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RESEARCH ARTICLE

Assessing *In Vivo* Biological control Activity of *Trichoderma Harzianum* Against *Fusarium Oxysporum* F. Sp*. Lycopersici* Causing Vascular Wilt Disease in Tomatoes

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A B S T R A C T

Tomato vascular wilt disease, caused by the pathogen *Fusarium oxysporum* f. sp. *lycopersici*, is a major challenge for tomato production, leading to significant crop losses and economic impact. Biological control using isolates of *Trichoderma harzianum* has emerged as a promising alternative to chemical fungicides, offering an environmentally friendly approach to disease management. In this study, we examined the effectiveness of seed coating with *T. harzianum* isolates in controlling *F. oxysporum* f. sp. *lycopersici* in tomatoes. Tomato seeds were coated with *T. harzianum* two weeks prior to inoculation with the pathogen in a controlled greenhouse environment. Disease incidence, plant growth, and flowering were monitored to assess the efficacy of the treatment. The results demonstrated that seed coating with *T. harzianum* effectively controlled the pathogen, leading to a marked reduction in disease incidence (index = 1.6). Additionally, treated plants showed enhanced growth factors, including improved germination and flowering. This study confirms the potential of *T. harzianum* as a biological control agent, particularly when applied before pathogen inoculation. The significance of this research lies in its potential to improve tomato yields and reduce economic losses due to vascular wilt disease. By providing an alternative to chemical fungicides, this method offers environmental and public health benefits while supporting sustainable agricultural practices.

Keywords: *Trichoderma harzianum, Fusarium oxysporum* f.sp. *lycopersici, in vivo*, antagonism, stimulation, Algeria.

INTRODUCTION

Tomatoes are widely acknowledged as a critical crop for maintaining food security, being cultivated across a vast expanse of 3 million hectares worldwide utilizing both indoor and outdoor farming methods (Abdollahipour *et al.,* 2020; Yogalakshmi *et al.,* 2021; Aynalem *et al.,* 2022). In the northern African country of Algeria for instance tomato cultivation holds immense importance with over 26,000 hectares dedicated to its plantations (FAO, 2022). Nonetheless, tomato crop productivity faces challenges due to a variety of pests including microbial diseases (Duressa, 2018).

One of the most destructive pathogens affecting tomato plantations is Fusarium wilt, caused by *Fusarium oxysporum* f. sp. *Lycopersici* Yogalakshmi *et al.* (2021). This disease has resulted in significant economic losses globally due to its ability to severely damage tomato crops. It

manifests as yellowing lower foliage, inhibited growth, leaf wilting, and ultimately plant death (Prihatna *et al.,* 2018). The disease obstructs the plant's ability to transport water and nutrients effectively, thereby reducing yield (Li *et al.,* 2022; Srinivas *et al.,* 2019).

Current control methods for *F. oxysporum* are limited, with most efforts focused on preventing the disease rather than treating it (Perveen and Boukhari, 2012). Furthermore, the use of fungicides to control fungal diseases poses health risks and environmental concerns (Yassin *et al.,* 2021). Therefore, finding new, sustainable alternatives to manage Fusarium wilt remains crucial.

The aim of the current study was to evaluate the effectiveness of *Trichoderma harzianum* isolates as a biological control agent for *Fusarium oxysporum* f. sp. *lycopersici*. The study focused on coating tomato seeds with *T. harzianum* and adding it to the soil to mitigate the impact of Fusarium wilt on tomato crops.

The *Trichoderma* spp genus has an anti-fungal activity against *Fusarium* spp including the production of antibiotics, parasitizing other fungi, and competing for nutrients and space (Pavlovskaya *et al.,* 2020). These abilities make *Trichoderma* spp valuable for integrated pest management programs, offering a sustainable alternative to chemical fungicides and promoting healthier agricultural practices (Nagamani *et al.,* 2017).

MATERIALS AND METHODS

Seed biological treatment with *T. harzianum* as a biological control agent: Tomato seeds (*Solanum esculentum* L.) were first sanitized by soaking them in ethanol for 5 minutes to remove surface contaminants. Next, they were thoroughly rinsed with sterile distilled water for 10 minutes to remove any pesticide residues used in seed treatment, following the methods described by Benhamou *et al.* (1997) and Marzano *et al.* (2013). After sanitation, the seeds were submerged in a spore suspension of *T. harzianum* for 15 minutes. This treatment aims to allow the seeds to absorb the biological control agent for increased resistance against pathogens. The seeds were then partially dried and coated with talc powder to maintain the integrity of the biological control agent on the seed surface. Finally, the treated seeds were stored at a controlled temperature of 4 ± 1°C to preserve their viability and ensure the effectiveness of the treatment. Four tests were conducted to assess the efficacy of the treatments:

Control test (CT): Healthy, untreated seeds were used as a baseline for comparison with other tests.

Pathogen-inoculated substrate using untreated seeds (Fol): This test involved inoculating a substrate with a pathogen (presumably *Fusarium oxysporum* or another common tomato pathogen) and planting untreated seeds to observe the impact of the pathogen on untreated seeds.

Pathogen-inoculated substrate utilizing seeds coated with *T. harzianum* (Fol+Th): In this test, the pathogen-inoculated substrate was planted with seeds that had been treated with *T. harzianum*, aiming to observe the effects of the biological control agent on pathogen resistance.

Substrate containing *T. harzianum* (Th): This test used seeds treated with *T. harzianum* planted in a substrate containing the biological control agent, aiming to examine how the agent affects seed growth and resistance within a controlled environment.

In vivo* experiments assessing the effectiveness of *T. harzianum* as a biological control agent against pathogen *F. oxysporum* f. sp*. Lycopersici: Live cultures of *F.oxyporum* f. sp. *lycopersici* and *T. harzianum* were obtained from the collection of the Department of Agronomy (University of Chlef). These fungi were stored at a temperature of +4°C in a refrigerator to maintain their viability and slow down their growth, preventing overgrowth and contamination. The study was conducted at the experimental station of the University of Chlef (Lard Elbeida). A Petri plate containing a strain of *T. harzianum* cultivated on Potato dextrose agar (PDA) medium was placed in 1000 ml of sterile distilled water and stirred for 15 minutes to prepare the *T. harzianum* spore suspension to ensure even distribution of the biological control agent. A sterile cheesecloth screen was used to filter the mycelium, separating it from the fungal liquid. This process produced a retained spore concentration of 2.4×10^6 spores/ml.

Employing a seven-day-old culture in 1000 ml of previously autoclaved, the *F. oxysporum* f. sp*. lycopersici* inoculums were multiplied in Erlenmeyer flasks for 25 minutes at 120°C and 1 bar of pressure. After that, the flasks were shaken and incubated for seven days at 23°C (Sivakumar *et al.,* 2000) to give 2.3×10^6 spores/ml. To guarantee germination, the pots were incubated for four to five days at 28°C in an oven. Seedlings were placed into cell trays with pasteurized soil after they had pre-germinated. A glass greenhouse cell at 26°Cwith 12-hour photoperiod was employed to cultivate the seedlings and to facilitate optimal growth conditions (Hibar *et al.,* 2005; Marzano *et al.,* 2013). When tomato seedlings reached a stage of two evenly spaced leaves, they were transplanted (Woo *et al.,* 1996).

The seedlings were transferred into 11 x 11 x 6 cm^3 plastic pots each filled with 150g of soil and put in a greenhouse.

T. harzianum was introduced to the soil 15 days prior to the pathogenic agent's introduction to establish a beneficial presence, by giving 50 ml/pot concentration and 1.9×10^6 spores/ml (i.e., 0.3 ml/g of soil) spore concentration. Subsequently, the pathogenic agent (*F. oxysporum* f.sp *lycopersici*) was added to the soil at a concentration of 0.2×10^6 spores/ml using the same volume (50 ml/pot). Three seedlings were placed per pot after seven days. Ten milliliters of fungicide (Benomyl) were added following the pathogenic agent inoculation of the substrate (i.e. the soil). Control tests used untreated tomato seeds to provide a baseline for evaluating the efficacy of the biological control agent against the pathogen.

Score Rating Index of Fusarium Wilt in Tomato: According to Sivan *et al.* (1987), the plants undergoing testing should be monitored daily. The final readings for the inoculation and coating tests were obtained 53 days after seedling transplantation and 75 days after coated seed was sown, respectively. This time frame was selected to allow adequate growth of the plants and manifestation of disease symptoms. Abdelaziz *et al.* (2023), noted that disease symptoms became apparent after a period of 45 days. The Cal *et al.* (1995) symptom rating system for Fusarium wilt, which was caused by *Fusarium oxysporum* f. sp. *lycopersici,* was employed to evaluate disease severity. The score rating range consisted of:

1: Healthy plant, all leaves are green
2: Yellowing of the first leaf
3: Yellowing of basal leaves
4: Yellowing of some upper leaves and death of basal leaves
5: Wilt of some upper leaves and death of basal leaves
6: Dead plant

Based on this scoring, the disease index (DI) was evaluated according to the attack index recommended by Koudri and Zentou, (2006).

$$DI = \frac{Fi \times i}{N} \times 100$$

DI= Disease index

i : represents each degree of the scoring scale (e.g., 0 for healthy, 1 for mild symptoms, 2 for moderate symptoms, etc.).

F_i: is the number of plants at each degree ii.

N: is the total number of plants observed.

Using this approach provides a standardized way to quantify disease severity and compare different treatments' efficacy.

STATISTICAL ANALYSIS

ANOVA (Analysis of Variance) was employed to assess the impact of the experimental factors on the measured outcomes with a significance level set at $P < 0.05$. Six replications and fully randomized design ensured robustness and minimized the influence of confounding variables, enhancing the reliability of the results obtained through statistical analysis with the STAT.ITCF program.

RESULTS AND DISCUSSION

The use of the Fusarium wilt index in assessing the effectiveness of *T. harzianum* as a biological control agent against pathogen *F. oxysporum f. sp. Lycopersici:* In the test involving tomato seed coating with *T.harzianum*, no symptoms of Fusarium wilt disease were observed on plants inoculated with the pathogen. This can be attributed to the low quantity of pathogen *F. oxysporum* f.sp *oxysporum* spores (20 ml/150g of soil). Seedlings inoculated with both the pathogen (*F. oxysporum*) (Fol) and the antagonistic fungus (*T. harzianum*) (Th) had the lowest disease index of 1.6 for the Fol+Th (Figure 1). Seedlings treated only with the pathogen (Fol) had the highest index of 3.5compared to an index of 2.1 for plants transplanted into soil inoculated with the pathogen and treated with the fungicide Benomyl (i.e., Fol+Be). In control tests without *T. harzianum* or *F. oxysporum f. sp. lycopersici*, no symptoms of Fusarium wilt were observed.

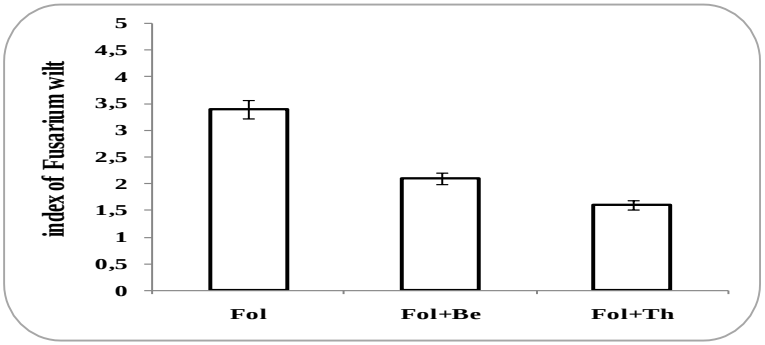

Figure 1. Index of the vascular wilt disease of tomato (Fol: *Fusarium oxysporum* f.sp *lycopersici*; Fol+Be: *Fusarium oxysporum f.sp lycopersici*+ Benomyl; Fol+Th: *Fusarium oxysporum* f.sp *lycopersici*+ *Trichoderma harzianum*)

The current results relating to the ability of *T. harzianum* to protect tomato seedlings showed that inoculation of the soil with this biological control agent (*T. harzianum*) significantly reduced the infectious power of the fungal pathogen *Fusarium oxysporum f.sp lycopersici* towards the tomato plant. Previous studies have shown that this phenomenon was probably due to the presence of numerous *T. harzianum* spores at each infection site of the plant (Bellahcene, 1990). Yassin *et al.* (2021), specified that *T. harzianum* can penetrate and then lyse the mycelium of the agent responsible for Fusarium. *Trichoderma* species have demonstrated a pervasive presence across various soil types and have been acknowledged for their potential efficacy as antagonistic or biological control agents against phytopathogenic microorganisms, as elucidated by Shahid *et al.* (2014) and Mazrou *et al.* (2020).

The infection of plants by the filamentous fungal pathogen *F. oxysporum* f.sp *lycopersici* could be explained by the penetration of the hyphae of the pathogen without contacting with *T. harzianum* or by the presence of a small quantity of spores of the antagonist; the infection sites of the host plant were possibly not saturated with *T. harzianum*. The inhibitory effect of *Trichoderma* sp against diverse plant pathogens may mediated via a range of mechanisms, encompassing antibiosis, mycoparasitism, competition for nutrients and habitat, and triggering systemic resistance in host plants (Ghazanfar *et al.,* 2018; Macías-Rodríguez *et al.,*2020). Furthermore, previous research has shown that the application of *T. harzianum* in the growing medium significantly reduced the incidence of Fusarium wilt during the growing season and consequently a significant increase in the total yield was obtained (i.e., 18.8%) (Hibar *et al.,* 2005). For this reason, in the last years, there has been a substantial rise in the utilization of *Trichoderma* species for biocontrol purposes (Yogalakshmi *et al.,* 2021). Larkin and Fravel (1998) documented that the application of *Trichoderma harzianum* in granular form resulting in a reduction in the severity of Fusarium wilt by *F. oxysporum* f.sp. *lycopersici* by approximately 62 to 68% in tomato plants.

The *in vivo* study carried out by Abdelaziz *et al.,* (2023), confirmed that *T. harzianum* can reduce the pathogenic power of *F. oxysporum* on pepper plants. Biological control relies on the employment of microorganisms to effectively diminish the proliferation of pathogenic parasites, particularly those inhabiting soil, to mitigate their adverse impacts, as reported by Attia *et al.,* (2022) and Daigham *et al.,* (2023). *In vitro*, *T. harzianum* was very effective against the agent of vascular wilt of tomatoes. Nevertheless, it can be argued that extrapolating findings obtained in a Petri dish on agar medium to a greenhouse setting poses challenges due to the distinct conditions governing fungal development.

Bellahcene, (1990) suggested that the antagonistic or biological control effectiveness of *Trichoderma* against the fungal *F. oxysporum* might be partially attributed to its antibiotic activity. Yogalakshmi *et al.,* (2021) reported that *Trichoderma* demonstrated remarkable antimycotic efficacy against *F. oxysporum* f.sp*. lycopersici*. *Trichoderma* has also demonstrated effectiveness in diminishing both the occurrence and severity of the disease within non-sterile soil conditions (Nguyen *et al.,* 2023).

Germination rate measurements of tomato seeds: Analysis of variance revealed significant differences for the tomato seed coating factor (*p*-Value=0.00001). Figure 2 shows that the germination rate reached the maximum for seeds coated with *T. harzianum* (Th) compared to control test (CT) and soil inoculated with *F. oxysporum* (Fol). In addition, seeds coated with *T. harzianum* and inoculated with the pathogen (Th+Fol) had a higher germination rate than other tests (i.e., CT and Fol). Indeed, Windham *et al.,* (1986), showed that the addition of *T. harzianum* and *T. koningii* to the soil increased the percentage of germination of tobacco and tomato seeds. Some authors have suggested that this phenomenon may be due to secretions of specific molecules by *T.harzianum* stimulating seed germination (Celar and Valic, 2005; Vitti *et al.,* 2022).

Numerous studies have highlighted the stimulatory effect of *Trichoderma* on the germination of tomato seeds. Indeed, Inbar *et al.,* (1994), reported that in addition to their bio-control activities, *Trichoderma* species can promote plant growth. Once established, this genus can have a stimulating effect on the plant in the absence of pathogenic fungi (Caron, 2002). The best results obtained were on the percentage of germination and the number of flowers produced per plant. The germination rate of seeds coated with *T. harzianum* reached the maximum (i.e., 100%) compared to the control tests which were only 90%. The current study results partly matched those obtained by Silvy and Guy, (2002) and Datnoff and Pernezny, (1998). Liton *et al.,* (2019), and Abdelaziz *et al.,* (2023) also noted that *T. harzianum* played a substantial role in improving soil fertility and increasing crop productivity.

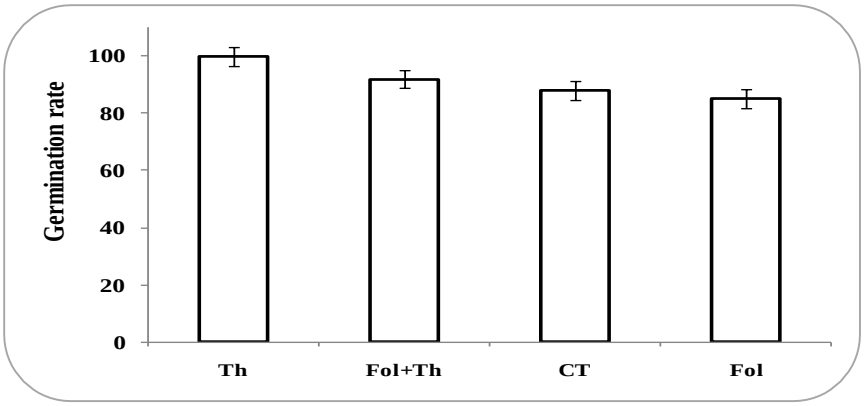

Figure 2. Germination rate measurement of tomato seeds (Th: *Trichoderma harzianum*; Fol+Th: *Fusarium oxysporum* f.sp *lycopersici*+ *Trichoderma harzianum*); CT: Control test; Fol: *Fusarium oxysporum* f.sp *lycopersici*)

Assessment of flowering rate of tomato seedlings: The analysis of variance revealed significant results (*p-Value*=0.001). The Newman Keuls test allowed for the classification of the three groups: Th>Fol+Th> Ct>Fol. The current results showed that coating tomato seeds with *T.harzianum* stimulated the plant to produce more flowers compared to other tests (Figure 3). Hibar *et al.,* (2006) reported that the use of biological fungicide of *T. harzianum* stimulated tomato plants to produce more fruits of better quality compared to those treated with the fungicide Hymexazol.

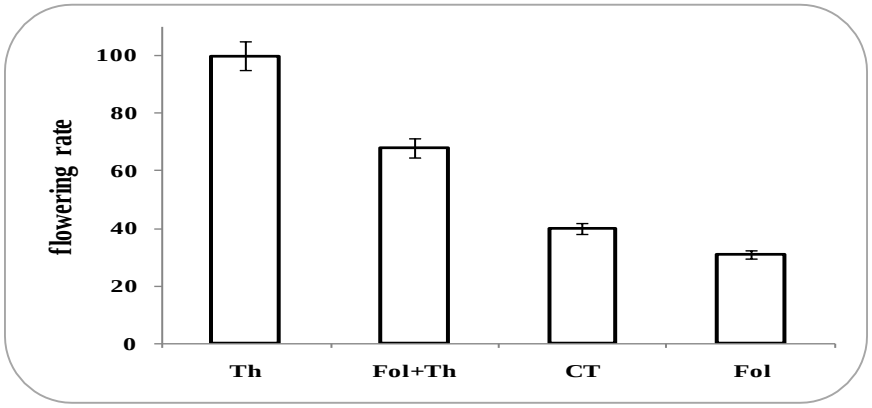

Figure 3. Flowering rate of tomato seedlings (Th: *Trichoderma harzianum*; Fol+Th: *Fusarium oxysporum* f.sp *lycopersici*+ *Trichoderma harzianum*); CT: Control test; Fol: *Fusarium oxysporum* f.sp *lycopersici*)

CONCLUSION

The study successfully demonstrates the effectiveness of *T. harzianum* as a biological control agent against *F. oxysporum* f. sp. *lycopersici* in tomatoes. By introducing *T. harzianum* into the growing soil, the incidence of vascular wilt disease was significantly reduced, leading to improved seed germination and vegetative growth. These findings highlight the potential of T. *harzianum* as a sustainable and environmentally friendly approach to managing Fusarium wilt in tomato crops.

The practical implications of this research are significant for farmers and agriculturalists aiming to enhance tomato production while reducing reliance on chemical fungicides. The use of *T. harzianum* not only provides an effective means of disease management but also supports overall plant health, potentially leading to higher yields and better-quality crops.

Future research directions should focus on gaining a deeper understanding of the mode of action of *T. harzianum* and its interactions with other soil microbes and environmental factors. Exploring these relationships could lead to the development of more robust and integrated biological control strategies. Additionally, further studies should assess the long-term effects of *T. harzianum* application on soil health and plant-microbe interactions.

In conclusion, the study contributes valuable insights into the biological control of soil-borne diseases in tomatoes. By advancing our understanding of the role of *T. harzianum* in agricultural systems, we can move closer to more sustainable and effective approaches to crop protection and production.

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