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RESEARCH ARTICLE

EFFICACY OF *TRICHODERMA HARZIANUM* AS A BIOLOGICAL CONTROL AGENT AGAINST *FUSARIUM OXYSPORUM* IN TOMATOES (*SOLANUM ESCULENTUM* L.)

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ABSTRACT

Fusarium oxysporum f.sp lycopersici is a soil borne pathogen of economic importance that reduces quality and quantity of tomatoes. Various fungicides have been utilized in the control of the pathogen however, resistance still remains a problem. However, biological control has been found as a promising alternative that suppresses *F. oxysporum f.sp lycopersici*. *In vitro* and *in vivo* experiments were carried out to determine the efficacy of *Trichoderma harzianum* as a bio-control agent against *F. oxysporum.* In the *in vitro* experiment, treatments (*Trichoderma* only, Dual culture, *Fusarium* only and Mancozeb) were laid in a randomized complete block design to evaluate radial mycelial growth of *F. oxysporusm*. *Trichoderma harzianum* significantly ($p<0.001$) reduced radial mycelium growth to 9.88 mm which is an inhibition of 86.8 % in a dual culture. On the other hand, Mancozeb (positive control) moderately reduced radial mycelium growth by 32.58% hence less effective compared to the *Trichoderma* treatment. Under greenhouse conditions, plants treated with 4 g *T. harzianum* exhibited least disease severity and incidence of 17.67 % and 43.7 %, respectively. High severity of 57.33 %, 80.67 % and incidence of 79.2 % and 85.4 %, respectively were recorded for positive and negative controls. In addition, 4 g *T. harzianum* treated plants recorded significantly higher plant height and total yield per plant for three weeks of harvest than the positive and negative control treatments. In conclusion, 4 g *T. harzianum* suppressed *F. oxysporum* development in tomato plants and further research can be done to study its effect on tomato plants under field conditions.

KEYWORDS

Biological control, *Fusarium oxysporum,* Tomatoes, *Trichoderma harzianum*

1. INTRODUCTION

Tomato *(Solanum esculentum* L.) is cultivated either as a field or greenhouse crop in different seasons throughout the year (Akrami and Yousefi, 2015). The vegetable is well known for its richness in nutrients especially vitamin B, vitamin C and some minerals including phosphorus, potassium and magnesium (Sundaramoorthy and Balabaskar, 2013). According to a study, tomato is a crop of high economic value to most growers, both smallholder and commercial farmers (Barari, 2016). People consume tomato as a cooked vegetable, used raw in salads and as raw material in the processing industry for example in the making of tomato sauce and tomato puree (Mwangi et al., 2011; Choga et al., 2021). However, its production is affected by several biotic and abiotic factors.

Disease infections are regarded as the chief yield reducing biotic factor in tomato production (Agrios, 2005; Choga et al., 2021). Recent studies have indicated that soil borne diseases such as early blight (*Alternaria solani*), late blight (*Phytophothora infestans*), bacterial wilt (*Ralstonia solanecerum*) and Fusarium wilt (*Fusarium oxysporum*) cause reduction in yield and quality of tomatoes (Barari, 2016). However, *Fusarium oxysporum f. sp lycopersici* (W.C. Synder and H.N Hans) which causes Fusarium wilt disease remains the most threatening pathogenic fungi which results in high economic yield losses ranging from 10 to 90 % in tomatoes depending on environmental factors (Singh and Kamal, 2012). Economic yield loss can be attributed to symptoms caused by *Fusarium oxysporum* such as yellowing of leaves, browning within the stem, wilting, browning of roots, decreased root system and necrosis which reduce photosynthesis in infected plants (Tomar et al., 2017). *Fusarium oxysporum* is a soil borne pathogen that produces resting spores (chlamydospores) that can persist in the soil for a very long time, hence the use of cultural and chemical control measures are less effective against the pathogen (Bharat and Sharma, 2014; Repalle, 2015).

Some researchers reported that the use of synthetic fungicides to control *F. oxysporum* has detrimental effects on non-target organisms such as beneficial microbes in the soil (Sundaramoorthy and Balabaskar, 2013). Furthermore, the toxicity of synthetic fungicides persists for a long time in the soil thereby increasing environmental pollution (Senthilkumar et al., 2011). Therefore biological control which is a potential alternative strategy which involves the use of pathogenic antagonists to control different pathogens without causing environmental pollution and promotion of resistant biotypes due to the injudicious use of fungicides currently obtaining in the smallholder sector (Alwathnani et al*.,* 2012).

Biological control using *T. harzian*um has been widely used in the control of many pathogens and proved to be effective and efficient as control is achieved without causing pollution to the environment (Ghazalibiglar et al., 2016). *Trichoderma harzianum* possesses attributes of a good biological agent such as ability to reproduce in large numbers and the ability to survive under unfavorable conditions through competition for nutrients and space with fungal pathogens, antibiosis, fungistasis as well

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as myco-parasitism (Howell, 2003; Ramezani, 2009; Sood et al., 2020). This agent also enhances the growth of treated plants by improving germination, improving fertilizer and improving the efficiency of the root system for increased nutrient uptake in tomato plants (Sood et al., 2020). A group of researchers reported an increase in tomato plant growth of 50% due to the use of *Trichoderma* in controlling *F. oxysporum* (Ghazalibiglar et al., 2016). In Zimbabwe, there are no reports about the efficacy of *T. harzianum*. The objective of the study was to determine the effectiveness of *T. harzianum* in controlling *Fusarium oxysporum f. sp lycopersici* populations *in vitro* and *in vivo* in tomato plants growing under greenhouse conditions.

2. Materials and Methods

2.1 Study site

The study was carried out in a Laboratory and greenhouse experiments at the University of Zimbabwe's Department of Plant Production Sciences and Technologies (17.18^0, 31.05^0 and altitude 1260 m) during 2017-2018 season.

2.2 *In vitro* evaluation of the effect of *Trichoderma harzianum* on growth of *Fusarium oxysporum* f. spp *lycospesici*

2.2.1 Experimental procedure

Trichoderma and *Fusarium* fungal isolates were obtained from the Department of Plant Production Sciences and Technologies Pathology culture collection. Two different media were prepared, Potato dextrose agar (PDA) and PDA supplemented with Mancozeb (75% WP). The two media were prepared separately following the manufacturer's instructions. A stock solution of Mancozeb was prepared by mixing 7.5 g active ingredient (*a.i*) of Mancozeb with 10 ml of distilled water. The prepared fungicide stock solution measuring 400 µl was diluted with 1litre of distilled water and was used to prepare modified PDA. Both media were autoclaved for 15 minutes at 121 ^{0}C and 15 psi. The mixture was left to cool for approximately ten minutes after which 20 ml of media was poured in each Petri dish aseptically. The media was left to set before use. Pure cultures of purified isolates of *T. harzianum* and *F. oxysporum* were mass propagated aseptically on PDA in 90 mm diamter Petri dishes. The antagonistic effect of *T. harzianum* isolates on *F. oxysporum f. spp lycospesici* was evaluated in sterile 90 mm diamter Petri dishes. A sterile cork-borer was used to cut mycelial disc measuring 5 mm in diameter from pure cultures of *T. harzianum* and *F. oxysporum* and the two fungal discs were placed 45 mm apart in dual cultures. The treatments were: 1) *T. harzianum,* 2) dual culture of *T. harzianum* and *F. oxysporum f.sp lycospesici*, 3) *F. oxysporum* only and 4) *F. oxysporum* on PDA supplemented with Mancozeb at a concentration of 400 ppm (positive control). The Petri dishes were laid out in a Randomized Complete Block Design (RCBD) with four blocks and each treatment was replicated six times. The plates were incubated at 25 ± 2°C for seven days. Asceptic conditions were observed throughout the whole procedure.

2.2.2 Data collection

Data was collected on the radial diameter of fungi at 1, 3, 5 and 7 days after culturing using a ruler. Inhibition percentage was calculated as per formula described by (Sharma et al., 2011).

$$\text{Inhibition }\% = \frac{\text{Colony diameter in control plate } (F.\ oxysporum\ only)}{\text{Colony diamater in treated plate}(Trichoderma \text{ and } Fusarium)} * 100$$

2.3 Greenhouse Experiment

2.3.1 In vivo evaluation of the effects of Trichoderma harzianum on disease development of Fusarium oxysporum on tomato plants

The experiment was laid out in a Randomized Complete Block Design (RCBD) with six treatments (*T. harzianum* different concentrations) and three replications. These concentrations were as follows: 1g, 2 g, 3 g and 4 g *T. harzianum*, with chemical treatment of Mancozeb and Control.

2.3.2 Experimental procedure

Certified tomato seeds of Floridade variety were purchased from Prime Seed-Co company. To prepare inoculum, spore suspension for the *in vivo* experiment was prepared from sporulated *F. oxysporum f. sp lycospesici* strains. A sterilized spatula was used to collect spores from 14-day old cultures by scrapping the PDA surface. The number of spores were determined using a hemocytometer. The spores were diluted in sterile distilled water to a final spore concentration of 1.0 x 10^6 CFUml^{-1}. *Trichoderma harzianum* was weighed at different concentrations that are 1g, 2g, 3g and 4g from 14-day old cultures. Pots measuring 24 cm diameter top diameter, 18 cm bottom diameter and 20.5 cm height were disinfected with 10% sodium hypochlorite to reduce contamination from other undesirable pathogens. These pots were three quarter filled with media (red loam soil and pine bark at a ratio of 2:1) sterilized in the oven at 80°C for 48hours. Three tomato seeds were sown at a depth of 0.5 cm in each pot. An equal amount of autoclaved sterile water measuring 300 ml was added to each pot. Tomato seedlings were thinned to one seedling per pot 10 days after emergence (DAE). *Trichoderma harzianum* at 1, 2, 3 and 4g concentration and Mancozeb treatments were applied on 14 DAE. Ten milliliters of *F. oxysporum* was applied to the pots 14 days after applying *Trichoderma* and Mancozeb treatments. Equal amounts of 250 ml sterile water were applied four times per week in each pot.

2.3.3 Data collection

Data was collected on several parameters which included plant height, number of leaves, disease incidence and severity. Plant height (cm) was measured weekly from one to four weeks after inoculation (WAI) with *F. oxysporum* using a measuring tape. The number of leaves per plant were counted weekly from 1-4 WAI. Disease incidence was measured weekly by counting the number of plants showing symptoms of *F. oxysporum* from 1-4 WAI. This was calculated using the formula below:

$$\text{Disease incidence} = \frac{\text{number of infected plants}}{\text{total number of plants assessed}} \text{ x } 100$$

Disease severity was assessed at weekly intervals starting from 2 WAI using a rating scale of 1 to 5. The rating was based on yellowing, wilting and necrotic symptoms on the leaves and stem where 0 = healthy plants with no symptoms, 1= 1-2 leaves showing yellowing, wilting or necrosis (1-25%), 2= 3-5 leaves showing yellowing, wilting or necrosis (26-50%), 3= 6-8 showing yellowing, wilting or necrosis 51-75%, 4= 9-11 leaves showing yellowing, wilting or necrosis (76%-100%) and 5=dead plants. Yield characteristics such as the number of flowers per plant and number of fruits per plant were also assessed by counting at four weeks after inoculation with *F. oxysporum*.

2.3.4 Data analyses

Data were tested for normality and homogeneity of variance using Ryne-Joiner and Barlett's tests, respectively. Data were subjected to analysis of variance using Genstat version 14. Mean separation was done using Fisher's protected least significant difference (LSD) test at 5% significance level.

3. Results

3.1 Effect of *T. harzianum* on radial mycelium diameter of *F. oxysporum in vitro.*

Trichoderma harzianum significantly ($p < 0.05$) reduced mycelial growth of *F. oxysporum* from 1-4 DAI (Table 1). The lowest mycelium diameter of 9.88 mm was recorded for *F. oxysporum* in the dual culture at seven days after inoculation. There was no significant difference among *F. oxysporum* pure culture, dual culture and positive control on day one after inoculation. Mycelial diameter decreased as the inhibition percentage increased in dual culture (Table 2).

Table 1: Effect of *T. harzianum* in dual cultures on the mycelium diameter (mm) of *F. oxysporum*

Treatment	Mycelium diameter (mm) at 1 DAI	Mycelium diameter (mm) at 3 DAI	Mycelium diameter (mm) at 5 DAI	Mycelium diameter (mm) at 7 DAI
Trichoderma only	15.21^b	74.75^d	90^d	90^d
Dual culture	8.42^a	19.96^b	18.63^a	9.88^a
Fusarium only	9.13^a	31.58^c	56.15^c	75.92^c
Mancozeb	8.87^a	16.00^a	24.17^b	32.58^b

Means bearing different letters within a column are significantly different at p<0.05.

Table 2: Effect of *T. harzianum* on calculated inhibition percentage against *F. oxysporum invitro*

Day	Inhibition %
1	6.06
3	36.8
5	66.4
7	86.8

Data represents calculated inhibition percentage using formula (C- CT / C) * 100.

3.2 Effects of *Trichoderma harzianum* activity on plant height and number of leaves in tomato plants against *F. oxysporum*

Trichoderma harzianum had a significant ($p<0.05$) effect on the height of tomato (Table 3). The plant height increased as the amount of *Trichoderma* added increased, 4g *T. harzianum* showed the highest height every week. There were no significant ($p>0.05$) differences at week 1 in all treatments (Table 4). The number of leaves increased with time from week 2 and reached a peak at week 3. There was strong evidence of increased number of leaves in plants treated with 4 g *T. harzianum* with the highest value (14.16) (Table 4).

Table 3: Effect of *Trichoderma harzianum* and Mancozeb activity on plant height of Floridade tomato variety under greenhouse conditions

Treatment	Plant height (cm) at 1 WAI	Plant height (cm) at 2 WAI	Plant height (cm) at 3 WAI	Plant height (cm) at 4 WAI
1g *T. harzianum*	29.41^{b}	37.14^{b}	43.57^{b}	49.10^{b}
2g *T. harzianum*	30.12^{b}	37.99^{b}	43.02^{b}	49.6^{b}
3g *T. harzianum*	33.67^{c}	44.69^{c}	52.32^{c}	65.06^{d}
4g *T. harzianum*	35.83^{c}	49.22^{d}	56.89^{d}	70.07^{e}
Mancozeb	29.90^{b}	38.92^{b}	45.21^{b}	53.05^{c}
Control	25.44^{a}	34.32^{a}	38.57^{a}	43.72^{a}

Means bearing the different letters within a column are significantly different $p > 0.001$, LSD= 2.664.

Table 4: Effect of *Trichoderma harzianum* and Mancozeb on number of leaves from week 1 to 4 after inoculation of *Fusarium oxysporum f. sp lycopersici* on tomato plants under greenhouse conditions

Treatment	No of leaves 1 WAI	No of leaves 2 WAI	No of leaves 3WAI	No of leaves 4 WAI
1g *T. harzianum*	7.66^{ns}	11.00^{b}	11.08^{ab}	8.91^{ab}
2g *T. harzianum*	7.83^{ns}	12.00^{ab}	11.91^{b}	9.75^{b}
3g *T. harzianum*	7.83^{ns}	12.58^{bc}	13.75^{c}	12.00^{c}
4g *T. harzianum*	8.25^{ns}	13.08^{c}	14.16^{c}	12.50^{c}
Mancozeb	8.16^{ns}	12.00^{ab}	11.66^{bc}	9.41^{ab}
Control	7.66^{ns}	10.75^{a}	10.53^{a}	8.16^{a}

Means bearing different letters within each column are significantly different ($p < 0.001$), LSD at 5 % = 1.2727. ns mean they were no significant differences.

3.3 Evaluation of the efficacy of *Trichoderma harzianum* on disease severity against *Fusarium oxysporum* in tomato plants

It is apparent from the graph (Figure 1) that disease severity increased with time from week 1 to 4. Interestingly, 4 g *T. harzianum* recorded the lowest disease severity score of 17.67 % as shown by a small area under the disease progress curve (Figure 1). This was significantly different ($p < 0.001$) from positive control Mancozeb with a value 57.33%. The negative control recorded the highest disease severity at 80.67 %. However, there were no differences between 2 g *T. harzianum* and Mancozeb treatments.

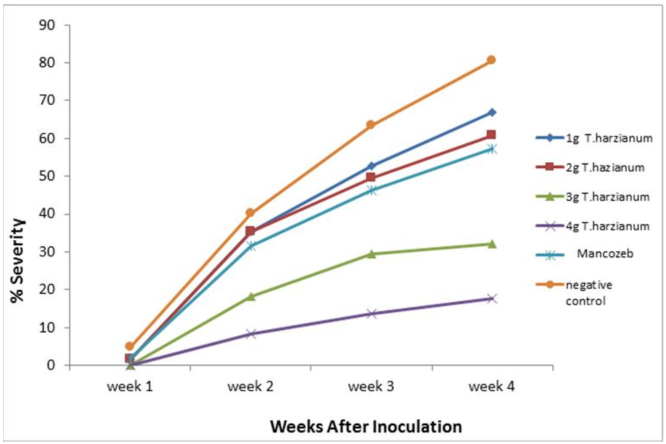

Figure 1: Percentage disease severity at week 1, 2, 3 and 4 after inoculation with *F. oxysporum f sp lycpersici* in tomato plants controlled by *T. harzianum* and Mancozeb

3.4 Efficacy of *Trichoderma harzianum* at different concentrations on percentage disease incidence as well as total yield of tomatoes against *Fusarium oxysporum* f spp *lycospesici*

Plants treated with 4 g *T. harzianum* recorded the lowest disease incidence value of 43.7 % which was significantly different ($p < 0.001$) from positive control (Mancozeb) which recorded 79.2 %. However, the negative control was statistically similar to Mancozeb, 1 g *T. harzianum* and 2 g *T. harzianum*. Treatments 1 g, 2 g *T. harzianum* and Mancozeb performed the same (Figure 2).

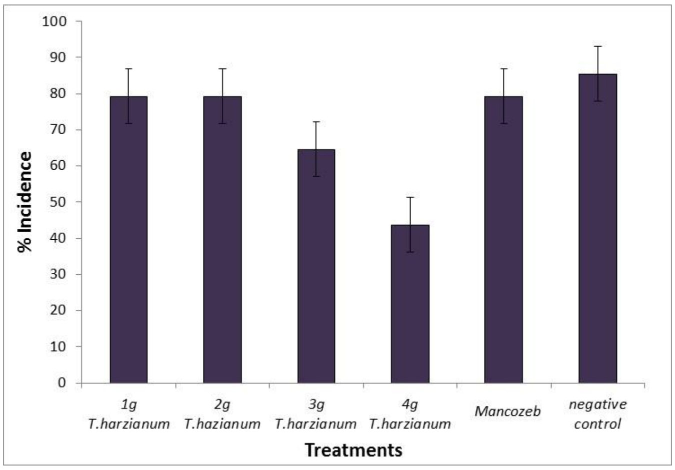

Figure 3: Efficacy of *T. harzianum* at different concentrations and Mancozeb treatments on total yield produced per plant at three weeks of harvesting. Data presented show means of three replicates. Error bars represent LSD at 5 % = 0.103 to separate means.

4. Discussion

4.1 Effects of *Trichoderma* on the radial mycelium diameter

The genus *Trichoderma* has been reported by several scientists to effectively act as a biological antagonist against several pathogens *in vitro* (Bokhari and Perveen, 2012). From the results, *Trichoderma harzianum* greatly reduced the mycelium diameter of *Fusarium oxysporum f spp lycospesici.* The results can be attributed to the presence of an inhibition zone that developed around the pathogen. This inhibition zone can be a strong evidence of the release of antibiotics such as trichodermol, harzianum, trichodernin and hazianolide which have the ability to restrict the growth of *F. oxysporum* in dual culture (Ramezani 2009; Bokhari and Perveen, 2012). The results are supported by other researchers who reported an inhibition percentage of *Fusarium oxysporum* spp *in vitro* of 53 %, 68.22 % and 51.4 %, respectively (Alwathnani et al., 2012; Sundaramoorthy and Balabaskar, 2013). Furthermore, *Trichoderma spp* produced antibiotics which reduced mycelium growth of *F. oxysporum phaseoli* in dual culture. In addition, (Zhao et al., 2014) clearly illustrated that the genus *Trichoderma* has the ability to secrete cell wall degrading enzymes such as chitins, 1.3, glucases and 1.6 glucases which target the cell wall of *F. oxysporum* causing loss of cell integrity hence restricting the mycelial growth of the pathogen. As observed from the microscopic studies *T. harzianum* grows towards *F. oxysporum* causing hyperparasitism by coiling its hyphae onto the hyphae of *F. oxysporum*, resulting in the death of the pathogen (Ommati and Zaker, 2012). This generally showed the efficacy of *T. harzianum* in controlling *F. oxysporum*.

4.2 Effects of *T. harzianum* on disease severity and incidence.

Disease development and symptom expression caused by *F. oxysporum* can be reduced by *T. harzianum* as a bio-control agent (Harman, 2005). The results of the current study revealed that *T. harzianum* at 3 g and 4 g per pot decreased disease severity and incidence compared to chemical and untreated treatments. These results are in agreement with who (Barari, 2015) reported mean disease severity of 37.74 % under *T. harzianum* treatments whereas in the positive control a value of 90-95 % severity was recorded. This is a clear illustration that *T. harzianum* showed strong evidence in the ability to suppress *F. oxysporum* infection in tomato plants. Furthermore, the potential of *T. harzianum* was also reported by (Moosa et al., 2017) who indicated greatly reduced disease severity with 41% and subsequently disease incidence by 55%. A group of researchers reported that *T. harzianum* as a soil borne antagonist exhibits different modes of action that suppress diseases development caused by soil borne pathogens particularly *F. oxysporum* in host plants (Gajera et al., 2013). The modes of action of *T. harzianum* include the ability to parasitize phytopathogens. It can also detect their presence in the soil rhizosphere, moving towards them and directly attacking the pathogens by winding around the hyphae of the pathogen (Gajera et al., 2013; Bokhari and Perveen, 2012). The reduced disease severity observed in the current study could be attributed to the fact that *T. harzianum* forms aspersoria and cell wall degrading enzymes such as chitinases, 1, 3 and 1,6 glucanases that act upon pathogen cell wall resulting in loss of cell wall integrity (Sharma et al., 2011; Gajera et al., 2013).

Additionally, reduced disease severity could also be explained by the findings of (Howell, 2003) who clearly illustrated that when combinations of hydrolytic enzymes and antibiotics are released into the *B. cinema* and *F. oxysporum,* there is a high occurrence of synergism in which cell wall degradation is highly needed to establish effective control of the pathogens. Other mechanisms employed by *T. harzianum* include high competitive ability for nutrients in the soil and the ability to produce effective sidephores which act on chelating iron to stop the activity of other pathogens (Saba et al., 2012). However, results from the study contradict with the findings of other researchers who found out that Mancozeb suppressed disease development moderately compared to *T. harzianum* treated plants (Doni et al., 2014; 2009). The differences in responses in Mancozeb could be due to differences in pathogen virulence since a highly virulent strain of *F. oxysporum* was used in the current study. The prevailing environmental conditions were highly favourable for disease development, and this reduced the effectiveness of Mancozeb hence the symptom development.

4.3 Effects of *T. harzianum* on the growth parameters and total yield

Several studies have clearly illustrated that *T. harzianum* does not only reduce disease development of *F. oxysporum* but it increases growth parameters such as plant height, number of leaves, number of flowers and concomitantly total yield of different crops including tomatoes (Zhang et al., 2018). The results from the current study showed that plants treated with high concentrations of *T. harzianum* increased all tomato growth and yield traits. These findings are in agreement with (Zhang et al., 2018) who found that plants treated with *T. harzianum* recorded increased flower number and leaf area than control in potato against *F. oxysporum*. Some researchers found out that there was an increase in the plant height and total yield of tomatoes in plants treated with *T. harzianum* (Sundaramoorthy and Balabaskar, 2013). This could be as a result of *T. harzianum* producing plant growth promoters such as auxin, cytokinin and gibberellin which play a vital role in rapid cell division thereby increasing the growth of the plant (Saba et al., 2012). In addition, *T. harzianum* modifies the host plant roots to efficiently uptake more nutrients to be utilized for increased leaf area, more flowers and finally increase in fruit number and size which constitute the total yield (Ommati and Zaker, 2012).

5. Conclusion

T. harzianum suppressed radial mycelium growth of *F.oxysporum f.sp lycospesici* and is associated as the production of antibiotics which inhibit growth of the fungus. *T. harzianum* at 3g and 4g reduced disease severity and disease incidence of *F. oxysporum f.sp lycopersici* in Floridade tomato variety. Furthermore, this biocontrol agent significantly increases total yield of tomatoes which demonstrates the effectiveness of *T. harzianum* in controlling *F. oxysporum* in tomatoes.

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Author Contributions

EN, planned and designed the project; TN, and GOV participated in the sampling, laboratory and greenhouse analysis; RJT, GE and MW conducted data analysis and drafted the manuscript. All authors wrote and discussed the manuscript.

Conflict of Interest

The authors declare that they do not have any conflict of interest.

Ethical Approval

This study does not involve any human or animal testing', and was approved by the Department of Crop Science, University of Zimbabwe.

Data Availability Statement

The data that support the findings of this study area available from the corresponding author upon reasonable request.

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