

Article

Sustainable Management of Soil-Borne Bacterium *Ralstonia solanacearum* In Vitro and In Vivo through Fungal Metabolites of Different *Trichoderma* spp.

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Abstract: The efficacy of traditional control measures for the management of plant pathogens is decreasing, and the resistance of these pathogens to pesticides is increasing, which poses a serious threat to global food security. The exploration of novel and efficient management measures to combat plant disease is an urgent need at this time. In this study, fungal metabolites from three *Trichoderma* spp. (*T. harzianum*, *T. virens* and *T. koningii*) were prepared on three different growth media (STP, MOF and supermalt (SuM)). The fungal metabolites were tested in vitro and in vivo from March–April 2020 under greenhouse conditions in a pot experiment utilizing completely randomized design to test their management of the bacterial wilt disease caused by *R. solanacearum* in tomato plants. The effect of the fungal metabolites on bacterial cell morphology was also investigated through scanning electron microscopy (SEM) analysis. In vitro investigation showed that the fungal metabolites of *T. harzianum* obtained on the STP medium were the most effective in inhibiting in vitro bacterial growth and produced a 17.6 mm growth inhibition zone. SEM analysis confirms the rupture of the cell walls and cell membranes of the bacterium, along with the leakage of its cell contents. Generally, fungal metabolites obtained on an STP medium showed higher activity than those obtained on the other two media, and these metabolites were then evaluated in vivo according to three application times (0 days before transplantation (DBT), 4 DBT and 8 DBT) in a greenhouse trial to examine their ability to manage *R. solanacearum* in tomato plants. Consistent with in vitro results, the results from the greenhouse studies showed a level of higher anti-bacterial activity of *T. harzianum* metabolites than they did for the metabolites of other fungi, while among the three application times, the longest time (8 DBT) was more effective in controlling bacterial wilt disease in tomato plants. Metabolites of *T. harzianum* applied at 8 DBT caused the maximum decrease in soil bacterial population (1.526 log cfu/g), resulting in the lowest level of disease severity (area under disease progressive curve (AUDPC) value: 400), and maximum plant freshness (with a resulting biomass of 36.7 g, a root length of 18.3 cm and a plant height of 33.0 cm). It can be concluded that *T. harzianum* metabolites obtained on an STP medium, when applied after 8 DBT, can suppress soil bacterial population and enhance plant growth, and thus can be used as a safe, environmentally-conscious and consumer-friendly approach to managing bacterial wilt disease in tomato plants and possibly other crops.



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1. Introduction

The tomato is the most consumed vegetable crop, and is ranked as the second most important after the potato. Because of its potential health benefits and economic importance,

the tomato's total cultivation area and level of production is increasing globally. Approximately 182.3 Mt of tomato fruits are produced annually on approximately 4.85 million hectares of land. China is the world's leading producer of tomatoes, accounting for 32.6% of the world's tomato production [1]. Due to having several health-promoting compounds, tomatoes can easily be incorporated into a balanced diet as sources of nutrients [2]. Tomatoes can be used as fresh fruits, as well as in many processed forms, including sauces, juices and soups [3,4]. The nutritional characteristics of tomato plants can be explained by the presence of health-promoting compounds, such as carotenoids, vitamins and phenolic compounds [2,4–6]. Tomatoes also have a range of other nutritionally important metabolites, including ascorbic acid, sucrose, hexoses, malate and citrate [4].

Tomato production faces several challenges globally. Several factors (such as management strategies, diseases, cultivar selection, pests, etc.) can affect tomato yield and production, the most important of which are diseases [7,8]. Among the effects caused by diseases, the bacterial wilt caused by *Ralstonia solanacearum* is one of the most serious symptoms afflicting the tomato plant, causing huge losses in yield worldwide in warm temperature, sub-tropical and tropical regions [9–12]. In China, bacterial wilt disease commonly occurs in the eastern and southern regions rather than in the western and northern regions. The 30 provinces listed in the study of Jiang, G. et al. have reported high incidences of bacterial wilt [13]. Depending on the soil temperature and crop season, the disease incidence in tomato fields ranges between 10% and 95% [14–16].

R. solanacearum is a Gram-negative soil-borne plant pathogenic bacteria that causes huge losses to agriculture worldwide [17]. The bacterium has a wide host range, infecting more than 200 plant species. Infection starts via the penetration of root tissues, then aggressive colonization occurs on the root system of the infected plant and leads to a systemic spread of the pathogen, producing shoot symptoms [18,19]. *R. solanacearum* can live in soil for a long time in several environments [20,21]. The bacterium's soil-borne nature, survivability in different environments, genetic diversity and wide host range make it difficult to manage. However, an integrated disease management approach combining host plant resistance with cultural and biological control measures appears to be effective. Although attempts have been made to manage *R. solanacearum* with different level of success, there is still a great opportunity to solve this problem by finding a stable solution.

A potential solution for the management of plant pathogens (which is also environmentally-conscious and consumer-friendly) is the utilization of bioactive metabolites/compounds obtained from microorganisms [22]. Among microorganisms, fungi are special in their production of a variety of anti-microbial metabolites. Several fungus genera are well known for their capacity to produce organic acids, antibiotics, enzymes, vitamins and polysaccharides [23]. Among fungi, the genus *Trichoderma* contains fungal species that are well-studied biocontrol agents today [24,25]. Bioactive metabolites secreted by *Trichoderma* spp. can be useful to several industries, as well as to the agricultural and medical fields. These *Trichoderma* metabolites, including terpenes, pyrones, gliotoxin, gliovirin, peptaibols and polyketides, exhibit anti-microbial activities against several plant pathogenic bacteria, yeasts and fungi [26–28]. The metabolite production ability of the fungus is generally species/isolate dependent. A given *Trichoderma* species can secrete various metabolic compounds and, in a similar manner, a given compound can be secreted by different *Trichoderma* species [29]. Moreover, different isolates of the same species can produce different compounds [30]. Recently, soil applications of *Trichoderma* spp. in the form of fungal suspension were evaluated for their ability to manage *R. solanacearum* in potato crops [31,32]. However, the efficacy of *Trichoderma* metabolites in the management of the bacterial wilt of tomato plants has not been reported yet. In this study, the metabolites were prepared from three *Trichoderma* spp. on three different growth media and evaluated in vitro for their anti-bacterial potential against *R. solanacearum*. Those metabolites that showed higher activity were further evaluated in vivo to test their management of the bacterial wilt disease caused by *R. solanacearum* in tomato plants at three different application times in a greenhouse trial. Scanning electron microscopy (SEM) analysis was also conducted to observe the morphological destruction

of the bacterial cell at a cellular level, and soil bacterial population was investigated under the influence of fungal metabolites.

2. Materials and Methods

2.1. Fungal and Bacterial Cultures

The pre-identified preserved fungal cultures of *T. harzianum* (T180), *T. virens* (T136) and *T. koningii* (T176) and a bacterial culture of *R. solanacearum* (RS13) were procured from College of Life Science, Agriculture and Forestry, Qiqihar University in 2020. The fresh culture of bacteria was obtained from a lysogeny broth (LB) medium incubated at 28 °C for 24–48 h, while fresh fungal cultures were obtained from a potato dextrose agar (PDA) medium incubated at 28 °C for 7–10 days [33].

2.2. Fungal Metabolites Preparations

Three fungal species were grown on three liquid media to produce anti-bacterial metabolites. The detailed recipe of the media is given in Table S1. From the freshly prepared fungal culture, five plugs were added to a 100 mL fungal seed medium (with a composition of 10 g of yeast extract, 4 g of agar, 40 g of maltose and 10 g/L of bacto-neopeptone) in one 500 mL flask and kept on shaking incubation for 4 days at 25 °C [33]. Then, 5 mL of the obtained culture broth was used to inoculate each of the flasks containing 100 mL of the three liquid media used in this study and incubated at 25 °C for 2 weeks. After 2 weeks, the fungal metabolites were extracted using the extraction solvent ethyl acetate [33]. After drying the extraction solvent, the fungal metabolites were collected, preserved at 4 °C and used for their anti-bacterial potential against *R. solanacearum*.

2.3. In Vitro Test

The anti-bacterial efficacy of the fungal metabolites was checked using the agar well diffusion method [34]. The fungal metabolites were dissolved in methanol to a concentration of 150 mg mL⁻¹. In a petri plate, the LB medium, with 0.5 mL of bacterial suspension (10⁸ cfu), was poured and allowed to cool to solidification. A total of five wells of uniform size were made in the solidified LB medium. Three wells were filled with 10 µL of the fungal metabolites obtained from the three growth media, one well was filled with a positive control (streptomycin (an antibiotic)) and one well was filled with a negative control (methanol). The plates were incubated at 28 °C for 24 h, and anti-bacterial activity was evaluated by measuring the inhibition zone. The test was repeated once, with five replicates in each test, and the results were presented as mean values.

2.4. Morphological Observation of the Bacterial Cells

The morphological destruction of the bacterial cells was evaluated using scanning electron microscopy (SEM). The bacterial samples were prepared by cutting small pieces of agar (with a thickness of 5 mm) from both the untreated control and from the inhibition zone of the best treatment and fixed in 2.5% (v/v) glutaraldehyde for one hour. After being washed with the phosphate buffer, the agar pieces were fixed in 1% (w/v) osmium tetroxide. The samples were then washed in a phosphate buffer and dehydrated in 30%, 50%, 70%, 90%, and 95% ethanol series, for 15 minutes each. After this, the samples were subjected to 100% ethanol and CO₂. The fully dried samples were coated with gold sputtering and used for SEM analysis [35].

2.5. In Vivo Test

The fungal metabolites obtained on the STP medium (showing maximum in vitro anti-bacterial activity) were tested in vivo for their ability to manage *R. solanacearum* in tomato plants under greenhouse conditions. In brief, plastic pots with a diameter of 20 cm were filled with 1 kg of sterilized soil per pot. Thirty-five mL of a bacterial suspension (10⁸ cfu mL⁻¹) was poured in the center of each pot. For each of the three *Trichoderma* species tested, 15 mL of the fungal metabolites (9% w/v) obtained after a fungus cultivation on the

STP medium were applied to the soil at three positions in the center of each pot 3 days after bacterial inoculation of the soil. One healthy tomato seedling of uniform size (25 days old) was transplanted into each pot. The fungal metabolites were applied separately at three different application times (i.e., 0, 4 and 8 days before transplantation (DBT)). The irrigation and fertilizer requirements were fulfilled according to the horticultural recommendations for tomato plants [36]. The experiment was repeated once, with five replications of the experiment conducted without any modification.

2.6. Data Parameters

The experiment was terminated after the 60th day since the transplantation was completed and the data on different plant growth parameters (i.e., fresh biomass, root length, plant height, bacterial population in soil and disease severity) were recorded.

2.7. Bacterial Population in Soil

To measure the number of bacteria in soil, four samples of soil cores were collected randomly from a 10–12 cm depth in each pot, and a composite sample was made by mixing the four samples together [37]. Three sub-samples were made from the composite samples and diluted serially up to 10^{-7} . The 100 μ L suspension from each sub-sample was poured on a petri plate containing 25 mL of the selective media tetrazolium chloride nutrient agar TZCNA [38]. The bacterial colonies with an off-white color and red center were counted and soil bacterial population was presented in terms of cfu g^{-1} of soil.

2.8. Area under Disease Progressive Curve (AUDPC)

The disease severity of the bacterial wilt affecting the tomato plants was recorded using a specified scale and converted to % disease severity [39]. The disease severity was measured 5 times every 12 days and converted to an AUDPC value using the AUDPC formula [40].

2.9. Statistical Analysis

Results were presented as mean values \pm standard deviation. Analysis was done using ANOVA with IBMSPSS Statistix 20 software. Factorial arrangements were applied to both in vitro and in vivo data. Least significant difference (LSD) test was conducted to indicate significant differences among the treatments [41].

3. Results

3.1. In Vitro Test

Fungal metabolites of the three *Trichoderma* spp. (*T. harzianum*, *T. virens* and *T. koningii*) obtained on three different growth media (STP, MOF and supermalt (SuM)) were tested for in vitro anti-bacterial activity against *R. solanacearum*. Significant differences in anti-bacterial activity were observed among the treatments (Figures 1 and 2). Among different fungal species, the fungal extracts of *T. harzianum* showed the highest anti-bacterial activity, and across different media, the fungal metabolites obtained on the STP media showed the highest anti-bacterial activity. The fungal metabolites obtained on the MOF and SuM media exhibited similar anti-bacterial activity from all three fungal species. After that of the positive control (streptomycin), the maximum growth inhibition zone was produced by the fungal metabolites of *T. harzianum* obtained on the STP media, followed by the *T. virens* fungal extracts obtained on the STP media. Growth inhibition zones of 17.6 mm, 13.1 mm and 12.6 mm were produced by *T. harzianum* metabolites obtained on the STP, MOF and SuM media, respectively. The inhibition zones for *T. virens* metabolites obtained on the STP, MOF and SuM media were recorded as 13.2 mm, 9.6 mm and 8.1 mm, respectively, while *T. koningii* metabolites obtained on STP, MOF and SuM media produced 10.3 mm, 7.4 mm and 7.2 mm growth inhibition zones, respectively. The negative control methanol did not show any activity and produced no inhibition zone.

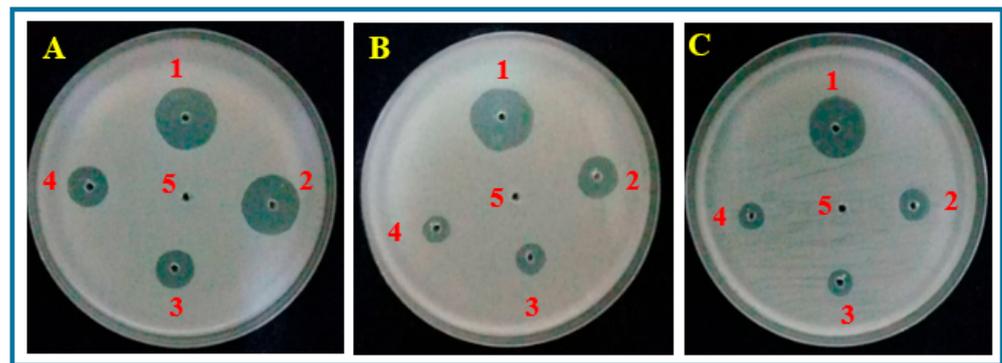


Figure 1. Zones of inhibition produced by fungal metabolites of *Trichoderma* spp. obtained on different growth media. **A:** *T. harzianum*, **B:** *T. virens* and **C:** *T. koningii*. 1: Streptomycin (positive control), 2: STP media, 3: MOF media, 4: Supermalt (SuM) media and 5: Methanol (negative control).

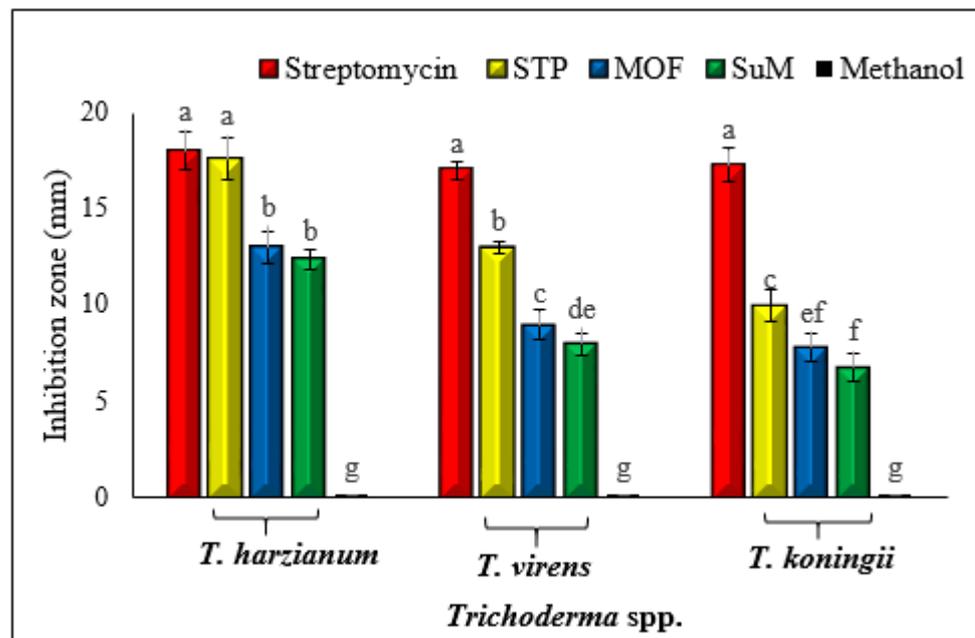


Figure 2. Anti-bacterial activity of fungal metabolites of *Trichoderma* spp. obtained on different growth media. Each value is a mean of five replicates \pm standard deviation. Means with similar lettering shows no significant difference among the treatments ($p \leq 0.05$), as per Fisher's protected least significant difference (LSD) test.

3.2. Bacterial Cell Morphology

The morphological alterations in bacterial cells were observed through SEM analysis. The treated and untreated bacterial cells exhibited a clear difference in their morphology (Figure 3). The morphology of bacterial cells treated with fungal metabolites (the most effective treatment of which was *T. harzianum* metabolites obtained on STP media) underwent tremendous destruction. The cell content leakage, cell wall degradation and membrane breakdown can be easily observed. The bacterial cells belonging to control group (without any treatment) showed normal rod-shaped uniform bacterial morphology without any interference.

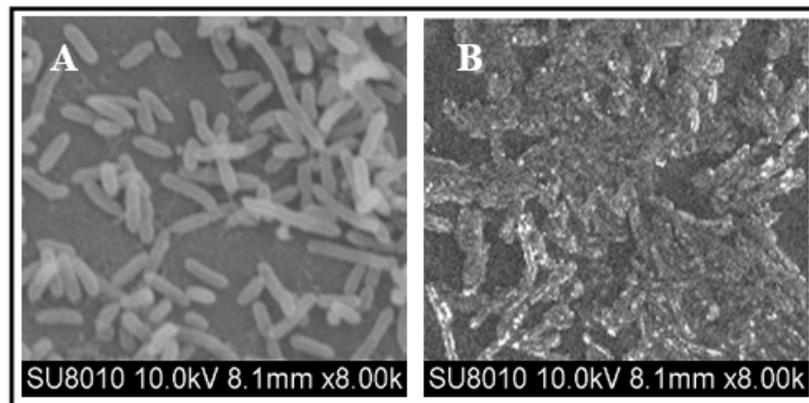


Figure 3. Scanning electron microscopy (SEM) micrograph of *Ralstonia solanacearum* cells (A) in the control without treatment and (B) under the influence of *T. harzianum* metabolites obtained on STP.

3.3. Plant Growth

In a green house experiment, the fungal metabolites of three *Trichoderma* spp. (*T. harzianum*, *T. virens* and *T. koningii*) obtained on an STP medium and applied at three different times (0, 4 and 8 DBT) were evaluated for their ability to manage bacterial wilt disease in tomato plants. The results showed significant differences both for *Trichoderma* spp. and application times in terms of affecting plant growth parameters such as fresh biomass, root length and plant height (Figure 4). Among the three application times, 8 DBT gave the best results, and among the three fungal metabolites, the *T. harzianum* metabolites gave the best results. The maximum fresh biomass (51.3 ± 3.6 g), root length (27.4 ± 2.4 mm) and plant height (46.3 ± 3.1) were achieved when *T. harzianum* metabolites were applied at 8 DBT. The minimum effect on plant growth was noticed for the treatment combination of *T. koningii* and 0 DBT that gave 25.2 ± 1.7 g, 10.5 ± 0.9 cm and 16.5 ± 2.1 cm in fresh biomass, root length and plant height, respectively. A similar trend in the results was noticed in the repeated experiment, where the application time 8 DBT and the fungal metabolites of *T. harzianum* gave the best plant growth as compared to other application times and fungal metabolites (Tables 1–3, Experiment II).



Figure 4. Tomato plants grown in soil inoculated with *R. solanacearum* and treated with fungal metabolites applied at 8 days before transplantation (DBT). 1: Control (inoculated and untreated), 2: *T. koningii* metabolites, 3: *T. virens* metabolites and 4: *T. harzianum* metabolites.

Table 1. Effect of fungal metabolites applied at different application times on fresh biomass (g) of tomato plants grown in soil inoculated with *R. solanacearum*.

Application Time (DBT)	<i>Trichoderma</i> spp.			
	<i>T. harzianum</i>	<i>T. virens</i>	<i>T. koningii</i>	Control
Experiment I				
0	32.1 ± 3.1 c	27.3 ± 2.5 de	25.2 ± 1.7 e	24.4 ± 1.8 e
4	40.3 ± 3.1 b	33.4 ± 3.2 c	27.7 ± 2.7 de	24.2 ± 1.9 e
8	51.3 ± 3.6 a	39.7 ± 2.9 b	30.4 ± 2.8 cd	25.6 ± 2.5 e
Experiment II				
0	35.5 ± 2.8 ef	33.2 ± 3.1 ef	30.3 ± 2.8 f	28.5 ± 2.3 f
4	46.1 ± 2.9 b	41.6 ± 3.2 c	35.4 ± 3.4 ef	29.3 ± 2.2 f
8	59.2 ± 3.8 a	47.2 ± 3.5 b	38.6 ± 3.2 de	27.4 ± 2.2 f

Each value is a mean of five replicates ± standard deviation. Similar lettering shows no significant difference among the treatments ($p \leq 0.05$) as per Fisher's protected LSD test.

Table 2. Effect of fungal metabolites applied at different application times on root length (cm) of tomato plants grown in soil inoculated with *R. solanacearum*.

Application Time (DBT)	<i>Trichoderma</i> spp.			
	<i>T. harzianum</i>	<i>T. virens</i>	<i>T. koningii</i>	Control
Experiment I				
0	17.4 ± 2.1 c	14.3 ± 1.4 ef	10.5 ± 0.9 gh	9.2 ± 1.1 hi
4	20.6 ± 1.6 b	16.4 ± 1.3 cd	12.2 ± 1.0 fg	9.1 ± 0.8 hi
8	27.4 ± 2.4 a	22.3 ± 2.1 b	15.6 ± 1.6 de	8.1 ± 0.9 i
Experiment II				
0	19.2 ± 2.3 c	16.6 ± 2.1 d	14.3 ± 1.7 de	12.4 ± 1.4 e
4	23.4 ± 2.1 b	19.7 ± 1.8 c	16.2 ± 1.4 d	13.5 ± 1.2 e
8	28.7 ± 3.1 a	23.3 ± 2.4 b	19.6 ± 1.8 c	12.8 ± 1.0 e

Each value is a mean of five replicates ± standard deviation. Similar lettering shows no significant difference among the treatments ($p \leq 0.05$) as per Fisher's protected LSD test.

Table 3. Effect of fungal metabolites applied at different application times on plant height (cm) of tomato plants grown in soil inoculated with *R. solanacearum*.

Application Time (DBT)	<i>Trichoderma</i> spp.			
	<i>T. harzianum</i>	<i>T. virens</i>	<i>T. koningii</i>	Control
Experiment I				
0	23.9 ± 2.6 d	18.3 ± 2.4 e	16.5 ± 2.1 e	15.8 ± 1.8 e
4	40.6 ± 3.3 b	33.3 ± 2.4 c	26.3 ± 1.9 d	17.2 ± 1.2 e
8	46.3 ± 3.3 a	38.5 ± 3.1 b	32.3 ± 2.6 c	15.0 ± 1.5 e
Experiment II				
0	25.6 ± 2.6 d	23.2 ± 2.5 de	19.6 ± 3.2 e	20.3 ± 2.1 e
4	39.2 ± 2.8 b	31.3 ± 2.7 c	25.5 ± 2.2 d	19.2 ± 1.3 e
8	47.3 ± 3.1 a	40.6 ± 2.2 b	32.2 ± 2.5 c	18.1 ± 2.1 e

Each value is a mean of five replicates ± standard deviation. Similar lettering shows no significant difference among the treatments ($p \leq 0.05$) as per Fisher's protected LSD test.

3.4. Soil Bacterial Population

At the beginning and the end of the experiment, the soil bacterial population was counted. The difference in the number of bacteria from the beginning to the end is classified by the decrease in bacterial population. The results showed that soil treatment with fungal extracts significantly affected the soil bacterial population. The three application times of

the fungal extract also showed significant differences regarding the decrease in soil bacterial population. Among application times, the 8 DBT was the most effective at suppressing soil bacterial population, especially when combined with *T. harzianum* metabolites, which were the most highly active of the fungal metabolites in suppressing soil bacterial population. *T. harzianum* metabolites applied at 8 DBT showed a maximum decrease of 1.526 log cfu/g in soil bacterial population, followed by the decrease exhibited by *T. viresns*. Meanwhile, among all the treatments, *T. konigii* metabolites applied at 0 DBT exhibited the lowest decrease (0.686 log cfu/g) in soil bacterial population (Figure 5A). Similar results were shown by the treatments when the experiment was repeated (Figure 5B).

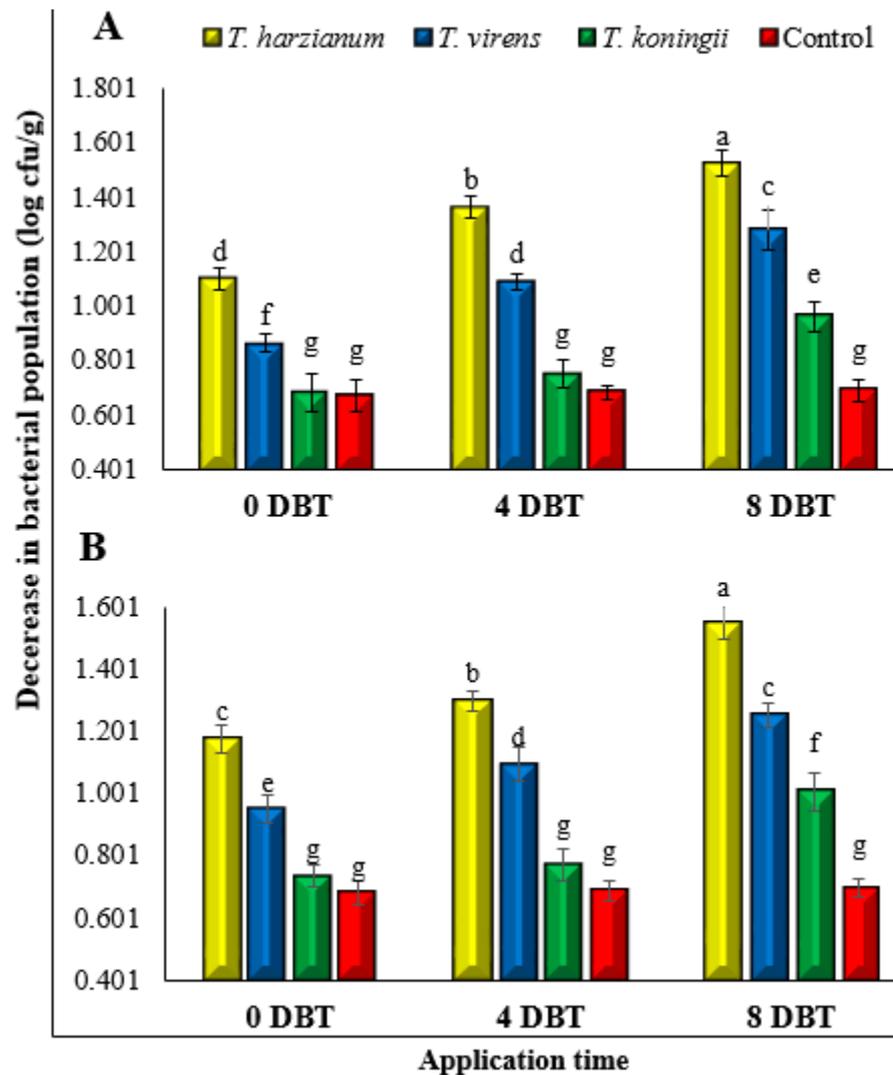


Figure 5. Decrease in soil bacterial population (in log cfu/g) affected by fungal metabolites of *T. harzianum*, *T. viresns* and *T. konigii* obtained on the STP medium applied at different application times (0 DBT, 4 DBT and 8 DBT). Each value is a mean of five replicates \pm standard deviation. Means having similar lettering shows no significant difference among the main effect ($p \leq 0.05$) as per Fisher's protected LSD test. **A:** Experiment I. **B:** Experiment II.

3.5. AUDPC

The data recorded on disease severity of the tomato plants grown in soil treated with fungal extracts at different application times were changed to AUDPC. Results showed significant differences among the treatments. The plants grown in untreated control soil showed the maximum AUDPC value and died completely. Compared to the control, the

plants grown in treated soil exhibited lower AUDPC values. Among fungal spp., the metabolites of *T. harzianum* were the most effective in lowering the AUDPC value, and among application times, 8 DBT was the most effective in lowering the AUDPC value. The fungal metabolites of *T. harzianum* applied at 8 DBT had the lowest AUDPC value (400). The maximum AUDPC value (1750) was shown by plants grown in soil treated with *T. koningi* applied at 0 DBT (Figure 6A). The experiment was repeated without any modification and similar results were obtained (Figure 6B).

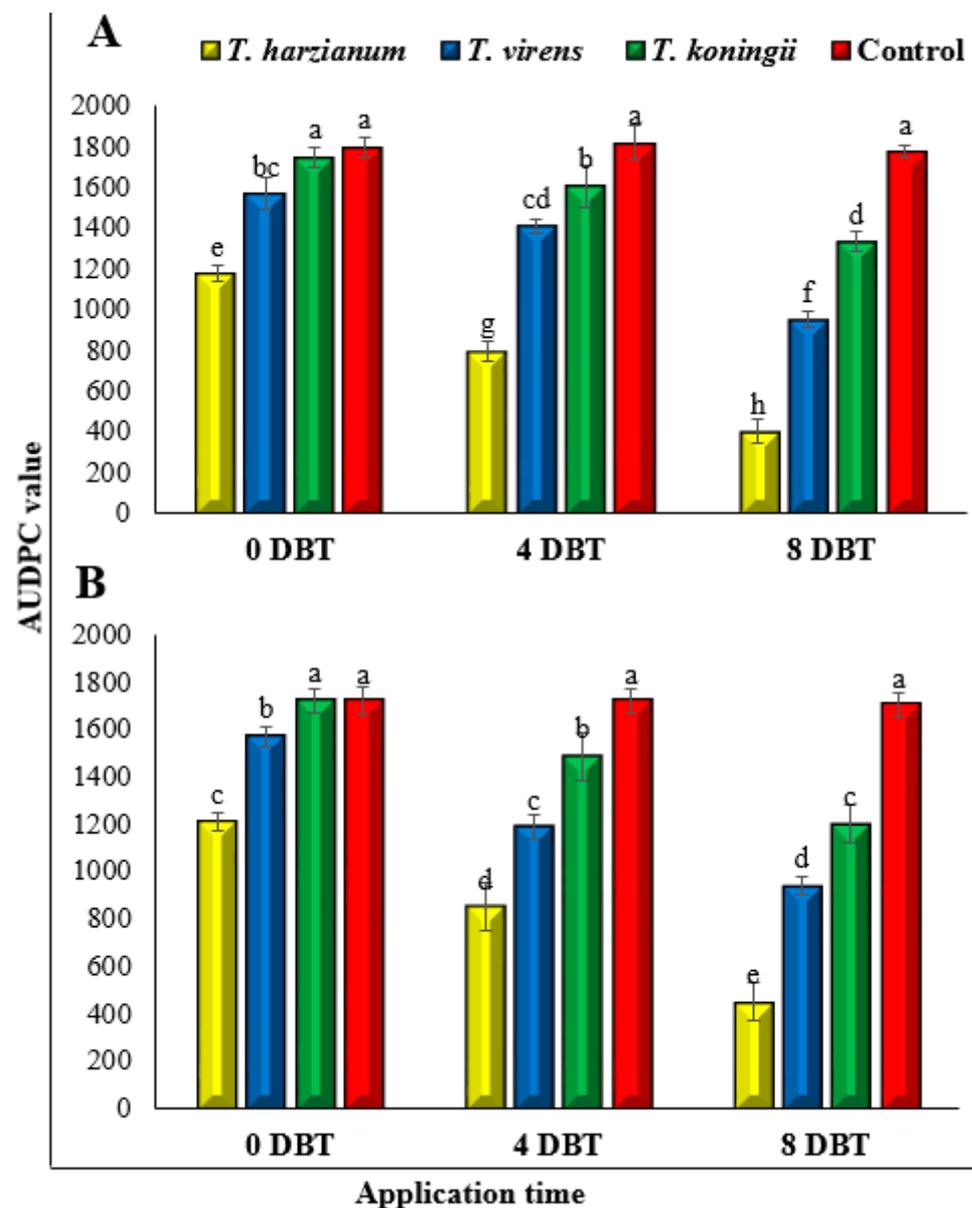


Figure 6. The area under disease progressive curve (AUDPC) affected by the fungal metabolites of *T. harzianum*, *T. virens* and *T. koningi* obtained on an STP medium applied at different application times (0 DBT, 4 DBT and 8 DBT). Each value is a mean of five replicates \pm standard deviation. Means having similar lettering shows no significant difference among the main effect ($p \leq 0.05$) as per Fisher's protected LSD test. **A:** Experiment I and **B:** Experiment II.

4. Discussion

Managing phytopathogens through the metabolites of biocontrol agents that are biologically active is a potentially environmentally-conscious and consumer friendly approach [22]. In this study, the metabolites of three *Trichoderma* spp. obtained on three different growth media, were tested for their anti-bacterial potential in managing *R. solanacearum* both in vitro and in vivo in tomato plants.

The results from in vitro evaluation showed the strong bioactivity of the fungal extracts. All of the fungal metabolites were not equally active against *R. solanacearum*. Generally, the fungal metabolites of *T. harzianum* exhibited higher anti-bacterial activity, followed by the fungal metabolites of *T. virens* and *T. koningi*. This is due to the fact that different species (as well as different strains) of the fungus produce different secondary metabolites (SMs). It is reported that different *Trichoderma* isolates exhibited different extents of anti-microbial activity against pathogens [32]. The results obtained in this study are in line with previously reported results, in which metabolites of different *Trichoderma* spp. were evaluated in vitro against different plant pathogens, and it was found that *T. harzianum* metabolites were more active than other species [33]. The anti-bacterial activity of the fungal metabolites could be attributed to the several anti-bacterial compounds present in the metabolites. Different bioactive compounds have previously been reported from *Trichoderma* spp. and used against plant pathogenic bacteria [42–45]. Several studies have also reported the ability of SMs to inhibit nucleic acid and cell wall synthesis and destroy membranes [46–48]. The lysosime was reported to cause membrane breakdown and cell content leakage, which lead to cell death [49]. The analysis of bacterial cell morphology in this study also confirms that these mechanisms (such as the disintegration of bacterial cell wall and membranes) can be easily seen in SEM micrographs.

The results from the in vivo tests indicated that fungal metabolites, when applied to soil infested with *R. solanacearum*, can suppress the bacterial population in soil, reducing the disease severity and improving plant growth. Consistent with the results of the in vitro tests, the fungal metabolites of *T. harzianum* were more active than the metabolites of other fungus. This is because the fungal metabolites of *T. harzianum* may have stronger anti-microbial metabolites against *R. solanacearum*. The ability of the anti-biotic production is usually isolate or species dependent. A specific anti-biotic can be produced by different fungal species, and, in a similar way, a specific fungal species can produce several bioactive compounds [29]. The secretion of anti-microbial compounds is one of the major anti-microbial mechanisms of a biocontrol agent. It is reported that the use of fungal metabolites against the pathogens exhibited similar results that the same processes achieved through corresponding micro-organisms [28]. The improved plant growth resulting from the application of fungal metabolites can also be explained on the basis of the plant growth promoting properties of *Trichoderma* metabolites. Along with direct antagonistic or parasitic activity against pathogenic microbes, *Trichoderma* spp. also secrete various compounds that alter the host metabolism positively and affect its development and growth. It was reported that plant seeds exhibited increased productivity when exposed to conidia of *Trichoderma* spp., which suggests that metabolites from *Trichoderma* spp. can act as signaling compounds in addition to promoting plant growth effects [50]. Several *Trichoderma* strains are capable of producing secondary metabolites involved in auxin-dependent mechanisms to enhance root growth and increase plant biomass [51,52]. The role of fungal metabolites (i.e., koninginins A, B, C, E and G isolated from *T. koningii* and 6-pentyl-alpha-pyrone isolated from *T. harzianum*) as growth regulators in wheat plants was reported by several studies [53–56]. Trichocaranes A, B, C and D produced by *T. virens* significantly affected the growth of etiolated wheat coleoptiles [57]. A sesquiterpene metabolite cyclonerodiol obtained from culture filtrate of *T. harzianum* and *T. koningii* demonstrated a regulatory effect on plant growth [58,59]. The direct toxic effect to the *R. solanacearum*, as well as the plant growth promoting effect of compounds present in the fungal metabolites, collectively contributed to enhanced plant growth. As discussed above, the anti-bacterial compounds affect the bacterial population in a variety of ways, including targeting membranes and

DNA and causing protein destructions [45,47,48]. This direct toxic effect of fungal metabolites contributes largely to a higher decrease in the soil bacterial population, resulting in a lower disease severity (or AUDPC) and the resulting enhanced plant growth.

It was noted that a longer application time (8 DBT) was superior to shorter application times (4 DBT and 0 DBT). This is because the longer application time helps in thorough mixing, as well as increasing the stability and compatibility of fungal metabolites in the soil, resulting in increased activity of the extract. Our results are in line with the findings of Khan et al. [60], where an organic soil amendment with different application times was used to test its management of *R. solanacearum* in tomato plants. The results suggested that a longer application time exhibited the best results because of the increased stability and compatibility of the treatments, which, in turn, release more anti-microbial compounds in the soil. The fungal extract was prepared on low cost media without the use of heavy mechanical or chemical involvement. The extract was active at a low concentration and effectively reduced soil bacterial population and improved plant growth. The fungi belonging to the *Trichoderma* genus are well known and widely used as biocontrol agents. Recently, the management of bacterial wilt disease was enacted through direct application of the *Trichoderma* spp. to the soil [31,32]. However, the metabolites were not evaluated beforehand for their ability to manage bacterial wilt disease in tomato plants and other crops. The results of this study indicated the potential of *Trichoderma* metabolites to manage *R. solanacearum* on tomato plants. However, such potential can be evaluated only in comparison with other protection techniques applied currently on tomato plants against this pathogen. This study will also provide a basis for the commercial preparation of *Trichoderma* metabolites in the market for the purpose of managing *R. solanacearum* in tomato plants and possibly other crops and, therefore, could be useful for global research and agricultural communities.

5. Conclusions

In this study, fungal metabolites of three *Trichoderma* spp. (i.e., *T. harzianum*, *T. virens* and *T. koningii*) obtained on three different growth media (STP, MOF and SuM) were tested in vitro for their anti-bacterial activity and in vivo for their anti-bacterial potential to manage bacterial wilt disease caused by *R. solanacearum* in tomato plants. Fungal metabolites of *T. harzianum* obtained on the STP medium were most effective in the management of *R. solanacearum*. *T. harzianum* metabolites caused the maximum decrease in soil bacterial populations, resulted in lowered disease severity and enhanced plant growth. The results from this study suggest that *T. harzianum* metabolites can be used as eco-friendly, cost-effective and efficient tools to manage *R. solanacearum* in tomato plants and possibly other plants.

Supplementary Materials: The following are available online at <https://www.mdpi.com/2071-1050/13/3/1491/s1>, Table S1: Composition of five growth media used for the production of fungal metabolites.

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