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Article

In vitro evaluation of the inhibitory capacity of three *Trichoderma* isolates on *Ralstonia solanacearum*

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ABSTRACT

Bacterial wilt in bananas, caused by *Ralstonia solanacearum* or Moko, limits crop production and threatens Ecuador. This study evaluated *Trichoderma* isolates in laboratory conditions as an innovative alternative to ensure sustainability in banana production. The four *R. solanacearum*. isolates were obtained from banana plants exhibiting disease symptoms and were characterized through morphological and biochemical tests. Four treatments were evaluated: three isolates of fungi from the genus *Trichoderma* (*Trichoderma viride*, T. *harzianum*, *T. asperellum*) and one consisting of a combination of the three isolates above. The inhibitory capacity of the *Trichoderma* isolates on *R. solanacearum* colonies was measured. A completely randomized design with three replicates was used, and general linear and mixed models were employed, with qq-plot graphs for normality and residual plots for variance homogeneity.

Furthermore, a Fisher's LSD test was conducted at a significance level of $\alpha = 0.05$. In the biochemical tests, the bacterial isolates exhibited specific characteristics of *R. solanacearum* in two bacterial isolates. In the inhibition tests, treatment four and treatment one (consortium of the three *Trichoderma* isolates and *Trichoderma viride*) showed the highest inhibitory potential, with 76.07% and 61.19%, respectively. The consortium of *Trichoderma* isolates demonstrated the highest inhibitory potential against *R. solanacearum*, with day 10 being the time with the highest percentage of inhibition (72.61%).

Keywords: Bacterial wilt, *Ralstonia solanacearum*, *Trichoderma*, inhibition

INTRODUCTION

In Ecuador, several species of Musaceae are cultivated, with bananas being the crop with the largest planted area, covering 165,080 hectares, followed by plantains with 145,501 hectares, and baby bananas or "oritos" with a cultivated area of 6,839 hectares [1–3]. Plantains are a prominent export crop and a fundamental staple for the country's food security and employment generation [4]. The central banana-producing provinces in

Ecuador are Manabí, Santo Domingo, Esmeraldas, Guayas, Los Ríos, Orellana, Morona Santiago, Napo, and Sucumbíos [1].

In the banana belt of Ecuador, where varieties such as Dominico and Barraganete are grown, banana producers, mostly small-scale farmers, face significant challenges caused by phytopathogenic fungi and pests such as the black weevil (*Cosmopolites sordidus*), nematodes of the genera *Pratylenchus* and *Helicotylenchus*, and Black Sigatoka (*Mycosphaerella fijiensis*). One of the most significant challenges is *Ralstonia solanacearum*, known as "Moko," a bacterium that colonizes and obstructs the plant's vascular system, leading to wilting and, eventually, plant death [5]. Despite being an essential crop for food security and the country's economy, this disease severely threatens plants.

Biological control has been adopted as part of integrated pest management to promote sustainable agriculture practices. In this context, fungi of the genus *Trichoderma* have shown effectiveness in controlling phytopathogenic fungi in different crops. *Trichoderma* spp., microscopic facultative anaerobic fungi, are naturally found in the soil and other environments with decomposing organic matter and plant residues [6,7].

This research aims to evaluate the potential of biological control using three *Trichoderma* isolates (*T. viride, T. harzianum, T. asperellum*) to inhibit the growth of *Ralstonia solanacearum* under laboratory conditions. The focus is reducing dependence on agrochemicals and minimizing negative environmental impacts. Additionally, using Trichoderma spp. as a biological control agent is expected to improve the plantain crop's productivity and mitigate problems caused by *R. solanacearum* in this critical sector. Through this research, we seek to provide scientific evidence of the effectiveness of biological control with *Trichoderma* spp. as a viable and sustainable alternative for managing *R. solanacearum* in the plantain crop. The results will strengthen integrated pest management strategies and promote more environmentally friendly agricultural practices, benefiting banana producers and food security in the country.

MATERIALS AND METHODS

The study was conducted at the Plant Protection Laboratory, Central Experimental Station of the Amazonia of the National Institute of Agricultural and Livestock Research (INIAP). This institution is located in San Carlos, La Joya de los Sachas Canton, Orellana Province, at 280 meters above sea level.

Obtaining *Trichoderma* isolates

The isolates identified as *Trichoderma Viride, Trichoderma Harzianum*, and *Trichoderma Asperellum* were provided by the Tropical Pichilingue Experimental Station and preserved in Eppendorf tubes with sterile distilled water, following the methodology described by [8]. The main objective of this methodology is to maintain the viability, authenticity, and purity of the microorganisms.

Activation of *Trichoderma* isolates

The following steps were carried out to activate the Trichoderma isolates preserved in sterile water: the microtubes containing the *Trichoderma* strains were shaken in an isolation chamber. Then, 0.5 ml of each isolate was taken using a micropipette with previously sterilized tips. These samples were poured into the center of a Petri dish containing PDA (Potato Dextrose Agar) culture medium supplemented with lactic acid. The Petri dishes were incubated at a temperature of 27°C for 10 days, allowing the *Trichoderma* isolates to develop and grow actively.

Obtaining the pathogenic bacterial isolate

The following steps were followed for bacterial isolation from plant tissues with disease symptoms: portions of affected tissue were taken and superficially disinfected with a 4% sodium hypochlorite and 70% (v/v) alcohol solution. Subsequently, they were washed with sterile distilled water. Approximately 5 mm² each, tissue fragments were cut and macerated in a mortar using sterile purified water. The resulting suspension was spread on Petri dishes containing nutrient agar using a previously sterilized Drigalski spatula. After 48 hours

of bacterial growth, a loopful of bacterial growth was transferred to a selective culture medium called "selective media from South Africa" (SMSA) modified. The Petri dishes were incubated at 30°C for 48 hours. During this time, typical colonies of slightly fluid reddish-purple color with a pink center were selected using criteria described by [9-10], and other relevant studies.

Characterization of pathogenic bacteria

Biochemical and physiological methods in plant microbiology studies were widely used to characterize the isolated bacteria based on the techniques described by Goszczynska and colleagues [11]. These methods included Gram staining to determine cell morphology, the use of 3% KOH to identify Gram-negative or Gram-positive bacteria, the oxidase test to verify the production of the oxidase enzyme, the catalase test to detect the presence of the catalase enzyme, the nitrate reduction test to evaluate the bacteria's ability to reduce nitrate, growth at 41°C to determine thermotolerance, and salt tolerance by growth in media with NaCl concentrations of 1%, 2%, and 3%. Additionally, the fluorescence test was performed to detect the production of fluorescence, an essential criterion for identifying *Ralstonia* strains. These biochemical and physiological tests are fundamental for determining and characterizing isolated bacteria, as they provide essential information about their metabolic characteristics and ability to survive in different environmental conditions.

Conservation of bacteria

The methodology for conserving bacterial isolates described by [8] was used to maintain the bacteria's viability, authenticity, and purity over time. Purified bacterial isolates were cultured on Petri dishes with a nutrient agar medium. Then, sterile test tubes with nutrient agar medium were prepared and inclined at a 45° angle to allow the medium to solidify. The bacteria were streaked onto the inclined tubes and left to rest for one day at room temperature. Finally, the test tubes were preserved through freezing, ensuring the preservation of the bacterial isolates for future use.

Experiment management

To perform the inhibition tests, the methodology described by Andrade [11], was used with some modifications. First, in a laminar flow cabinet, enriched potato-dextrose-agar and nutrient agar media were dispensed in 90 mm Petri dishes. For the dual confrontation, a 5 mm diameter disc of each *Trichoderma* isolate and the bacterial isolates were placed opposite each other on the surface of the culture medium. As for the consortium confrontation, using a sterile punch, a 5 mm disc was set at three points of the Trichoderma isolates, and one disc was set at the center of the bacterial isolate that tested positive for Ralstonia. The Petri dishes were incubated at room temperature, and the interaction between the antagonistic organism and the pathogen was recorded every 24 hours. This recording allowed the observation and evaluation of the effects of *Trichoderma* isolates on the bacterial isolates, both in the dual confrontation and the consortium.

Inhibition tests of bacterial isolates

Tests were conducted to measure the inhibitory effect of three *Trichoderma* isolates (*T. viride, T. harzianum*, and *T. asperellum*) on two bacterial isolates positive for *Ralstonia solanacearum*. Five treatments were established: T1 (*T. viride*), T2 (*T. harzianum*), T3 (*T. asperellum*), T4 corresponding to the consortium of the three *Trichoderma* isolates, and T5 as the control, consisting of the unrestricted growth of bacterial isolates. A completely randomized design with three replications was used, with a Petri dish as the experimental unit (Table 1). This allowed for a systematic and controlled evaluation of the effect of different *Trichoderma* treatments on the bacterial isolates, providing precise information about the inhibitory potential of the *Trichoderma* isolates against *R. solanacearum*.

Study parameters

In evaluating inhibitory potential, the percentage of colony growth inhibition (PCGI) was determined using a modified formula based on the work of Suárez and Cabrales [12]. This formula allowed for the precise calculation of the inhibition degree of bacterial colony growth in the presence of different *Trichoderma* treatments. In this way, the inhibitory effect of *Trichoderma* isolates on *R. solanacearum* isolates could be quantified objectively and comparatively.

$$\mathbf{PI}=\frac{(Mt-Ma)}{Mt}*100$$

Where:
Ma: Ralstonia solanacearum, inhibited.
Mt: Mycelium of free growth of Ralstonia solanacearum (control).

Data analysis

In the statistical analysis, the *Trichoderma* isolates and the number of evaluations over time were considered fixed effects, while repetitions were treated as random. The data obtained were analyzed using the statistical software InfoStat version 2015. General linear and mixed models [13], were employed, and model assumptions were verified through qq-plot graphs for normality and residual plots against predicted values for variance homogeneity. Additionally, a Fisher's LSD test with a significance level of $\alpha= 0.05$ was conducted to compare the means. To assess interactions between the antagonistic organism and the pathogen, the Image Tool program version 3.0, developed by Wilcox et al. (2002) in collaboration with the University of Texas, was used. Compatible with Windows 7, this program allowed for measurements using photographs as reference.

RESULTS

Characterization of *Ralstonia solanacearum* isolates

In the characterization of four bacterial isolates obtained from banana tissue exhibiting Moko symptoms, two bacteria (isolates 3 and 4) with distinctive morphological characteristics such as a reddish appearance, mucous texture, and positive biochemical tests for *Ralstonia solanacearum* were identified. These results are presented in Table 1, and the appearance of the bacteria can be observed in Figure 2.

Tests	Reaction
Gram Staining	Gram-negative
Potassium Hydroxide Test (3% KOH)	Positive
Catalase	Positive
Nitrate Test	Positive
Tolerance to 1% and 2% Salts	Positive
Tolerance to 3% Salts	Negative
Growth at 41 °C	Negative
Fluorescence Test	Negative

Table 1. Biochemical Test Results for Two Bacterial Isolates.

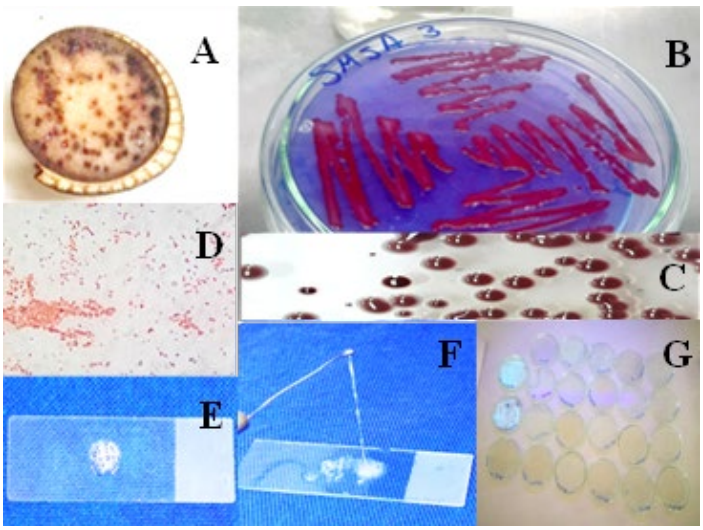


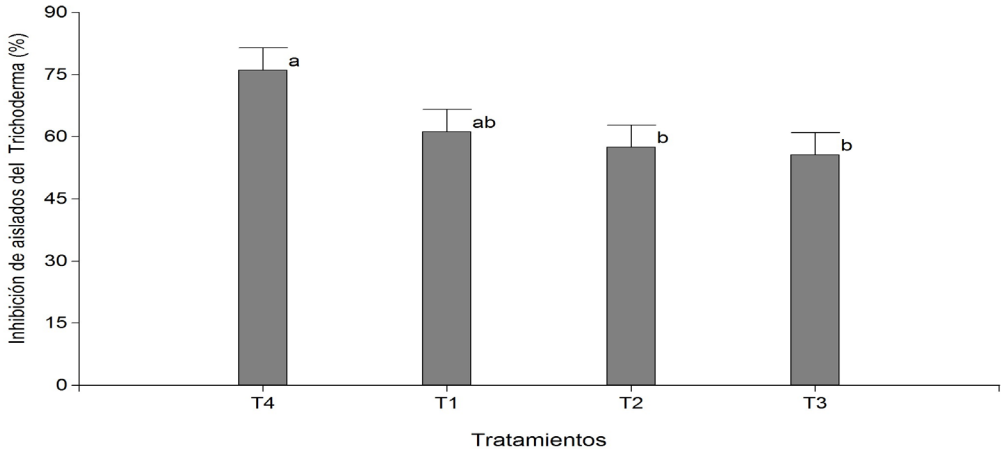
Figure 1. Response of the characterization of Ralstonia solanacearum. A; symptomatic tissue of the disease. B; Cultivation was performed in the SMSA culture medium. C; characteristics of the Ralstonia colony in SMSA medium. D; Gram stain (-). E; Catalase test (+). F; Potassium Hydroxide Test (+). G; Fluorescence test (-).

The results obtained in this research corroborate Torres's previous findings [14]. who also found similar characteristics in bacterial colonies' morphology and polysaccharide production. Furthermore, the results of this research are consistent with Mendoza's reports [15]. *they were* regarding the microscopic characteristics of Ralstonia solanacearum, such as its rod-shaped motile form and pink staining in the Gram stain using fuchsin. These results support the identification of the bacterium as Gram-negative, as determined by the 3% KOH test.

In summary, the results of the morphological and biochemical characterization of the bacterium in this research are consistent with previous findings and confirm the similarity of the isolated bacterium to Ralstonia. According to the study conducted by Pawaskar [16]. it aligns with the results of identifying Ralstonia solanacearum based on morphological and biochemical characteristics, including Gram staining, KOH test, colony coloration, starch hydrolysis, and cellulose decomposition.

Inhibition tests

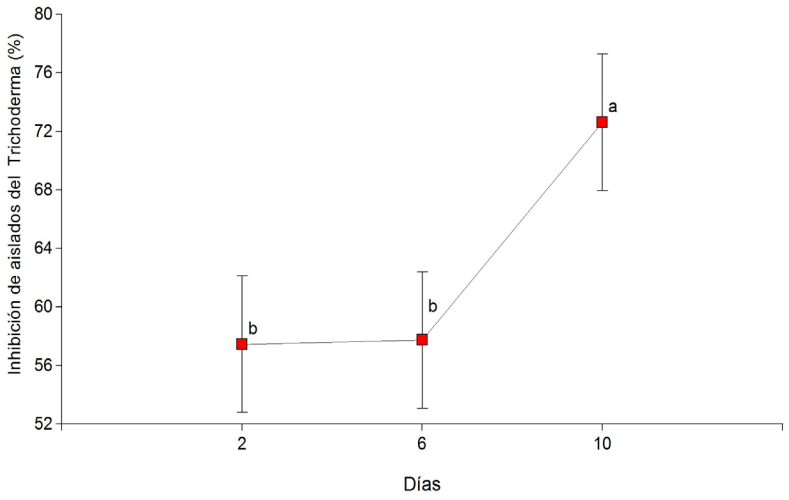
In Figure 2, it can be observed that the evaluated *Trichoderma* isolates exhibited a remarkable ability to inhibit the growth of the studied bacterial colonies. These results are consistent with those previously obtained by other researchers [17–19]. It further supports the effectiveness of *Trichoderma* as a control agent. The treatments showed significant differences ($P < 0.0001$), indicating variability in the inhibitory potential of the different *Trichoderma* isolates. Specifically, treatment four, consisting of the consortium of the three *Trichoderma* isolates, and treatment one, showed the highest inhibitory potential with a percentage of colony growth inhibition of 76.07% and 61.19%, respectively. These values were statistically different from treatments two and three, which had inhibition percentages of 57.19% and 55.65%, respectively. The presented results demonstrate that the evaluated Trichoderma isolates can inhibit the growth of the studied bacterial colonies, with the consortium of the three isolates and an individual isolate being the most effective in this inhibition.



Means with a typical letter are not significantly different ($p > 0.05$)

Figure 2. Percentage of inhibition of Trichoderma isolates on Ralstonia sp. isolates (T1 *Trichoderma viride*, T2 *Trichoderma harzianum*, T3 *Trichoderma asperellum*, and T4 consortium of three Trichoderma isolates).

When analyzing the effect of days on inhibition, significant differences in days are observed ($P < 0.0118$), with day 10 achieving the highest percentage (72.61%), being statistically different from days six and two, which reached the lowest percentage (57.73% and 57.45%, respectively) (Figure 3).



Means with a common letter are not significantly different (p > 0.05).

Figure 3. Percentage of inhibition of Trichoderma genus isolates on potential *Ralstonia solanacearum* isolates over time.

DISCUSSION

The in-depth analysis of the data presented, when compared to those of other authors, reveals that the results obtained in this research are consistent with previous studies conducted by Alvarez [20], Yendyo [21] and Goszczynska [22]. These studies have demonstrated that *Trichoderma* genus isolates possess a significant antibiotic effect due to the production of thermostatic metabolites. These metabolites have been associated with an increase in root collar height and diameter, which correlates with the results obtained in this research regarding the inhibitory capacity of the consortium of *Trichoderma* isolates.

Furthermore, a study by other researchers (23-24) (25) found that applying metabolites from *T. harzianum* resulted in maximum inhibition of the soil bacterial population and reduced disease severity. These findings are similar to the results obtained in this research, where bacterial inhibition was observed starting from the tenth day after the application of *Trichoderma* isolates.

On the other hand, the findings of Ceballos [7], Align [26] and Sutarman [27], support the results of this research by demonstrating that crude extracts of *Trichoderma* sp. have highly significant effects on the in vitro inhibition of colonies of different species of *R. solanacearum*. These results strengthen the evidence of *Trichoderma*'s antibacterial activity against the bacterium *R. solanacearum*.

According to the study by Khan [28], it aligns with the results obtained in this study, revealing that *Trichoderma* spp. Isolates significantly inhibited bacterial growth and cell damage by *R. solanacearum*.

CONCLUSIONS

This scientific study has significantly advanced our understanding of the interaction between Trichoderma isolates and Ralstonia solanacearum under laboratory conditions, as suggested in the article's title: "In vitro Evaluation of the Inhibitory Capacity of Three Trichoderma Isolates on Ralstonia solanacearum." Based on our results and concerning the objectives set forth, we have conclusively demonstrated that Trichoderma, especially the consortium of the three Trichoderma isolates and the T. viride isolate, possess a remarkable inhibitory potential against R. solanacearum. These findings strongly support using Trichoderma as an effective biological control agent in managing this phytopathogenic bacterium in banana crops. Furthermore, our

results pave the way for future research and applications in this field, potentially significantly benefiting farmers and promoting sustainable and safe agricultural practices for food production.

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