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Trichoderma virens - based formulation for the control of *Ralstonia solanacearum*, the causal agent of tomato bacterial wilt in Côte d'Ivoire

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ABSTRACT

The bacterial wilt disease caused by *Ralstonia solanacearum* is a severe threat to tomato production in Côte d'Ivoire. Genetic resistance is currently the most reliable control method. Unfortunately, it is challenged and overcame by the genetic variability and phenotypic plasticity of the bacterial strains. In this study, a powdery formulation based on *Trichoderma virens* FTR *in-vivo* was tested on two strains of *R. solanacearum* as an alternative control method. 50, 100 and 200 g of the product were applied to 3 kg of sterilized soil before the transplanting of two tomato cultivars (Petomech and Lindo). The product was applied once and twice; the second time was performed prior to bacterial inoculation to the root system. The results showed that the 200 g of the product applied in once and twice was more effective than the other treatments, reducing the disease severity by 71.44 and 92.78%, regardless of bacterial strain and tomato cultivar, respectively. The *T. virens*-based formulation is, therefore, noteworthy for the biocontrol of *R. solanacearum*.

INTRODUCTION

Bacterial wilt caused by *Ralstonia solanacearum* is one of the major phytosanitary problems in the world (Mansfield et al., 2012). In Côte d'Ivoire, bacterial wilt disease is the main threat to tomato cultivation. The disease was first observed in 1984 in southern eggplant fields (Declert, 1987) and is encountered today in almost all production areas leading the abandonment of farms. Furthermore, N'guessan et al. (2012) have reported a high genetic and phenotypic variability of the pathogen strains. Three of the four phylotypes have been characterized. The strains displayed significant variability in pathotypes (Lebeau, 2010). Different disease management methods have been implemented. These control measures are mainly based on cultural practices, chemical applications and use of tolerant varieties. However, genetic resistance stands as the most effective management strategy (Hayward,

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1991). Unfortunately, the high genetic plasticity in the disease causative agent results in the evolution of new pathotypes that overcome the built vertical resistance, making it challenging to breed for durable genetic resistance (Lebeau, 2010). Biological control could be a potential alternative. Particularly, disease suppressive soil made via microbial inoculation shows up as a promising, low-cost, eco-friendly and effective control method. Suppressive soils are a good candidate for alternative methods to chemical use which led to the emergence of resistance in the pathogen strains and environmental and public health issues (Lugtenberg et al., 1991).

Several tests have been carried out on the antagonism of microbial isolates against *R. solanacearum* in the laboratory or greenhouse conditions (Cao et al., 2018; Kheirandish and Harighi, 2015; Ceballos et al., 2014). There is a wide range of biocontrol agents of *R. solanacearum* such as *Pseudomonas putida*, *Pseudomonas fluorescens*, *Streptomyces* spp., *Trichoderma* sp., the genus *Bacillus* spp., and *Paenibacillus macreans* (Vanitha et al., 2009). The fungus *Trichoderma* spp. has also been reported antagonist and parasite against many plant pathogens (Elsharkawy et al., 2012) and are now the most studied and applied biological control agents (Alka et al., 2018). *Trichoderma* spp. strains have induced systemic resistance in plants against several pathogens, improved plant photosynthetic activity and promoted plant growth (Li et al., 2018). Beneficial microorganisms have gained considerable attention as an eco-friendly and cost-effective tool to trigger plant induced systemic resistance (ISR) and to promote plant growth for sustainable agricultural production (Abdelrahman et al., 2016).

Konappa et al. (2018) have reported that the application of *Trichoderma asperellum* isolates delayed the development of wilt, effectively reduced the incidence of the disease, increased fruit yield and improved plant growth of susceptible tomato variety Arka Meghali under the field conditions. Yendyo et al. (2018), from field data, revealed that single inoculation of *Trichoderma* spp. and *Pseudomonas fluorescens* prevented respectively 92 and 96% of *R. solanacearum* infection, and the dual inoculation prevented 97% of infection in tomato plants (*Solanum lycopersicum* L. var. Manisha).

In Côte d'Ivoire, a biological control approach using a formulation based *Trichoderma virens* has been considered in the suppression of sclerotinia in tomatoes. *In vivo* evaluations by amendment with different doses of a formulation of *T. virens* in one or two applications on naturally infested Songon soils and artificially inoculated soils with an isolated strain in the Songon market garden perimeter. The results showed that the incidence of sclerotinia is reduced in both types of soil amended with *T. virens* formulation. The dose of 200 g of the formulation per 3 Kg of soil in one or twice application was more effective in both reducing disease and stimulating plant growth. In addition, transplants on soils treated with *T. virens* have better vegetative growth compared to transplants on control soils (N'guessan et al., 2019).

In this context, this formulation has been tested to control bacterial wilt caused by *R. solanacearum* which constitutes a real constraint in tomato cultivation.

MATERIALS AND METHODS

Plant material

The plant material consisted of two tomato cultivars: Petomech and Lindo. The seeds were provided by the seed company Semivoire (Abidjan, Côte d'Ivoire).

Bacterial strains

Two Ivorian strains of *R. solanacearum,* RUN 1743 and RUN 1744 of phylotype I; sequevar 31, were used. They were isolated respectively in the localities of Daloa (Center Cote d'Ivoire) and Man (West Cote d'Ivoire) (N'Guessan et al., 2012).

Fungal material

A powdery formulation based on *T. virens* FTR (10^6 spores/mL), from the Industrial Research Unit (URI) on essential oils from the Scientific and Innovation Pole of the Felix Houphouët-Boigny University (Abidjan, Cote d'Ivoire), was used during this experiment.

Establishment of the nursery

50, 100 and 200 g of the powdery formulation were mixed with 3 kg of a sterilized potting soil before filling up blister plates with the substrate (N'guessan et al., 2019). The different substrates have been labelled respectively T0_50; T0_100; T0_200. The tomato seeds were sown at the rate of two or three seeds per cell and watered on alternate days. Another blister plates have been filled up with sterilized soil and tomato seeds under the same conditions without adding the powdery formulation.

Substrate preparation and transplanting of plants

The soil from the market gardening zone of Songon (South Cote d'Ivoire) previously sterilized for 1 h in an autoclave at 121 degrees under a pressure of 1 bar was mixed or not the powdery formulation of FTR. Two types of FTR applications have been performed. The first application was to mix the soil with the same doses as in the nursery

(50, 100 and 200 g for 3 kg of soil). These transplanting substrates were labelled, respectively T1_50, T1_100, and T1_200. For each dose, two lots of subculture substrates were placed in well-sealed plastic bags. Seven days after the first application, a second application of FTR at the same doses was carried out on only one of the batches. These pots that have been inoculated twice have been rated T2_50, T2_100, and T2_200. Batches of subculture substrates without FTR represented the control treatment and were labelled T0 n or T0 in. Transplanting of the tomato plants was performed with 21-day-old plants.

Bacterial pathogen inoculation of plants

The bacterial inoculum was prepared from 24 h-old bacterial colonies grown on solid Casamino acid-Peptone-Glucose (CPG) medium (Kelman, 1954) and then adjusted to a concentration of 10^8 CFU/mL in sterile water. The inoculation was made 7 days after plant transplanting. 10 ml of inoculum was poured around the previously scarified roots. For each treatment, ten tomato plants were used. Plants grew in greenhouse following a randomized complete block design. The average temperature and relative humidity in the greenhouse were respectively 28°C and 90%. The experiment was repeated twice. Experimental treatments were labelled as follows:

- Non-bacterized plants growing on untreated substrates (Negative control noted T0_n)
- Bacterized plants growing on untreated substrates (Positive control noted T0_in)
- Bacterized plants growing on substrate mixed with FTR at the nursery (T0_50, T0_100, and T0_200)
- Bacterized plants growing on subculture substrate mixed once with FTR (T1_50, T1_100, and T1_200)
- Bacterized plants growing on subculture substrate mixed twice with FTR (T2_50, T2_100, and T2_200)

Disease monitoring and latent infection assessment

Disease symptoms were monitored from the 3rd day after inoculation to the 28th day. Symptoms' severity was assessed on alternate day according to the following rating scale (Coupat-Goutaland et al., 2011).

0: No symptoms, 1: one leaf wilted, 2: two or three withered leaves; 3: all withered leaves; 4: bent or dead stem of the plant.

At the end of the test, the pathogen isolation from stems was carried out on asymptomatic plants to check for the presence and translocation of *R. solanacearum*. Briefly, 2 - 3 cm of plant stem were cut above the crown and surface-disinfected with alcohol 70%. The fragments were transferred to 5 mL of sterile water and left for 2 h at room temperature to promote the release of the bacterial colonies in the water. 50 µL of the extracts were spread on modified SMSA medium (Elphinstone et al., 1996). The Petri dishes were then incubated at 28°C for 3 to 4 days. Asymptomatic plants were rated positive for latent infection when the characteristic colonies of *R. solanacaerum* were observed (He et al., 1983). Data from latent infections were used to calculate the Colonization Index (CI). Three parameters were calculated after 28 days of observation:

- The wilting index (WI) reflects the incidence of the disease with the rating scale score 3 and 4.

$$\mathrm{WI}\ (\%) = \frac{\mathrm{N3} + \mathrm{N4}}{\mathrm{N}} \times 100$$

WI, Wilting index; N3, number of plants with note 3; N4, number of plants with note 4; N, total number of plants observed.

- The colonization index (CI) (Prior et al., 1996) was determined from latent infections.

$$\mathrm{CI}\ (\%) = \mathrm{WI} + (\mathrm{NS} \times \mathrm{RS})$$

WI, Wilting index, NS, asymptomatic plants rate; RS, infected asymptomatic plants rate.

- The reduction disease rate (RDR) (Winstead and Kelman, 1952) accounts for the effectiveness of the treatments in controlling the bacterial wilt. It is calculated from the disease index (DI).

$$\mathrm{RDR} = \frac{(\mathrm{DI\ witness} - \mathrm{DI\ test})}{\mathrm{DI\ Witness}} \times 100$$

DI witness, disease index from positive control; DI test, disease index of the treated substrates.

Statistical analyses

ANOVA was performed on the data, followed the post-hoc Newman-Keuls test at the statistical significance α = 5% using the software Statistica version 7.1.

RESULTS

Effect of treatments on wilting tomato plants

The wilting indices were higher for the control plants (T0_in) regardless of the tomato cultivar and pathogen strain (Figure 1-2). For all bacterized plants, the wilting index decreased with increasing FTR dose and number of

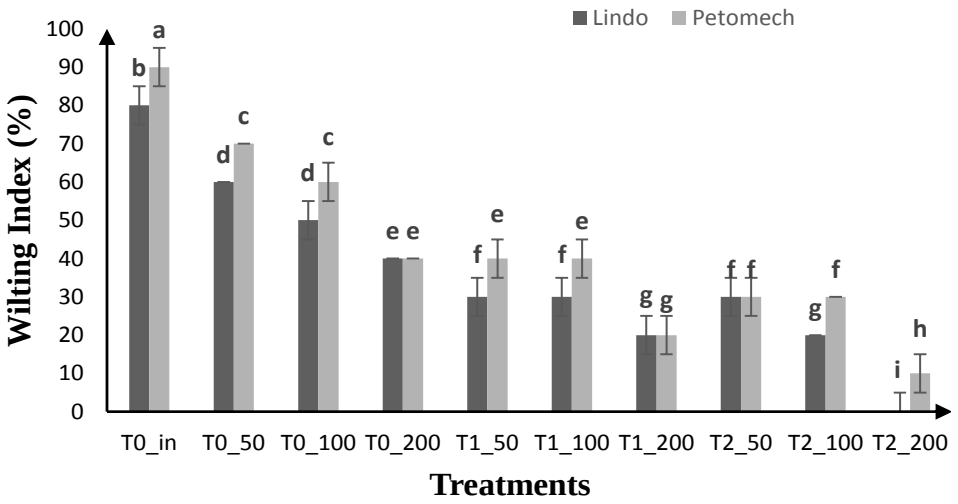

Figure 1. Effects of FTR on tomato wilting index due to *R. solancearum* RUN 1743.
50, 100 and 200 g, doses of FTR; T0_in, bacterized plants growing on untreated substrates; T0, bacterized plants growing on substrate mixed with FTR at the nursery; T1, bacterized plants growing on subculture substrate mixed once with FTR; T2, bacterized plants growing on subculture substrate mixed twice with FTR. Bars with by the same letter are not significantly different at the 5% significance level according to the Newman-Keuls test.

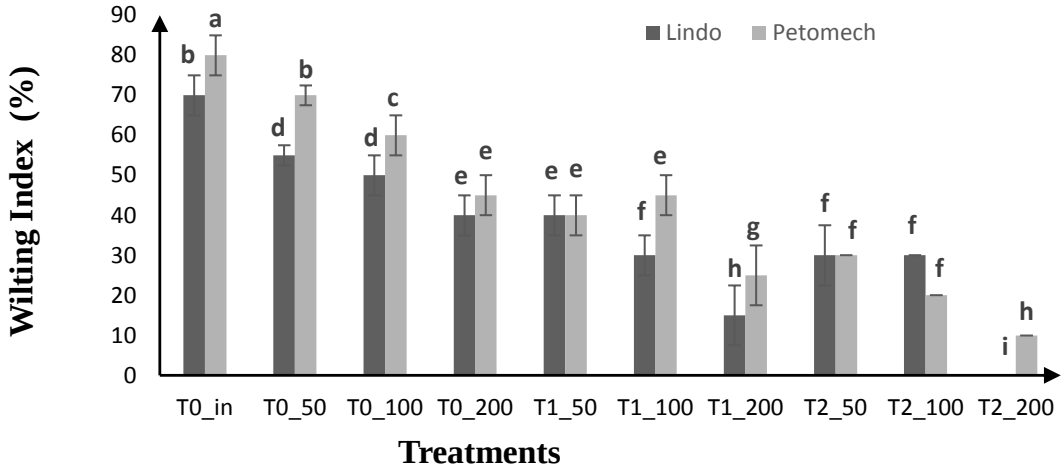

Figure 2. Effects of FTR on tomato wilting index due to *Ralstonia solancearum* RUN 1744.
50, 100 and 200 g, doses of FTR; T0_in, bacterized plants growing on untreated substrates; T0, bacterized plants growing on substrate mixed with FTR at the nursery; T1, bacterized plants growing on subculture substrate mixed once with FTR; T2, bacterized plants growing on subculture substrate mixed twice with FTR. Bars with by the same letter are not significantly different at the 5% significance level according to the Newman-Keuls test.

applications (Figure 1-2). Wilt indices were high with treatments made in nurseries: T0_50 and T0_100 (Figure 1-2). The lowest wilting indices were obtained with T2 treatments at a dose of 200 g/3 kg of soil regardless of tomato variety and pathogen strain. Interestingly, at this dose, the wilting index value was zero for the Lindo cultivar (Figure 1-2).

Effect of strain FTR treatments on the colonization index of tomato plants

The control plants (T0_in) presented the highest colonization indices (Figure 3-4). Plants treated in the nursery (T0) showed higher colonization rates than the T1 and T2 treatments. The highest colonization percentage

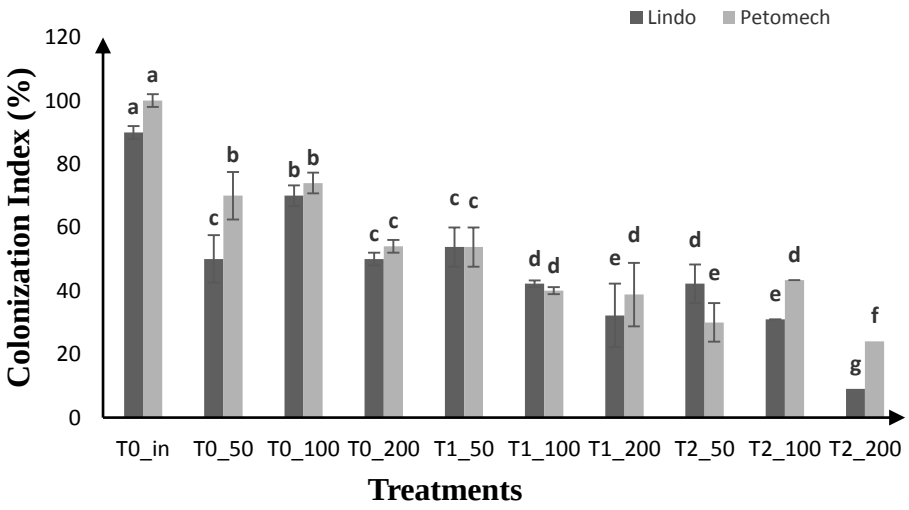

Figure 3. Effects of FTR on tomato colonization index due to *Ralstonia solancearum* RUN 1743.
50, 100 and 200 g, doses of FTR; T0_in, bacterized plants growing on untreated substrates; T0, bacterized plants growing on substrate mixed with FTR at the nursery; T1, bacterized plants growing on subculture substrate mixed once with FTR; T2, bacterized plants growing on subculture substrate mixed twice with FTR. Bars with by the same letter are not significantly different at the 5% significance level according to the Newman-Keuls test.

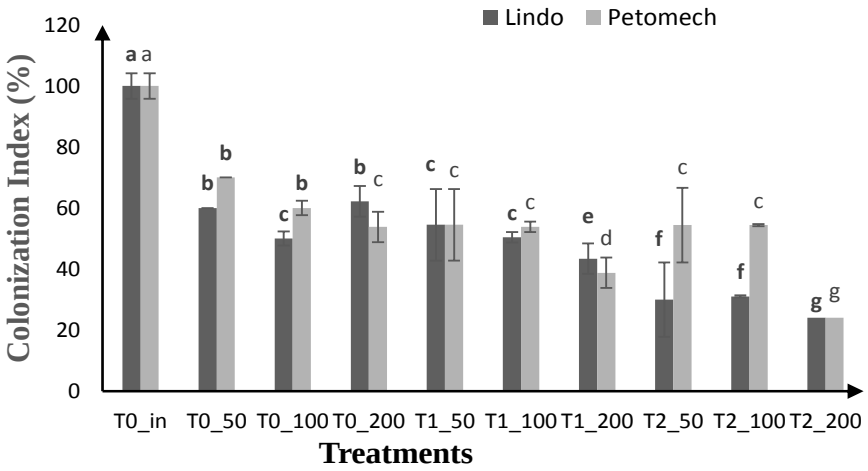

Figure 4. Effects of FTR on tomato colonization index due to *Ralstonia solancearum* RUN 1744
50, 100 and 200 g, doses of FTR; T0_in, bacterized plants growing on untreated substrates; T0, bacterized plants growing on substrate mixed with FTR at the nursery; T1, bacterized plants growing on subculture substrate mixed once with FTR; T2, bacterized plants growing on subculture substrate mixed twice with FTR. Bars with by the same letter are not significantly different at the 5% significance level according to the Newman-Keuls test.

(74%) was observed in the cultivar of Petomech plants challenged by the bacterial strain 1743 and transplanted onto the substrates at the dose of T0_100 (Figure 3). The colonization index values were relatively low when FTR formulation dose increased. Plants that received the T2 treatment showed the lowest colonization rates compared to those of the T0 and T1_i treatments. The lowest colonization percentage (9%) was observed with strain 1743 on Lindo cultivar protected by T2_200 treatment (Figure 3-4).

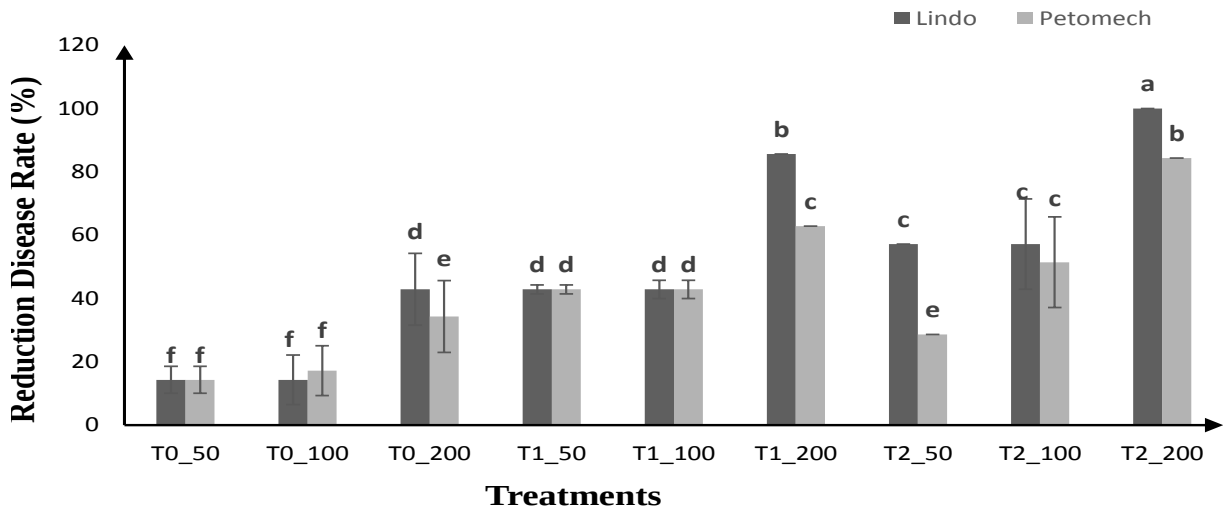

Figure 5: Reduction of tomato plants wilting according to treatments against infection of pathogen strain 1743. 50, 100 and 200 g, doses of FTR; T0_in, bacterized plants growing on untreated substrates; T0, bacterized plants growing on substrate mixed with FTR at the nursery; T1, bacterized plants growing on subculture substrate mixed once with FTR; T2, bacterized plants growing on subculture substrate mixed twice with FTR. Bars with by the same letter are not significantly different at the 5% significance level according to the Newman-Keuls test.

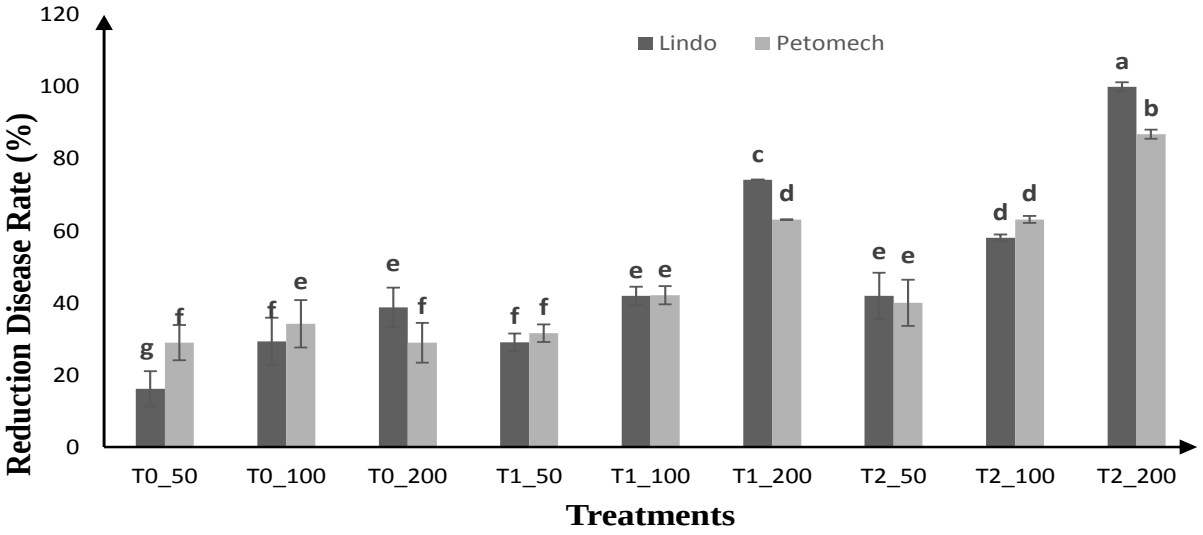

Figure 6. Reduction of tomato plants wilting according to treatments against infection of pathogen strain 1744 50, 100 and 200 g, doses of FTR; T0_in, bacterized plants growing on untreated substrates; T0, bacterized plants growing on substrate mixed with FTR at the nursery; T1, bacterized plants growing on subculture substrate mixed once with FTR; T2, bacterized plants growing on subculture substrate mixed twice with FTR. Bars with by the same letter are not significantly different at the 5% significance level according to the Newman-Keuls test.

Effect of strain FTR treatments on bacterial wilt disease reduction rate

The results showed that the higher the dose and the concentrations of FTR, the greater the reduction of wilt disease rate (Figure 5-6). The highest reduction percentages, 86.84 and 100%, were respectively recorded from T1_200 and T2_200 treatments regardless of tomato cultivar and pathogen strain (Figure 5-6). The lowest reduction percentages were recorded with the treatments T0_50 and T0_100 against the pathogen strain 1743 for the two cultivar (Figure 5) and T0_ 50 with the strain 1744 for the cultivar Petomech (Figure 6).

The effect of the various treatments in relation to the

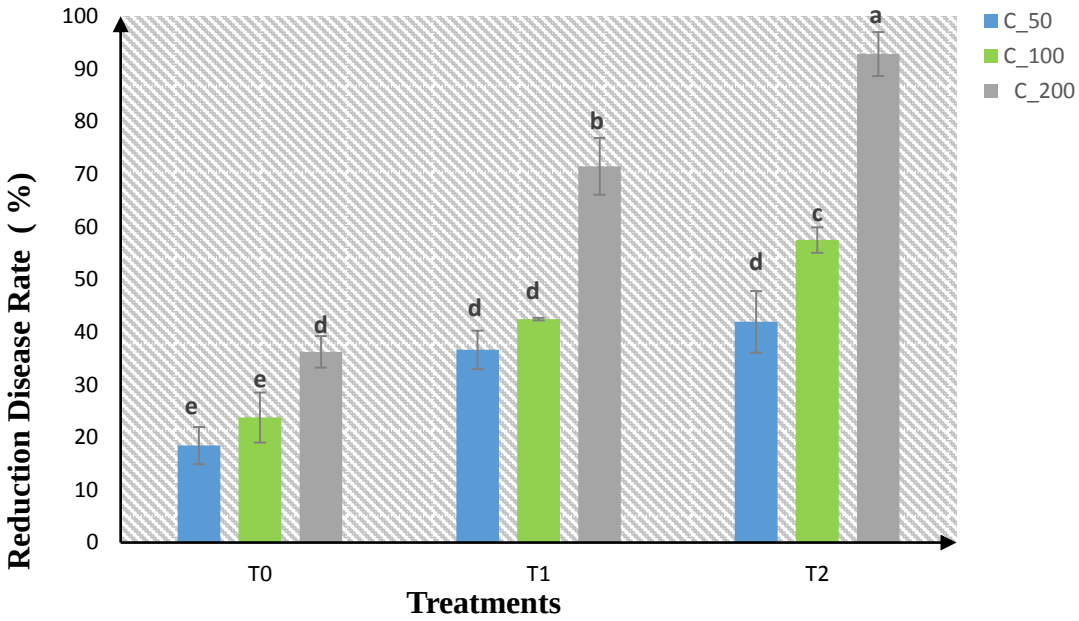

Figure 7. Effect of FTR treatments on the reduction rate according to strains and cultivars.
C_50, dose of 50 g of FTR for 3 Kg of soil; C_100, dose of 100 g of FTR for 3 Kg of soil; C_200 g, dose of 200 g of FTR for 3 Kg of soil; T0, Bacterized plants growing on substrate mixed with FTR at the nursery; T1, Bacterized plants growing on subculture substrate mixed once with FTR; T2, Bacterized plants growing on subculture substrate mixed twice with FTR. Bars with by the same letter are not significantly different at the 5% significance level according to the Newman-Keuls test.

strain-cultivar interaction has shown that the reduction of the disease rate was low for the T0 concentrations (50, 100 and 200 g FTR/3 kg of soil amended at the nursery), that is, 18.40; 23.73 and 36.19%. On the other hand, treatments T1_200 and T2_200 present the highest reduction rates, respectively 71.44 and 92.78% (Figure 7).

DISCUSSION

The use of the powdery *T. virens*-based formulation has significantly reduced the disease symptoms' development. Indeed, the various treatments showed that the protective effect of the fungus *T. virens* against *R. solanacearum* is related to the repetition of application and the doses used. T0 treatments performed in the nursery were more effective than plants without FTR treatments. However, they were less effective in reducing the disease compared to T1 and T2 treatments performed on subculture substrates. The T1 and T2 treatments, in the proportion 200 g/3 kg of soil, proved to be the most effective on the two strains with rates of 100% in the Lindo cultivar and 86.84% in the Petomech cultivar. Several studies indicated that *Trichoderma* spp. could effectively limit the pathogenicity of causal agents. Thus, mixing the biological control agent with the substrate at least two weeks before infection by the pathogen is a management strategy worthy of being promoted. During two weeks, *Trichoderma* multiplies and colonizes the rhizosphere allowing the symbiont to outcompete soil pathogen in occupying the ecological niche (Lebeau, 2010). Our results are consistent with those of Yedidia et al. (1999) who showed the protection of *T. harzianum*. The application of the latter to melon growing substrate would trigger plant defences' mechanism by inducing systemic or localized resistance. Poddar et al. (2004) also revealed the potential of *Trichoderma* to decrease chickpea wilt incidence. Treating chickpea seeds with three isolates of *Trichoderma* was very effective and therefore reduced the incidence of wilt from 71.7 to 92%. Findings of Konappa et al. (2018) have also pointed out that *T. asperellum* reduced the incidence of bacterial wilt. This study found out that in tomato fields infested with *R. solanacearum* and treated with isolates T4 and T8 of *T. asperellum*, wilt incidence decreased by 51.06 and 52.75% respectively. Meanwhile, the fruit yield increased, and the plant growth improved compared to the control plot. Hibar et al. (2005) also compared the fitness of tomato plants inoculated with a pathogenic strain of *F. oxysporum* and treated with *T. harzianum* with healthy uninoculated and untreated control plants. They found that the plants inoculated with the pathogen and treated with the antagonist exhibited greater vegetative and root development than the control plants. Plant growth and resistance stimulation are thought to be due to the

increase in the transfer of nutrients from the soil to the roots through colonization by *Trichoderma* sp. (Contreras-Cornejo et al., 2009). The effects of *Trichoderma* sp. on plants include the inducing of systemic or localized resistance. These fungi first colonize the epidermis of the roots, then the outer cortical layers and finally release bioactive molecules (Howell et al., 2000). As a result, plant growth, resistance, and nutrient supply are improved. Several modes of action have been proposed to explain the biological control of plant pathogens by *Trichoderm*a. These modes include the production of enzymes degrading pathogen cell wall and antibiotics as well as parasitism. *Trichoderma* spp. are very active producers of extracellular enzymes, and some of them have been implicated in the biological control of plant diseases (Harman, 2006). These explanations suggest that the resistance mechanism of tomato plants against bacterial wilt may be due to a degree of parasitism and the production of some degrading enzymes by *T. virens*. The study of the mechanisms involved in the antagonist relationship has revealed that *Trichoderm*a generally acts by releasing volatile and non-volatile organic compounds. The production of these defensing by *Trichoderma* species has been reported by Dennis and Webster (1971 a, b). These volatile and non-volatile compounds readily diffuse and inhibit the growth of various soil-inhabitant plant pathogens (Reddy et al., 2014; Wheatley, 2002).

Conclusion

Our work highlights the antagonist effect of *T. virens* FTR on the bacterial wilt disease caused by *R. solanacearum*. It appears that wilt reduction was related to the dose and the number of application of the formulation. Hence, the 200 g dose of FTR, applied once and twice to the subculture substrate, were the most effective in reducing the incidence of the disease by 71.44 and 92.78% respectively. These results are noteworthy as an alternative to control *R. solanacearum* threat to tomato production.

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