fresh shoot mass and root dry mass when co-inoculated with F. o (20.6 cm, 0.79 g and 0.15 g) or F. s (27 cm, 0.66 g and 0.17 g). Plants treated only with F. o had 10.17 cm and 14.00 cm height (0.31 g) shoot mass and (0.06 g) root mass. In addition, plants inoculated only with T. h showed the highest mean values for plant height, shoot dry mass and root dry mass (32.33 cm, 1.02 g and 0.18 g) compared to other treatments and control plants (Table 1). The increased growth response caused by *Trichoderma* isolate may be due to the modification of the rooting system (Chao et al., 1986). The results further indicate that *T. harzianum* had a significant role in reducing disease incidence. Therefore, the results of the study are similar to the findings of Abd- El-Khair et al. (2011), Otadoh et al. (2011) and Alwathnani et al. (2012).

Table 1: Effect of *T. harzianum*, *F. oxysporum* and *F. solani* on shoot height (cm), shoot and root dry mass (g) on tomato plants

| Treatments | Shoot height (cm) | Shoot dry mass (g) | Root dry mass (g) |
|-------------|-------------------|--------------------|-------------------|
| Control | 22.67a | 0.76a | 0.14a |
| T. h | 32.33b | 1.02c | 0.18b |
| F. o | 10.17c | 0.17d | 0.06c |
| F. s | 14.00d | 0.31e | 0.06c |
| T. h + F. o | 20.67e | 0.79a | 0.15d |
| T. h + F. s | 27.00f | 0.66b | 0.17be |

C: control; F. o: *F. oxysporum*; F. s: *F. solani* and T. h: *T. harzianum*.

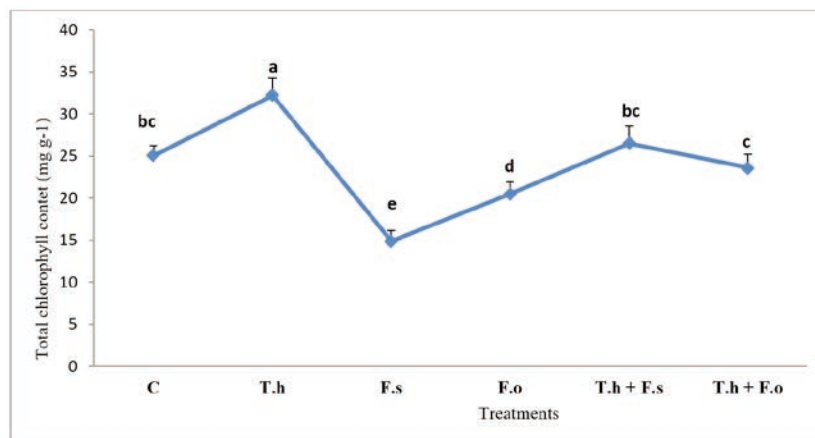


Figure 1: Effect of *T. harzianum* on chlorophyll content of the tomato plants inoculated with *F. oxysporum* and *F. solani*.

One of the important physiological indicators is the photosynthesis rate, which is related to the chlorophyll content in plant leaves in normal conditions. In the present study, the chlorophyll content was found to increase significantly in all plants treated with *T. h* compared to those inoculated with *F. s* or *F. o*. The chlorophyll content also significantly went up in plants treated with *T. h* + *F. s* (26.5) or *T. h* + *F. o* (23.5) in comparison to the *F. o* and *F. s* only (14.86 and 20.5) inoculated plants (Figure 1). Previous studies have claimed that applying biocontrol agents to infected plants increases mineral levels [(nitrogen (N), phosphorous (P), potassium (K) and magnesium (Mg)], chlorophyll biosynthesis and photosynthetic activity (Henry et al., 2009; Morsy et al., 2009; Alwathnani et al., 2012).

The total soluble protein content significantly decreased in tomato plants: *F. s* (0.082, 0.076 and 0.075) and *F. o* (0.084, 0.074 and 0.072) after 10, 20 and 30 days, respectively (Figure 2). The significant decrease in the

protein content in the tomato tissues as a result of the pathogen infection may be due to certain activities related to a hypersensitive response (Chandra and Bhatt, 1998). *Trichoderma harzianum* also increased the total protein content in the infected tomato plants (*T. h* + *F. s* and *T. h* + *F. o*). The defence reaction occurs due to an accumulation of pathogenesis-related (PR) proteins such as chitinase, phenylalanine ammonia lyase and peroxidase (Kloepper et al., 1992). This has also been previously reported by Houssien et al. (2010).

Tables 2 and 3, show that the activity of peroxidase and polyphenol oxidase, increased significantly compared to the controls. The activity increased during the treatment period from day 10 to day 30. Also *Fusarium* species are able to produce metabolites, which play a vital role in tissue browning due to their ability to oxidise phenols to quinones (Ramadoss, 1991). Except the tomato plant inoculated only with the antagonistic *T. harzianum*, there was an increase in the activity of the polyphenol ox-

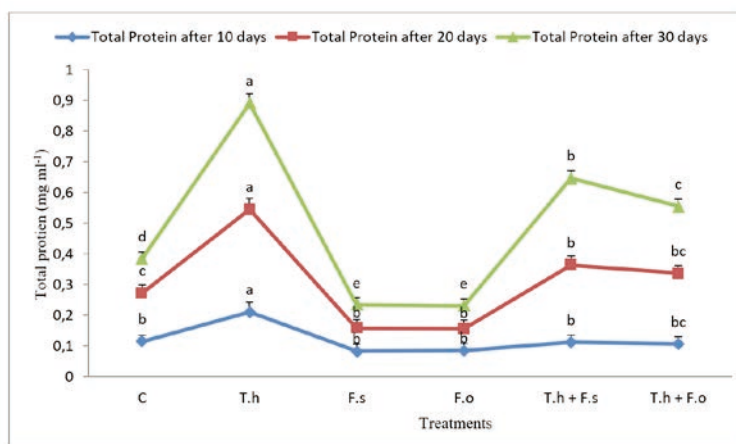


Figure 2: Effect of *T. harzianum* on the total protein content (mg g⁻¹) of the tomato plants inoculated with *F. oxysporum* and *F. solani*.

Table 2: Effect of *Trichoderma harzianum* on the peroxidase activity of the tomato plants inoculated with *F. oxysporum* and *F. solani*

| Treatments | Peroxidase activity min g ⁻¹ fresh mass of tomato leaves | | |
|------------|---|---------------|---------------|
| | After 10 days | After 20 days | After 30 days |
| Control | 0.18 a | 0.17 a | 0.15 a |
| T.h | 0.16 a | 0.43 b | 0.36 b |
| F.o | 0.25 b | 0.34 c | 0.59 c |
| F.s | 0.27 bc | 0.47 cd | 0.62 cd |
| T.h+ F.o | 0.27 bc | 1.20 f | 1.87 f |
| T.h+ F.s | 0.29 cd | 1.29 fg | 1.95 f |

Table 3: Effect of *T. harzianum* on the polyphenol oxidase activity of the tomato plants inoculated with *F. oxysporum* and *F. solani*

| Treatments | Polyphenol oxidase activity min g ⁻¹ fresh mass of tomato leaves | | |
|------------|---|---------------|---------------|
| | After 10 days | After 20 days | After 30 days |
| Control | 0.16 a | 0.18 a | 0.13 a |
| T.h | 0.17 a | 0.31 b | 0.17 a |
| F.o | 0.22 b | 0.35 ab | 0.69 b |
| F.s | 0.21 b | 0.39 bc | 0.75 bc |
| T.h+ F.o | 0.29 c | 1.28 d | 2.71 d |
| T.h+ F.s | 0.27 c | 1.29 d | 2.55 de |

idase and peroxidase up to the 20th day. After this time, the activity decreased or remained stable. At the initial stage of infection, there is probability of the host plant to secrete more defence enzymes (Ojha et al., 2012). The activity increased gradually from the 20th day up to the 30th day in the plants infected with T. h. + F. o. and T. h. + F. s.. The highest activities for both treatments 1.87 and 1.95 min g⁻¹ for fresh mass of peroxidase and 2.71 and 2.55 min g⁻¹ for fresh mass of polyphenol oxidase were recorded after the 30th day of inoculation. This is the most likely related to the fact that in tomato plants, when inoculated with a pathogen and an antagonist, the host plant secretes more of the phenol oxidase enzyme for defence, but at the later stages of infection, the antagonist blocks the activity of the pathogen. Various antioxidant enzymes, such as peroxidases and polyphenol oxidases can participate in the reactive oxygen metabolism of the species during infection (Morkunas and Gmerek, 2007). Other researchers have observed increased activity in these enzymes in the host tissues in response to pathogenic infections (Abo-Elyousr et al., 2008, Christopher et al., 2010; Ojha et al., 2012).

4 CONCLUSION

The experiments demonstrated that the *T. harzianum* is effective and can induce systemic and localized resistance against *Fusarium* infection in terms of changes of

the plants' polyphenol oxidase activity, peroxidase activity and the total phenolic content. These mechanisms help to develop resistance in tomato plants. The current study suggests using *T. harzianum* strain (UPM40) to manage *Fusarium* diseases in tomato plants.

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