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Behavior and use of quaternary ammonium-based disinfectants in biosafety protocols against *Fusarium oxysporum* f.sp. *cubense* Race 1 and tropical Race 4

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ABSTRACT

The banana is one of Colombia's main export products. However, production is seriously affected by Fusarium wilt of Banana (FWB), which is the most destructive disease caused by the fungus *Fusarium oxysporum* f.sp. *cubense* Tropical Race 4 (Foc TR4). Currently, management strategies focus on containment and biosecurity protocols to prevent its spread to territories that are free of this disease. This study aimed to evaluate 9 quaternary ammonium-based disinfectants (QAC) *in vitro* in Colombia on reproductive (microconidia and macroconidia) and resistance structures (chamydospores) of Foc Race 1 (R1) and Tropical Race 4 (TR4), with and without soil, to determine the influence of organic matter on the action of QACs. A method for inhibiting the action of QACs was standardized and evaluated at 1,200 ppm with a contact time of ≤ 30 seconds, while evaluating the soil-inoculum and soil-disinfectant interaction. The efficacy of QACs was 100% in the reproductive and resistance structures of Foc R1 and TR4 without soil and in the soil-inoculum interaction. However, in the soil-disinfectant interaction, only QAC4 controlled the pathogen at 100%. The presence of organic matter influenced the biocidal action of the QACs. Soil decreased the efficacy of the QACs and, therefore, must be removed from contaminated boots before treatments are applied.

Keywords: fungus, microconidia, macroconidia, chlamydospore, efficacy, banana, Fitosanidad.

INTRODUCCIÓN

The banana is one of the more important tropical fruits in Colombia and the world (Vásquez-Tirado and Cabrales-Herrera, 2020) and is a staple food in developing countries because of its high nutritional value (FAO, 2020). In that context, Latin American countries stand out as the main exporters, with Colombia ranked fifth for

exporting countries. According to data from the Banana Growers Association of Colombia (AUGURA, 2021), there are currently 52,270 ha, distributed in the Urabá region with 34,770 ha, which represents a productivity of 2,007 boxes*ha-1, followed by the states of Magdalena and La Guajira with 17,500 ha.

Fusarium wilt, one of the more devastating fungal diseases in the history of bananas, threatens production (Dita *et al*., 2018). *Fusarium oxysporum* f. sp. *cubense* (Foc), the causal agent of the disease, is a natural soil-dwelling fungus that causes destructive effects on plants, blocking the vascular system, preventing the absorption of water and nutrients, and causing wilting and then death (Dita *et al*., 2018). The presence of Foc TR4 in Colombia, specifically in La Guajira in 2019 (García-Bastidas *et al*., 2019), subsequently reported by the National Agricultural Health Service of Perú (Acuna *et al*., 2021) and recently in Venezuela (INSAI, 2023), have generated great concern and alerted the Latin American banana industry, launching various containment and prevention strategies. On the other hand, in Colombia (Urabá region) there are approximately 1000 ha with "Gros Michel" variety affected with Foc R1. Conventional management of Foc TR4 through the use of quaternary ammonium compounds (QACs) is an alternative with prevention protocols. QACs are surfactants that penetrate the membranes of microorganisms, destroying proteins and nucleic acids, which leads to cell death (Gerba, 2015). Finally, considering the threat that Foc TR4 has generated in the banana industry, the aim of this work was evaluating the biocidal action and effectiveness of commercial disinfectants available in the Colombian market in the absence and presence of soil on the survival of reproductive and resistance structures of different physiological races of *Fusarium oxysporum* f.sp. *cubense* to carry out a disinfection plan on banana farms and in strategic places, thereby containing this disease and determine the influence of organic matter and texture on its action.

MATERIALS AND METHODS

R1 and TR4 Isolates. The *F. oxysporum* f.sp. *cubense* Race 1 (Foc R1) strain IB, was provided by the fungal collection of the laboratory of the Banana Research Center (Cenibanano). The *F. oxysporum* f.sp. *cubense* Tropical Race 4 (Foc TR4) strain 190038, was provided by the Instituto Colombiano Agropecuario ICA. Both strains were cultivated on Potato Dextrose Agar (PDA), at a concentration of 100 ppm to prevent bacterial contamination and incubated at 27 °C for 7 days.

Inoculum production. Once the strain was grown, the mycelial clumps were rinsed with 10 mL of sterile distilled water (SDW) and filtered using a sterile miracloth to

separate the spores from the rest of mycelia. Spore concentration to Foc R1 and R4T was determined using a Neubauer hemocytometer and adjusted to 1 x 10^5 conidia. mL^{-1} before use in the disinfectant experiments. For the Foc R1 chlamydospores production, soil with a clay loam texture obtained from a conventional banana crop was used to prepare soil broth following the modified protocol described by (Nguyen *et al*., 2019). For the Foc TR4 chlamydospores production, the protocol developed by the National Banana Corporation of Costa Rica (CORBANA) and modified by (Izquierdo-García *et al*., 2021) was used.

Disinfectants. A total of 9 commercial disinfectants were tested (Table 1). Based on previous reports on effective QAC doses (Nguyen *et al*., 2019), the concentration of all QAC disinfectants was adjusted to 1,200 ppm. The contact times evaluated in the reproductive and resistance structures, with and without soil, were standardized at ≤ 30 seconds, simulating the time used by operators during the footbath disinfection process.

Table 1. QAC disinfectants and chemical characteristics evaluated *in vitro* against physiological races (R1 and TR4) of *Fusarium oxysporum* f.sp. *cubense*.

Disinfectant (Treatments)	Active Ingredient	QAC Generation
QAC1	Didecyldimethylammonium chloride 12%	4st
QAC2	Didecyldimethylammonium chloride 12%	4st
QAC3	Alkyldimethylbenzylammonium chloride 80%	3st
QAC4•	Di(octyl/decyl) chloride 1% + Benzalkonium chloride 13,5%	5st
QAC5•	Di(octyl/decyl) chloride 0,4 % + Benzalkonium chloride 1%	5st
QAC6	Dimethylbenzil ammonium chloride 12%	5st
QAC7	Quaternary ammonium mixture 10%	5st
QAC8	Quaternary ammonium mixture 10%	5st
QAC9	Quaternary ammonium mixture 8%	5st

To evaluate all situations that can be found in the field, three different tests were carried out simulating: i. Footbath with a QAC disinfectant solution free of soil contamination at a concentration of 1,200 ppm; ii. Footbath with a clean QAC disinfectant solution at 1,200 ppm and contaminated with soil; and iii. Footbath totally contaminated with soil. Each testing efficacy used macro, microconidia and chlamydospores from Foc R1 and TR4. For each of the tests, an aliquot of 100 µL was plated on PDA supplemented with 100 ppm chloramphenicol. All plates were

incubated at 27 °C for 48 h, and the number of CFU.mL^{-1} was estimated. For the CFU quantification, a 1 x 10^{-1} dilution was made to facilitate counting. The control treatment consisted of 100 µL of the inoculum suspension, 900 µL of SDW, and 1 mL of the inhibitor.

Inhibitor Standardization (Letheen Broth). To inhibit the effect of the QAC disinfectants after a contact time of ≤ 30 seconds Letheen-modified broth was used, composed of meat extract and soy peptone. Initially, six treatments were defined with different concentrations of the inhibitor (T1 = 43.8 g/L, T2 = 87.6 g/L, and T3 = 131.4 g/L) at a concentration of 1,200 ppm, T4 = 131.4 g/L at 2,400 ppm, T5 = 131.4 g/L SDW and T6 = Control.

Data and statistical analysis. The experiment design for each of the trials was completely randomized with the same number of replicates. For each of the treatments, three biological replicates were carried out with three experiment units. The CFUs formed in the culture medium, the concentration of the disinfectants (ppm), the assumptions of normality and the homoscedasticity were carried out using the Lilliefors (Kolmogorov-Smirnov) and Bartlett ($P<0.05$) tests. Since the data did not meet these assumptions, the Kruskal-Wallis rank sum test ($P<0.05$) was used to represent the significance of the differences. All analyses were performed with the statistical software R (v. 4.1.0), using the agricolae, nortest and ggplot2.

RESULTS AND DISCUSSION

Inhibitor Standardization (Letheen Broth). To determine the efficacy of QAC disinfectants against Foc R1 and TR4, initially, the inhibitor was standardized. The concentration of the Letheen broth presented the best efficiency at 131.4 g/L (3 times more than the recommended concentration), corresponding to T3, with growth of the pathogen. In treatments T1, T2 and T4, no growth of the pathogen was observed, indicating that the QAC continued to act during the incubation period (Figure 1).

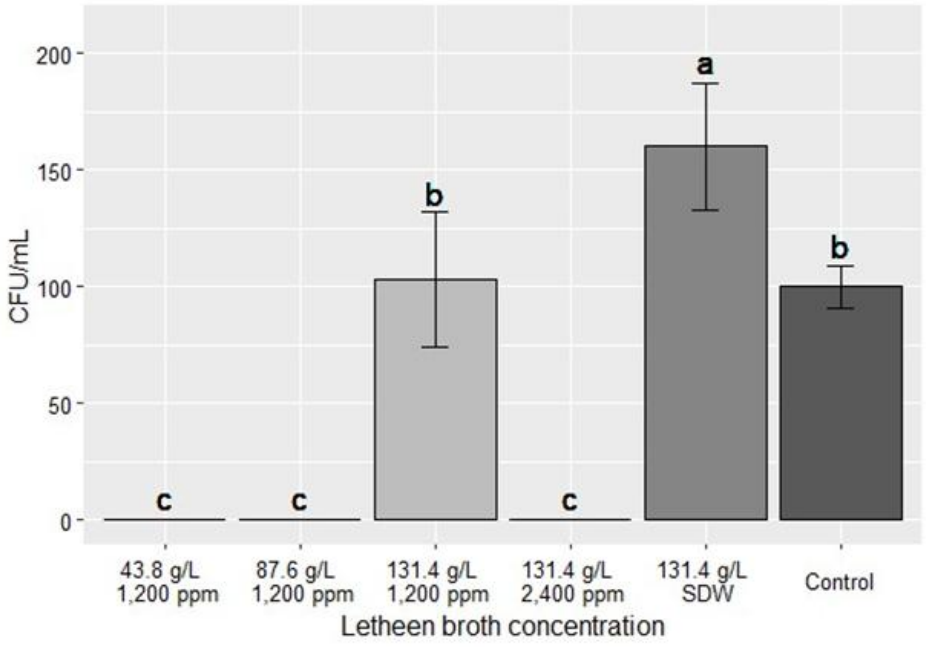

Figure 1. Average colony forming units (CFU) of *Fusarium oxysporum* f.sp. *cubense* R1 estimated for each treatment. Different letters denote significant between treatments according to Kruskal-Wallis non-parametric tests at 5% probability.

Efficacy of disinfectants. The QAC disinfectants evaluated at 1,200 ppm in the absence of soil showed 100% inhibition for the growth of microconidia, macroconidia (Figure 2A) and chlamydospores (Figure 2B) of both Foc strains with a contact time ≤ 30 seconds as compared to the control treatment. In the soil-inoculum interaction, the QACs showed 100% biocidal action in the reproductive structures of Foc R1 and Foc TR4, except for the QAC6 product, corresponding to the active ingredient dimethylbenzyl ammonium chloride at 12%, which allowed the growth of the reproductive structures of Foc R1 (Figure 2C). On the other hand, QAC6 and QAC7 did not fully control the Foc TR4 isolate in the resistance structures (Figure 2D). In the interaction between soil and disinfectant, the QAC4 product, with the active ingredient Di(octyl/decyl) chloride 12% + Benzalkonium chloride 25.5%, had an efficiency of 100% for the reproductive and resistance structures, as compared to the control and other QACs (Figure 2E, F).

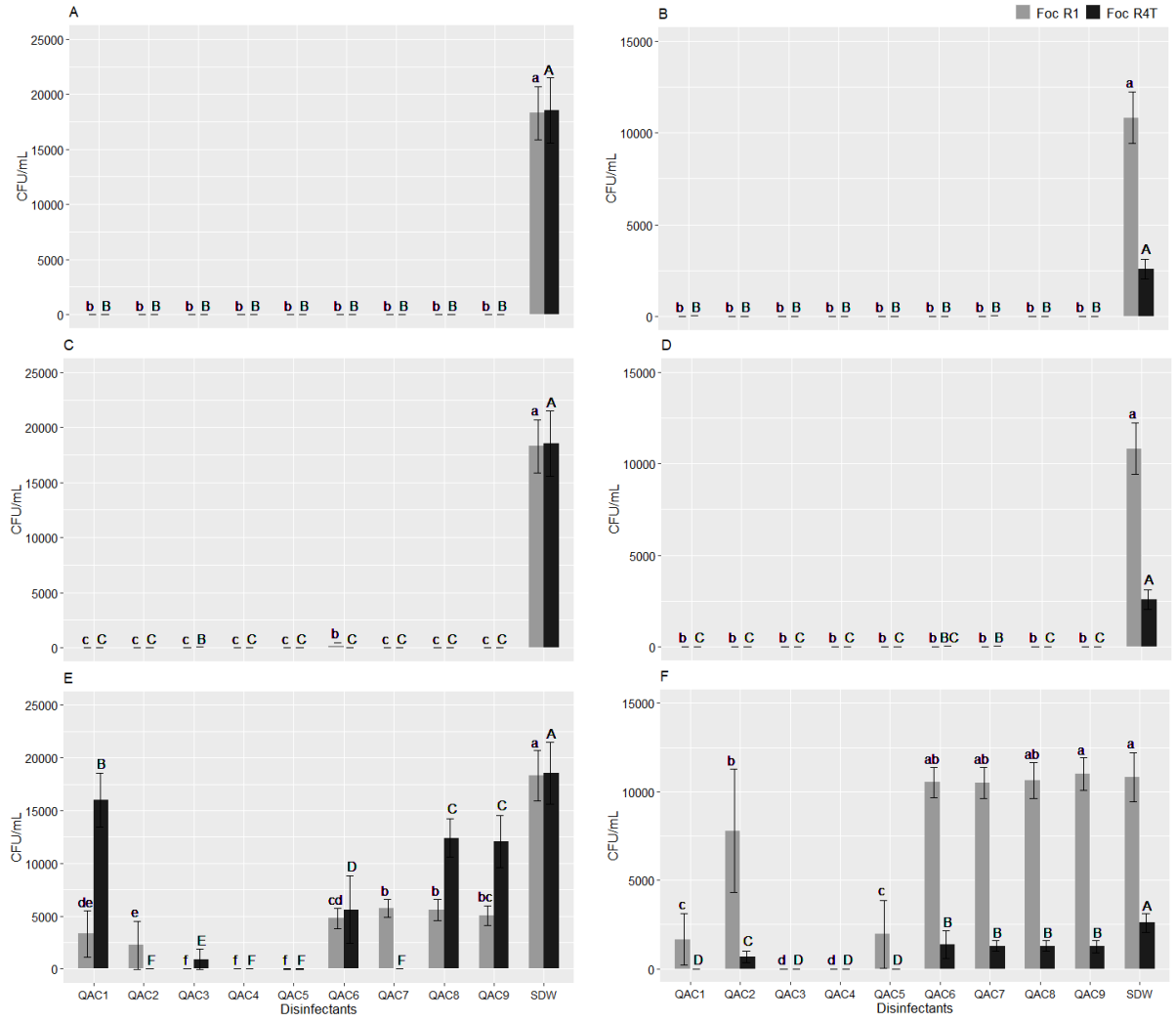

Figure 2. Efficiency of QAC disinfectants against propagules of *Fusarium oxysporum* f. sp. *cubense* R1 ( ) and TR4 (■) after ≤ 30 seconds of contact A, B. Testing efficacy in the absence soil C, D. Testing efficacy in the presence soil (soil-inoculum interaction) and E, F. Testing efficacy in the presence soil (soil-disinfectant interaction) A, C, E. Micro and macroconidia B, D, F. Chlamydospores. Small letters indicate differences between treatments against (R1), and Capital letters indicate differences between treatments against (TR4). The bars with the same letter do not show significant differences between treatments according to Kruskal-Wallis non-parametric tests at 5% probability.

The efficacy of 9 commercial quaternary ammonium-based disinfectants (QACs) were evaluated at 1,200 ppm with a contact time of ≤ 30 seconds (operator time in the footbath) against Foc R1 and TR4, with and without soil. In the absence soil, all QAC disinfectants inhibited the propagules of the pathogen. Similar results were obtained by (Nguyen *et al*., 2019; Izquierdo-García *et al*., 2021; Salacinas *et al*., 2022), with 100%

inhibition of reproductive and resistance structures of *F. oxysporum* f.sp. *cubense* when subjected to QAC disinfectants at concentrations of 1,200 ppm. In the presence of soil, the efficacy of the disinfectants was reduced. Indeed, for eight out of nine disinfectants, only QAC4, with the active ingredient Di(octyl/decyl) chloride 12% + Benzalkonium chloride 25.5%, showed an efficiency of 100% for reproductive and resistance structures of Foc R1 and TR4. QAC4 features two active ingredients at different concentrations, 12% Di-(Octyl/Decyl) Dimethylammonium Chloride and 25.5% Benzalkonium Chloride. A 1%, dilution generates a final concentration of 1,200 and 2550 ppm for Di-(Octyl/Decyl) chloride and benzalkonium chloride, respectively.

CONCLUSION

This study provides scientific evidence to recommend locally-available QAC disinfectants that limit the spread of Foc R1 and TR4. However, since evidenced for most product effectiveness against physiological races of *F. oxysporum* f.sp. *cubense* was not achieved in the presence of soil, washing boots, tools, equipment and essential vehicles used in banana production systems with water, before any disinfection process, is essential to avoid introducing this pathogen to areas that are free of this disease. On the other hand, the treatment water and final disposal of soil resulting from the washing and disinfection processes should be considered.

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