Reduction in populations of *Ralstonia solanacearum* race 2 in plantain (*Musa* AAB Simmonds) with extracts from *Trichoderma* spp. and antagonistic bacteria

Reducción de poblaciones de *Ralstonia solanacearum* raza 2 (Smith) en plátano (*Musa* AAB Simmonds) con aplicación de extractos de *Trichoderma* spp. (Alexopoulus y Mims) y bacterias antagonistas

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Abstract

Moko, bacterial disease caused by *Ralstonia solanacearum* race 2, affects plantain production in Colombia, with losses up to 100 %. The *in vitro* effect of crude filtrates of strains of *Trichoderma* spp. and two commercial products were evaluated versus *R. solanacearum*. *In vitro*, a filtrate was used (40 % in aqueous solution) for each strain of *Trichoderma* spp.; from *R. solanacearum* a dilution of 10^{-4} per petri dish was used, in a completely random design with three replications. For *Ralstonia solanacearum*, the number of colony forming units was determined. In greenhouse, the suspensions of *Trichoderma harzianum* (Agroguard ®), *T. viride* 14PDA3A, and Ecoterra® were applied to Dominico harton plantain plants, and 30 days later plants were inoculated with *R. solanacearum*, a randomized complete blocks design with four blocks was used. *R. solanacearum* severity was evaluated by leaf symptoms scale, of 0 to 6. *In vitro*, crude extracts of two strains and two commercial products of *Trichoderma* spp. inhibited 100% of *R. solanacearum*. *T. viride* and Ecoterra® treatments showed low levels of disease severity by *R. solanacearum* in plants (0.63 and 1.88 respectively).

Key words: Antagonism, Dominico Harton plantain, moko disease, *Trichoderma* spp.

Resumen

El moko, enfermedad causada por *Ralstonia solanacearum* raza 2, afecta la producción de plátano (*Musa* AAB Simmonds) en Colombia, ocasionando pérdidas hasta de 100%. *In vitro*, se evaluó el efecto de filtrados crudos de cepas de *Trichoderma* sp. y dos productos comerciales como posibles reductores de poblaciones de *R. solanacearum*. Para cada cepa de *Trichoderma* spp., se empleó filtrado (40% en solución acuosa) y para *R. solanacearum* se utilizó una dilución de 10^{-4} por caja de petri, en un diseño completamente al azar con tres repeticiones para determinar el número de unidades formadoras de colonia de *R. solanacearum*. En invernadero, se aplicaron suspensiones de *T. harzianum* (Agroguard®), *T. viride* 14PDA3A y Ecoterra® en plantas de plátano Dominico Hartón y 30 días después se inoculó *R. solanacearum* en un diseño de bloques completos al azar con cuatro repeticiones. La severidad del ataque de *R. solanacearum* se evaluó utilizando una escala de síntomas foliares de 0 a 6. *In vitro*, los extractos crudos de dos cepas y dos productos comerciales de *Trichoderma* spp. inhibieron el 100% de *R. solanacearum*. Los tratamientos con *T. viride* y Ecoterra® mostraron bajos niveles de control de *R. solanacearum* en plantas (0.63 y 1.88, respectivamente).

Palabras clave: Dosis-respuesta, *Meloidogyne,* mortalidad, *Radopholus*.

Introduction

In *Musa*, bacterial wilt or moko is the name of the disease caused by *Ralstonia solanacearum* (Smith) Race 2 (Yabuuchi *et al.*, 1995) Biovar 1 and 3, Phylotype 2 Sequevar 4 and 6 (Genin and Denny, 2012). Due to the genetic diversity of this plant pathogen, the group of microorganisms is denominated as *R. solanacearum* species complex (RSSC) (Fegan and Prior, 2006).

This complex induces a wilting that starts with yellowing and collapse of the youngest leaves and necrosis of the flag leaf, symptoms that progress toward the mature leaves and internally the vascular tissues become necrotic, especially those located near the central area of the pseudostem. Immature fruits of affected plants show yellow color and dry rot pulp forming a cavity; when early or before flowering infections occur, an abnormal cluster development occurs and in some cases the plant fails to generate them (De Oliveira and Silva *et al.*, 2000).

Moko has seriously reduced production in the major banana areas planted in Colombia, generating losses of up to 100% on some plantations where it occurs. The pathogen has spread in the departments of Tolima, Valle del Cauca, Huila, Caqueta, Amazonas, Putumayo and the Atlantic Coast (Belalcázar *et al.*, 2003). The most affected areas by the disease in Colombia are presented in the region of Urabá, Antioquia department, where large areas have been eradicated from infested banana crop (Castañeda and Espinosa, 2005; Londoño, 2012). In Meta and Caqueta the disease ravaged 20,000 hectares between 1970 and 1980 and in Quindio between 1999 and 2000, which generated losses of US $ 73,000, approximately (Obregón, 2007).

Because the chemical control of this disease has not been successful, work is currently done to develop management strategies designed to prevent the entry of the causal agent to plantations or prevent it from spreading to new areas, treating outbreaks to reduce the bacterial population and its spread to new areas (Álvarez *et al.,* 2013).

Fungi of the genus *Trichoderma* are widely used in the biological control of phytopathogenic antagonists presented in soil and seed (Alexopoulos and Mims, 1979). This biocontroller produce antibiotics and other secondary metabolites, with different mechanisms of action on the phytopathogenic microorganisms, some of them are: Pachibasin, which belongs to the octaketids; Trichodermin, from the group of monoterpenes or trichothecans; Trichorzianins, antifungal metabolites of high solubility that once located on the fungal spores can maintain its activity for extended periods (3-4 months); and gliotoxin, which has antibiotic activity against bacteria and fungi (Howell *et al.*, 2000).

The effect of *Trichoderma* spp., in inhibiting pathogens and improving plant nutrition occurs in differential way depending on the species used; for example, the significant growth of *Tagetes erecta* plants inoculated with *T. harzianum* and *T. aureoviride* was reported by Calvet *et al.* (1993), in contrast to the inhibitory effect of strains *T. pseudokoningii* on the growth of soybean plants (Martinez *et al.*, 2004).

Moreover, some bacteria of the genera *Pseudomonas* and *Bacillus* are efficient in controlling foliar and root diseases, due to its rapid colonization (Fernandez Larrea, 2001). *Pseudomonas* strains have properties against plant pathogens as they improve the absorption of minerals by the plant, such as iron, which after conversion to siderophores (low weight molecules with affinity for chelated iron (III)) becomes more plant available (Sharma and Johri, 2003) and less available for the pathogens; it is known that pathogens do not produce siderophores and can not take it from antagonists in its environment (Weller, 1988). Korsten *et al.* (1997) found that *B. subtilis* in pre- and post-harvest applications presented a similar effect compared to commercial

fungicides to control fungal diseases in avocado.

Materials and methods

Location

The trial was conducted in the laboratory and greenhouse of the Cassava Plant Pathology Program of the International Center for Topical Agriculture (CIAT), located in Palmira, Valle del Cauca (Colombia), at 3° 50' N and 76° 35' W, 980 m.a.s.l., with an annual rainfall of 1100 mm, relative humidity of 78% and average temperature 24.5 °C.

Laboratory Test

Under in vitro conditions crude extracts from *Trichoderma* strains were evaluated. To evaluate the effect, the strains 41TSM1, 19TSM3A (*T. virens*), 47PDA3A (*T. virens*) and CIAT 14PDA4 (*T. viride*) and the commercial products Agroguard® (*T. harzianum*), and Trichoplant® (mixed strains of *T. harzianum, T. viride, T. koningii* and *T. lignorum*) were used. Collection strains were characterized by Arango (2009), with amplification of the ITS region of ribosomal DNA (rDNA) by PCR with universal primers and ITS4-5 ITS1-4.

Samples of strains and each commercial product were cultured in liquid sterile -PD medium: Potato (200 g/l) and Dextrose ® (17 g/l) for 15 days. Crude extracts were sterilized by filtration through a nitrocellulose membrane with pore size of 0.22 μm. Evaluation of extracts was made at 40% concentration; to which 48 ml of structure-free filtrate was mixed with 72 ml of sterile PDA (39 g/l) to give a final volume of 120 ml, which was deposited in six Petri dishes (adapted from Bedoya *et al.*, 2000).

On the surface of solidified medium, which contained crude extracts of *Trichoderma* spp., using a bacteriological loop, a sample of 100 ml of 10^{-4} dilution of *R. solanacearum* (strain 78) was distributed. This dilution was obtained from a bacterial suspension of 0.2 absorbance (600 nm), corresponding to a concentration of about 1 x 108 CFU/ml (He et al., 1983). Negative and positive controls were prepared with sterile PDA and with and without *R. solanacearum*, respectively. A summary of the treatments is included in Table 1.

Table 1. Laboratory treatments employed

No.	Treatments
1	41TSM1 + *R. solanacearum*
2	19TSM3A *T. virens* + *R. solanacearum*
3	47PDA3A *T. virens* + *R. solanacearum*
4	14PDA3A *T. viride* + *R. solanacearum*
5	Agroguard®+ *R. solanacearum*
6	Trichoplant®+ *R. solanacearum*
7	Negative control: *R. solanacearum*
8	Positive control: PDA

The counting of colony forming units (CFU) of *R. solanacearum* per box was done daily, the values obtained in counts were calculated in CFU per microliter (CFU/μl) and the final results were expressed in CFU/ml of bacterial suspension. A completely randomized design with three replications was used, where the experimental unit consisted of a Petri dish.

Greenhouse test

For this test, two months old Dominico Harton plantain plantlets were used and planted in bags with 5 kg of a steam sterilized mixture of Oxisol soil of the experimental station CIAT-Quilichao and sand in a proportion 2:1. To study the inhibition effect on *R. solanacearum* the treatments with Agroguard® and 14PDA3A *T. viride* that showed consistent results in laboratory testing were chosen; in addition the product Ecoterra® (mixture of *Azotobacter chroococcum, Pseudomonas aeureofaciens, Bacillus licheniformis, B. megaterium and B. subtilis*) (Table 2) was included.

Initially, the antagonistic microorganisms T. viride and the commercial products Ecoterra® Agroguard® were applied, in order to promote the establishment and colonization of the

microorganisms in the soil and the radical system of banana seedlings. For each treatment 50 ml/plant were applied using Agroguard® concentrations and *T. viride* 14PDA3A 1 x 10^8 conidia/ml and 0.5 g of Ecoterra®, equivalent to a concentration of 1 x 10^8 CFU.

Table 2. Greenhouse treatments employed

No.	Treatments
1	Agroguard®+ *R. solanacearum*
2	Ecoterra ®+ *R. solanacearum*
3	14PDA3A *Trichoderma viride*+ *R. solanacearum*
4	Negative control: *R. solanacearum*
5	Positive control: Sterile water without *R. solanacearum*

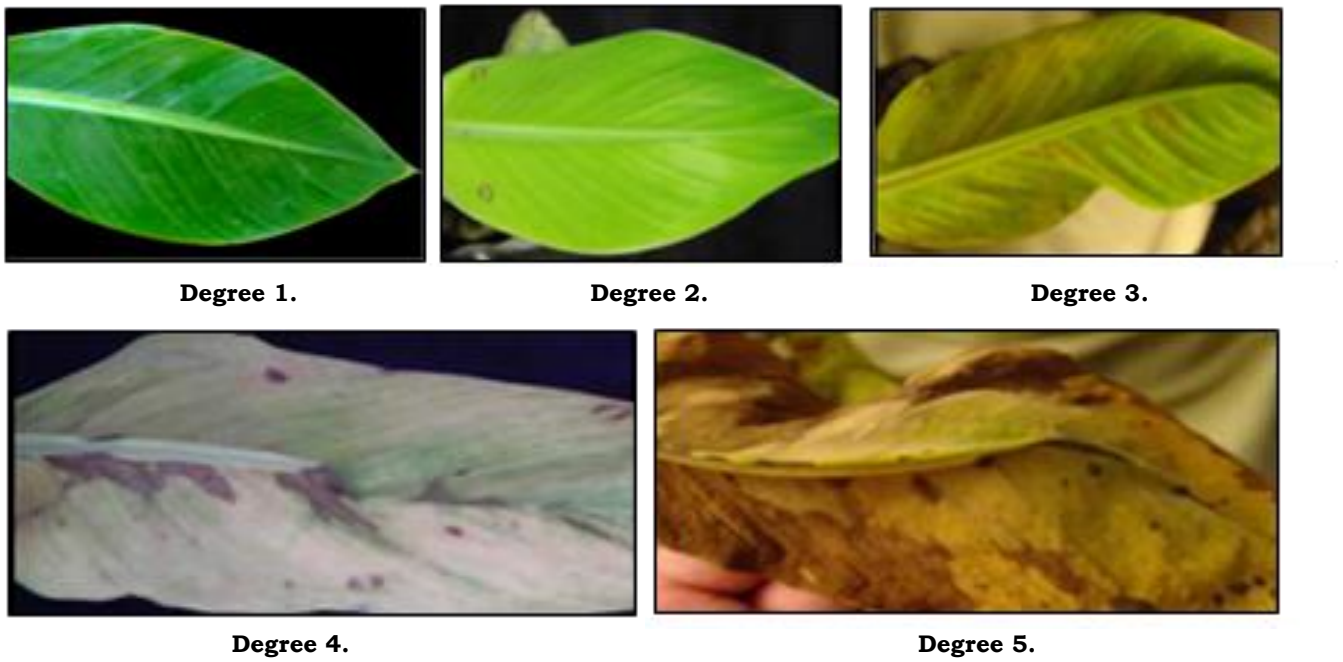
As inoculum, *R. solanacearum* (strain 78) was applied a month after the treatments above; for this, a wound was made in the plant root and above it a bacterial suspension of 0.1 absorbance (600 nm wavelength) equivalent to 1 x 10^5 CFU/ml 50 ml was applied, following the methodology used for tomato by Hernandez *et al.* (2005) and Deberdt *et al.* (1999). To measure the degree of disease a scale 0 (absence) - 6 (plant death) of foliar symptom evolution was used, proposed by Mesa and Triviño (2007) (Figure 1). Additionally foliar nutrient concentration was measured.

A randomized complete block design with four blocks and eight repetitions with a two plants as experimental unit was used. Data were analyzed by ANOVA and mean comparison tests, using SAS Statistical Analysis System Version 9.4 statistical package.

Results and discussion

Laboratory test

A highly significant effect (P <0.001) of the crude extracts of *Trichoderma* sp., *in vitro*



Degree 1. **Degree 2.** **Degree 3.**

Degree 4. **Degree 5.**

Degree	Simptoms
1	Flaccidity when touched.
2	Remarkable wilting, loss of bright green.
3	Remarkable flaccidity, yellowing occurring sometimes.
4	Yellowing with necrosis in some places, very advanced sagging, loss of formada.
5	Advanced necrosis, shows the severity of the disease, leaf shape lost.

Figure 1. Foliar damage symptom scale for *R. solanacearum* in banana plants. From Mesa and Triviño, 2007

inhibition of *R. solanacearum* (Table 3) was observed. The treatments that most inhibited the bacteria were 41TSM1, 14PDA3A, Agroguard® and Trichoplant® because they did not allow the growth of *R. solanacearum* colonies, compared to the negative control (2,920 CFU/ml).

Table 3. Analysis of variance for means of the effect of crude extracts of *Trichoderma* sp. in the inhibition *in vitro* of *R. solanacearum.*

Treatment	CFU[a]
41TSM1 + Rs[b]	0 c*
19TSM3A *T. virens* + *Rs*	5.720 ab
47PDA3A *T. virens* + *Rs*	8.853 a
14PDA3A *T. viride* + *Rs*	0 c
Agroguard®+ *Rs*	0 c
Trichoplant®+ *Rs*	0 c
Rs	2.920 bc
PDA	0 c

a. CFU: Average colony forming units of *R. solanacearum* per milliliter.
b. Rs/: Ralstonia solanacearum.
* Means with same letters do not differ significantly (P > 0.05), according to Tukey test.

The differences found on the effect of the strains of *Trichoderma* spp., correspond to the high specificity of this antagonistic phytopathogenic fungus, as some strains completely inhibited the *in vitro* growth of *R. solanacearum*, as e.g. 41TSM1, 14PDA3A, Agroguard® and Trichoplant®; while others like *T. virens* (19TSM3A and 47PDA3A strains) induced its growth.

This result agrees with those obtained by Asmaja (2005), who reported antibacterial activity of cell-free filtrate of *T. viride* against *R. solanacearum* which causes wilt in ginger.

Greenhouse test

In this test differences were observed among treatments on the development of leaf symptoms of moko on banana. Differences occurred on day 34 (P < 0.0385) and 40 (P < 0.0134) after inoculation with *Ralstonia solanacearum* (Table 4). At the end of the assessment, treatment differences were highly significant (P < 0.0084) being Agroguard® and *T. viride* (14PDA3A) the most contrasting, as they had the highest (3.63) and the lowest (0.63) degree of disease, compared to the negative control *R. solanacearum* (2.87). The result obtained with *T. viride* confirms its antagonistic capacity to counteract the effect of *R. solanacearum*, which was evident in the lower disease severity. This coincides with the results of Asmaja (2005).

Ecoterra® was also antagonistic to *R. solanacearum* as it delayed the appearance of symptoms and disease severity (1.88). With this product a lower symptom progression of the disease and further development of the roots (Photo 1) was observed. This is because the *Pseudomonas* strains that contains belong to the so-called plant growth promoting bacteria (PGPB) which stimulate the production of hormones such as auxin, gibberellins and

Table 4. Analysis of variance for the effect of the treatments with Agroguard®, Ecoterra® and *Trichoderma viride* (14PDA3A) on the development of leaf symptoms of moko caused by *R. solanacearum.* Scale 0 (absence) – 6 (death of plant).

Treatment	Days after inoculation									
	16	19	22	25	28	31	34 *	37 *	40 *	43 **
Agro® + Rs[a]	0.13 a[b]	0.25 ab	0.88 ab	1.25 a	2.75 a	3.00 a	3.25 a	3.38 a	3.50 a	3.63 a
Ecot® + Rs	0.00 b	0.00 b	0.13 ab	0.13 ab	0.75 ab	1.00 abc	1.38 abc	1.38 abc	1.75 abc	1.88
14PDA3A + Rs	0.00 b	0.00 b	0.00 b	0.00 b	0.13 b	0.13 bc	0.375 bc	0.5 ab	0.63 bc	0.63 bc
Rs	0.13 a	0.50 ab	1.00 ab	1.38 a	1.50 ab	1.50 abc	2.25 ab	2.38 ab	2.62 ab	2.87 ab
Witness	0.00 b	0.00 b	0.00 b	0.00 b	0.00 b	0.00 c	0.00 c	0.00 c	0.00 c	0.00 c

a Ralstonia solanacearum strain 78.
b Means with same letters do not differ significantly (P > 0.05), according to Tukey test.
* Significant differences.
** Highly significant differences.

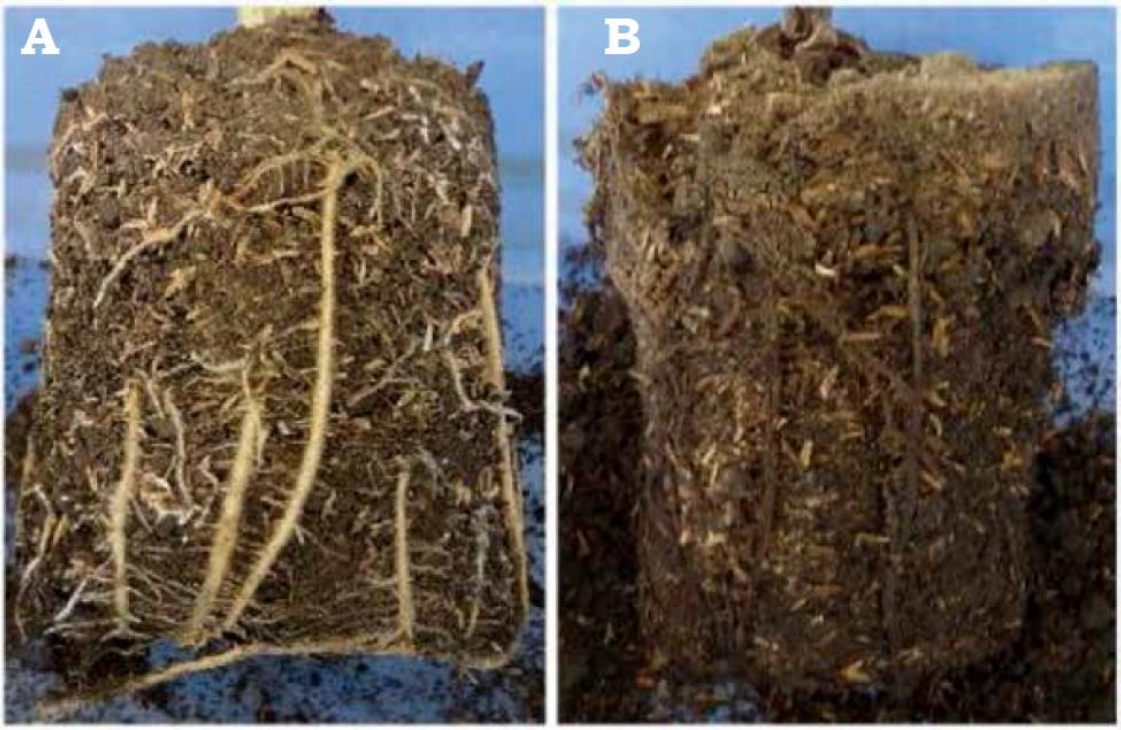


Photo 1. Root development in Dominico hartón plantlets treated with Ecoterra® (A) and untreated (B).

cytokinins, prompting root initiation and increasing the formation of roots and root hairs, feature that allows more area for plant nutrient absorption and tolerance to *R. solanacearum* infection (Table 5).

Even though the ANOVA showed no difference among treatments for foliar nutrient concentration, it was observed that the potassium concentration was higher in the treatment *T. viride* (60.1 g/kg) vs. the control (47.6 g/kg), which coincides with lower disease severity in plants treated with this strain, this shows that this nutrient promotes plant tolerance to attack by pathogens (Álvarez *et al.*, 2002).

However, the ability of *Bacillus* spp., and *Pseudomonas* spp., present in Ecoterra ® to solubilize nutrients such as phosphorus, by producing organic acids or by phosphatases (Kloepper *et al.*, 1989), this effect was not present in this soil since, according to the results of the chemical analysis, the concentration of this element was very low (13.72 mg/kg). Moreover, the higher iron content in the treatments with Ecoterra® coincides with the findings of Sharma and Johri (2003) who found that antagonist bacteria of the genus *Pseudomonas* improve the absorption of this element, which could influence the

Table 5. Foliar concentration of nutrients in plantles of Dominico hartón inoculated with Agroguard® (*T. harzianum*), Ecoterra®, and *T. viride* (14PDA3A).

Treatment	N[1]	P	K	Ca	Mg	Total S	Total B	Fe	Mn	Cu	Zn	Na
	g/k						mg/k					
Agro + Rs[a]	36.4	3.0	55.3	9.4	4.2	4.8	22.2	223.0	1.792.9	9.1	22.9	1.782.0
Ecot + Rs	37.3	1.9	51.3	11.5	2.9	3.1	17.1	230.9	3.565.0	9.9	27.3	1.574.8
T. viride+ Rs	35.0	2.3	60.1	12.2	4.1	3.3	23.7	208.3	2.104.8	10.4	29.4	1.260.4
Rs	36.4	3.0	55.3	9.4	4.2	4.8	21.3	223.0	1.792.9	9.1	22.9	1506.0
Witness	31.6	2.4	47.6	10.9	3.8	3.9	16.9	222.7	2.094.1	10.7	32.6	3.593.3

a. Rs: *Ralstonia solanacearum* strain 78

reduction of the amount of inoculum and pathogenic activity of *R. solanacearum*.

With the exception of zinc, all nutrients showed higher concentration in the treatments that received Ecoterra®, this mainly because the genera *Bacillus, Pseudomonas* and *Azotobacter* present in this product are the most important in agriculture for the transformation of organic and inorganic compounds that promote plant nutrition (Higuera, 2008).

The search for options to treat soils contaminated with high populations of *R. solanacearum* as a result of the devastation of highly susceptible hosts requires the exploration of new strategies, including the combined use of *T. viride* and Ecoterra® for field evaluations with natural populations of bacteria.

Conclusions

- *Trichoderma viride* and Ecoterra® are different alternatives to agrochemicals used conventionally to reduce populations of *R. solanacearum* in soil.
- *Trichoderma viride* was the best treatment to inhibit *R. solanacearum in vitro* and counter moko disease in banana plantlets under greenhouse conditions; however under field conditions the results still need to be evaluated over longer periods.
- Ecoterra® improves the root development of banana plantlets, resulting in improved plant growth and tolerance to biotic factors, including Moko disease.
- Reduced populations of *Ralstonia solanacearum* race 2 (Smith) on plantain (*Musa* AAB Simmonds) with application of extracts from *Trichoderma* spp. (Alexopoulus and Mims) and antagonistic bacteria.

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