

# Rhizosphere microorganisms, potential antagonists of *Fusarium* sp. causing agent of root rot in passion fruit (*Passiflora edulis* Sims)

Microorganismos rizosféricos, potenciales antagonistas de *Fusarium* sp. causante de la pudrición radicular de maracuyá (*Passiflora edulis* Sims)

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## Abstract

The passion fruit crop (*Passiflora edulis*) is very important for the Colombian economy. Nowadays this crop is affected by damping-off disease caused by *Fusarium* sp. So, it is necessary to look for alternatives which allow us to control the disease efficiently. The bacteria *Azotobacter* spp., *Azoosporillum* spp. and the fungi *Trichoderma* spp., were evaluated as a *Fusarium* sp. potential biocontrol in In vitro and In vivo test. The research was carried out in laboratory and nursery. The “dual test” showed that a wild isolate of *Trichoderma* spp. and a commercial product (*Trichoderma lignorum*), inhibited the mycelial growth of *Fusarium* sp. between 94.2 and 93.6% respectively. *Trichoderma* evaluation on passion fruit plantlets at three application times demonstrated that applying *Trichoderma* before *Fusarium* sp. appearance, decreased the disease occurrence between 75.0 and 50.0%, whereas applying *Trichoderma* after or simultaneously with the pathogen, the disease in the plantlets decreased until 25.0%. This suggests that inoculation of pregerminated seeds with biocontrol agents improved the protection of plants against the pathogenic and they are an important tool for management of diseases in plants of passion fruit.

**Key words:** *Azoosporillum* spp., *Azotobacter* spp., biocontrol, *Passiflora edulis*, *Trichoderma* spp.

## Resumen

El cultivo de maracuyá (*Passiflora edulis*), de gran importancia económica para Colombia, actualmente es afectado por la enfermedad del marchitamiento vascular causado por *Fusarium* sp. lo que hace necesario la búsqueda de alternativas que permitan un control eficiente de esta enfermedad. Aislados de las bacterias *Azotobacter* spp., *Azoosporillum* spp. y el hongo *Trichoderma* spp., fueron evaluados como potenciales biocontroladores de *Fusarium* sp. en pruebas in vitro e in vivo. Las pruebas de “test dual” evidenciaron que un aislado nativo de *Trichoderma* sp. y un producto comercial (*Trichoderma lignorum*), provocaron la inhibición del crecimiento micelial de *Fusarium* sp. entre 94.2% y 93.6%, respectivamente. La evaluación de aislados de *Trichoderma* sobre plántulas de maracuyá en tres momentos de aplicación indicó que la inoculación previa disminuyó el porcentaje de infección de las plantas entre un 75 y 50%, mientras que con aplicaciones después o simultáneamente con el patógeno, el porcentaje de infección disminuyó en 25%. Estos resultados indican que la aplicación de organismos de biocontrol en semillas pregerminadas mejora la protección de las plantas contra el fitopatógeno estudiado y son un recurso importante en el manejo preventivo de las enfermedades de maracuyá.

**Palabras clave:** *Azoosporillum* spp., *Azotobacter* spp., biocontrol, *Passiflora edulis*, *Trichoderma* spp.

## Introduction

Passion fruit production (*Passiflora edulis* Sims) in Colombia has an important place in the fruit exploitation. Among the departments with higher production is Huila with a cultivated area of 1635 ha. However, a phytosanitary problem in the crop is the vascular wilt known as "damping-off" caused by *Fusarium* spp., that is characterized by the partial yellowing of leaf, dwarf sprouts, reduction in plant growth and therefore production losses (Lozano-Tovar *et al.*, 2008).

*Fusarium* pathogenic strains show a high specificity level for their hosts and there are around 120 special forms known (Armstrong and Armstrong, 1981). The control of this pathogen is primarily done by broad spectrum fungicides as methyl bromide (Fravel *et al.*, 2003). However, these chemical control strategies have generated an emergence of resistant strains, in addition to the negative effects on public health and environment. This problem allows presenting the use of biological control strategies, important to recover the balance in the agroecosystem and to exploit the potential natural antagonist of some microorganisms, as fungi and rhizobacteria, against vulnerable pathogens (Avendaño *et al.*, 2006).

Among the most used microorganisms in biological control are the genus *Trichoderma*, *Pseudomonas*, *Bacillus*, *Paenibacillus*, *Azobacter* and *Azospirillum* (González *et al.*, 2004). *Trichoderma* is a beneficial fungus of free live, commonly found in soil and associated to plant roots. It is avirulent, able to produce antibiotics and lytic enzymes as cellulases, hemicellulases, xilases and chitinases of industrial interest to protect crops (Harman *et al.*, 2004).

*Trichoderma* species can perform indirect biocontrol on phytopathogenic fungi, competing for space and nutrients, modifying environmental conditions, stimulating plant growth and their defense mechanisms; also they can do biocontrol directly by mycoparasitism. These mechanisms can act on a coordinate fashion and its importance in the biocontrol processes depends on the *Trichoderma* strain, the fungus that is antagonist to, crop type and environmental conditions such as

nutrient availability, pH and temperature (Benítez *et al.*, 2004; Porras, 2000).

Root colonizing bacteria and their zone of influence are known as plant-growth promoting rhizobacteria (PGPR), have key functions in plants, such as biological control of pathogens by antagonistic effects or induction of systemic resistance, increment in the bioavailability of the mineral nutrients such as phosphate solubilization, nitrogen fixation or phytostimulation, antibiotic production, phytotoxins degradation and siderophores production (Mantilla, 2007).

Based on the stated above, the objective of this research was to evaluate rhizospheric microorganisms as potential antagonist of *Fusarium* spp., causing agent of root rot in passion fruit in the department of Huila, including the bacteria genus *Azotobacter*, *Azospirillum* and species of the fungi *Trichoderma*.

## Materials and methods

The research was performed in the laboratory of Soil Microbiology of the Colombian Corporation for Agricultural Research (Corpoica), in Nataima, Espinal-Tolima (Colombia).

### Biological material

The *Fusarium* sp. isolate 054 was used, it was obtained from sick passion fruit plants selected as highly pathogenic in previous tests (Ruiz *et al.*, 2010) and molecularly characterized (Sandoval-Lozano *et al.*, 2010). PDA (potato dextrose agar) pH 5.5 – 6.0 was used for its growth. The beneficial microorganisms *Azotobacter* spp. (isolates 015 and 028) and *Azospirillum* spp. (isolates 002 and 023) were obtained from the rhizosphere of healthy passion fruit plants and selected by their capacity of indolacetic acid production (Muñoz and Lozano-Tovar, 2007). For the *Trichoderma* genus were used: three native isolates (Tr001, Tr002 and Tr003) obtained from soils of Algerias and Rivera in the department of Huila (Muñoz and Lozano-Tovar, 2007), two commercial products (*T. lignorum* and *T. harzi-anum*) and a preformulated product (*Trichoderma* sp.). For antagonists growth specific media were used: Ashby for *Azotobacter* spp., semisolid NFB for *Azospirillum* spp. (Bashan,

1998) and agar-juice V-8 for *Trichoderma*. Passion fruit seeds were commercially obtained from the company Semillas del Pacífico ICA 00581 registry.

#### **in vitro antagonistical activity of *Trichoderma* spp. on a *Fusarium* sp. isolate**

This test was performed in PDA Petri plates. For this, a 5 mm diameter disc with a pathogen growth of 8 days was placed at 1 cm of the plate edge, in the opposite side was placed a disc with 6 days old *Trichoderma* spp. A completely randomized design was used with four replicates. It was measured: percentage of inhibition of mycelia growth of *Fusarium* sp., determined by the equation  $\%IC = (CC - CF)/CC \times 100$ , where  $CC$  = *Fusarium* sp. colony diameter growing without the presence of antagonists and  $CF$  = phytopathogen colony diameter growing in the presence of the antagonist (Avendaño *et al.*, 2006). Antagonistic capacity was determined by the scale proposed by *et al.* (1982) from 0 to 4, where: 0 = absence of invasion of the pathogen surface and 4 = total invasion of the pathogen surface and sporulation on it.

#### **Antagonist crude extracts effects on conidia germination of *Fusarium* sp.**

*Trichoderma* isolates were cultured in 500 ml of V8-pH 6 media, incubated for 6 days at 140 rpm and 28 °C. *Azotobacter* spp. and *Azospirillum* spp. were cultured in 250 ml of NFB modified media (Haahtela *et al.*, 1981) and were incubated for 48 h at 140 rpm and 28 °C. Fungi and bacteria biomass were separated by centrifugation at 3000 rpm for 15 min, supernatant was filter on Waltman® 40 paper and through a cellulose membrane Milipore® 0.22 µm. on agar plates was pipetted 1 ml of the filtrates, then 100 µl of  $1 \times 10^5$  conidia/ml of *Fusarium* sp. suspension were sowed in spots previously determined.

For control, 100 µl of distilled water were used. Treatments were distributed on a completely randomized design with three replicates. Percentage of inhibition of germination (%IG) was evaluated, which was determined 7 h after treatments were set by counting 100 spores (germinated or not), with the equation:  $\%IG = ((GC - GF)/GC) \times 100$ , where  $GC$  = spores germination on the control treatment,  $GF$  =

phytopathogen spores germination treated by filtering.

#### ***Trichoderma* spp. effect on the root rot development on plants inoculated with *Fusarium* sp. in mesh house**

The native isolates (Tr001, Tr002 and Tr003) were cultivated on PDA plates and incubated for 96 h. Spores were harvested in sterile distilled water and the concentration was adjusted to  $1 \times 10^6$  conidia/ml using Neubauer chamber, the commercial and preformulated products of *Trichoderma* were applied according the technical recommendation for product use. *Fusarium* sp. Isolate was multiplied in potato (*Solanum tuberosum*) slices and was incubated for six days at 28 °C, the concentration for plant inoculation was adjusted to  $1 \times 10^6$  conidia/ml (Ruiz *et al.*, 2010).

Commercial seeds of passion fruit (*Passiflora edulis*) were used, sowed on substrate 1:1:2 (burnt rice husk: rice husk: soil) sterile on autoclave at 15 psi for 1 h during two consecutive days. Three times of biocontrols application were evaluated: **moment 1**: pathogen was applied eight days after antagonist inoculation; **moment 2**: pathogen and antagonist inoculation at the same time and; **moment 3**: pathogen was applied eight days before the antagonist. Three controls were used: chemical (Mefenoxam 48% v/v, equivalent to 465 g/l Metalaxil-M); negative or blank were plants without inoculum and; positive were plants inoculated only with the phytopathogen. Observations on the disease development were done for four months by registering the external symptoms and plant mortality. A completely randomized design with four replicates was used. All the plantlets had a superficial cut at the root collar made with scalpel at the moment of pathogen inoculation.

#### **Evaluation of antagonistic microorganisms application on seeds pre-germinated and sowed on *Fusarium* sp. inoculated substrate**

Passion fruit seeds were disinfected with 0.8% sodium hypochlorite for one minute and washed three times with sterile water. Then, they were placed on water for 24 h to pre-germinate. Antagonistic microorganisms Azo-

*tobacter* spp. (015 and 028), *Azospirillum* spp. (002 and 023), *Trichoderma* sp. (Tr003) were cultures on specific media (Ashby, semisolid NFB and V8 juice). *Fusarium* sp. inoculum was obtained by the previously described methodology. A concentration of  $1 \times 10^6$  conidia/ml was used for *Trichoderma* spp. and *Fusarium* sp. Bacteria isolates concentration was adjusted colorimetrically to  $600 \times 10^6$  UFC/ml (Sutton, 2011). Two moments of application were evaluated: moment 1: previous inoculation of the antagonists to the pregerminated seeds and sowing of these 48 h after pathogen application to the substrate; moment 2: inoculation of the antagonists to the pre-germinated seeds and sowing eight days before the phytopathogen application to the substrate. Observations on the disease development were done for four months registering the external symptoms and plant mortality. The experiment had a completely randomized design with four replicates. Additionally, three controls were established as previously described.

### Analysis of results

Data were processed by analysis of variance and mean differences were determined by the Tukey's multiple range test at 95% ( $p < 0.05$ ) using the software Statistix 8 (2008).

## Results

### *Trichoderma* spp. antagonistic activity over *Fusarium* sp.

Antagonistic activity evaluation showed differences between treatments ( $p < 0.001$ ); the Tr003 isolate and the commercial product *T. lignorum* showed the highest percentages of mycelial growth inhibition of *Fusarium* sp.

with 94.2 y 93.6%, respectively. According to the scale proposed by Bell *et al.* (1982), the native isolate Tr003 was qualified as class 4 because it invaded the pathogen surface and sporulated over it (Table 1). Avendaño *et al.* (2006) in *in vitro* tests for antagonism with *Trichoderma* spp. found, similarly, inhibitions in growth of *F. oxysporum* with total invasion of the pathogen mycelium seven days after application. According to Tovar (2008) *Trichoderma* spp. isolates inhibited the growth of *R. solani* till 63.67% after 72 h. According to Melo and Faull (2000) *T. harzianum* and *T. koningii* inhibited between 79-82% of the mycelial growth of *R. solani* demonstrating different mechanisms of action where *T. harzianum* parasites and destroys *R. solani* mycelium and *T. koningii* produces considerable amounts of antibiotics.

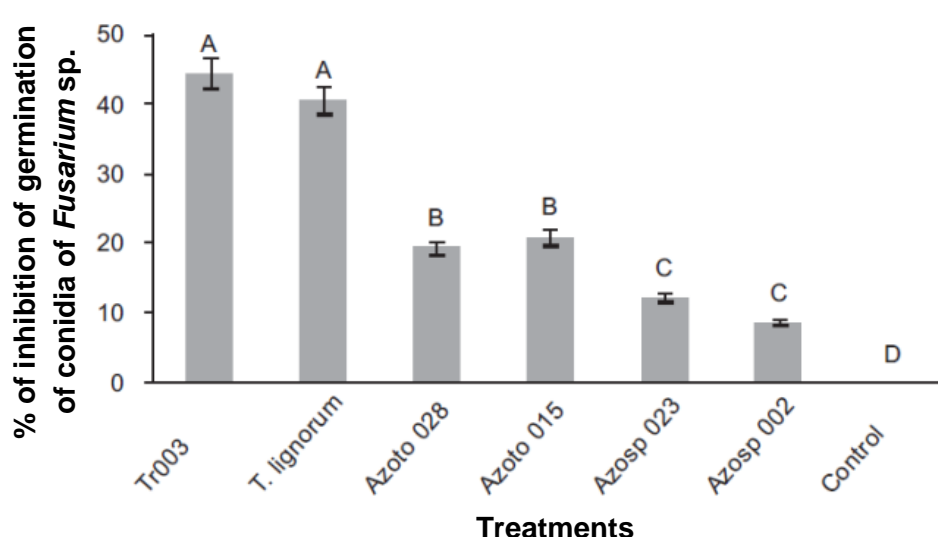
### Evaluation of the effect of antagonist raw extracts on conidia germination of *Fusarium* sp.

Statistical differences between treatments ( $p < 0.001$ ) were observed, differences between all the microorganisms against the control and differences among antagonists were found (Figure 1). The commercial product *T. lignorum* and the native isolate of *Trichoderma* sp. (Tr003) presented the highest percentage of inhibition on conidia germination of *Fusarium* sp., with 44.7 and 40.7%, respectively; whereas the observed inhibition with extracts of *Azobacter* and *Azospirillum* isolates was less than 20% (Figure 1). Inhibition of germination could occur by the variety of enzymes produced by *Trichoderma* spp. as glucanases, chitinases, exonucleases, proteases, and other highly toxic metabolites as harzianic acid, alamethicins, peptaibols,

**Table 1.** Percentage of inhibition of mycelia growth of *Fusarium* sp. In cultures of dual plate with *Trichoderma* spp. and valuation according to the scale of Bell *et al.* (1982).

Treatment	Inhibition (0)	Qualification (Scale of Bell <i>et al.</i> , 1982)
Isolate Tr003	94.2 a*	3.5 a
<i>T. lignorum</i>	93.6 a	3.3 ab
<i>T. harzianum</i>	79.4 b	2.8 abc
Isolate Tr001	78.7 b	3.0 abc
Isolate Tr002	77.4 b	2.3 bc
Pre-formulated	73.6 b	2.0 c

\* Values with same letters in column do not have statistical differences according to the Tukey's test ( $P < 0.05$ ).



**Figure 1.** Effect of raw extracts of antagonistic microorganisms on conidia germination of *Fusarium* sp. Values with same letters do not have statistical differences according to the Tukey's mean test at 95% ( $P < 0.05$ ).

antibiotics, 6-pentyl- $\alpha$ -pyrone, viridine, gliovirin, glisoprenins, heptelidic acid, among others (Benítez *et al.*, 2004). According to Michel *et al.* (2005) *Trichoderma* strains produce chitinases and glucanases and inhibit the reproductive potential of *F. oxysporum* conidia till 95% and inhibit mycelial growth in 34%

#### ***Trichoderma* spp. effect on root rot in passion fruit plants inoculated with *Fusarium* sp.**

In the study done in mesh house, the previous inoculation showed differences ( $p = 0.0003$ ) between the treatments. Native isolates of *Trichoderma* Tr002 and Tr003 reduced plant infection between 75 and 50%, respectively. Nonetheless, when applied simultaneously with the pathogen the infection reduction was only 25% and when applied eight days after pathogen inoculation there was no disease reduction. The *Trichoderma* sp. (preformulated) and *T. lignorum* products showed 100% of infection in all the moments of application (Table 2), indicating that some *Trichoderma* fungal strains reduce the disease only when are applied for prevention. Hernández *et al.* (1999) found similar results to the ones in this study when evaluating application times of *T. harzianum* for controlling *Dothiorella* sp., ob-

taining a better result when the antagonist was applied 24 h before the pathogen.

#### **Evaluation of the antagonistic microorganism application in pregerminated seeds of passion fruit sowed on *Fusarium* sp. inoculated substrates**

When pre-germinated seeds of passion fruit were treated with *Azobacter* spp., *Azospirillum* spp. and *Trichoderma* spp., eight days before the *Fusarium* sp. inoculation, difference between treatments were observed ( $p < 0.001$ ), being the *Trichoderma* treatments statistically equal to the blank as well as the *Azotobacter* isolate 015 and the *Azospirillum* isolate 002. The highest efficiency was achieved with *Trichoderma* (Tr003 and *T. lignorum*), which reached 100% protection (Table 3).

When the antagonistic were added to the seeds 48 h after pathogen inoculation statistical differences between treatments were established ( $p < 0.001$ ), thus the *Trichoderma* isolates were different to the positive control but equal to the blank. Its protection against the disease was 75 and 87.5% (Table 3), while the rhizobacteria and chemical control treatments were similar to the positive control and their protection varied from 12.5 to 50% (Table 3). González *et al.* (2004) observed that the

application of *T. harzianum* in melon seeds reduced the incidence of *F. oxysporum* between 37.5 and 46.3%. Rincon (1991) demonstrated the antagonistic effect of *Trichoderma* on *R. solani* in coffee nurseries, and obtained a 55% reduction in the disease

incidence when inoculating the substrate with the antagonistic fungus. Betancourt (1997) performed a study using *Trichoderma* Th003 strain against the phytopathogen *F. oxysporum* in pre-emergence tomato seeds and observed 66.94% of protection vs. control.

**Table 2.** Percentage of passion fruit plants affected by *Fusarium* sp. four months after pathogen inoculation and application of the different treatments (isolates and moments of application).

Treatments	Application moments		
	Previous inoculation of <i>Trichoderma</i> spp. (% infection)	Previous inoculation of <i>Trichoderma</i> spp. and <i>Fusarium</i> spp at the same time (% infection)	Previous inoculation of <i>Fusarium</i> spp. (% infection)
Positive control	100.0 a*	100.0 b	100.0 b
<i>Trichoderma</i> sp. (preformulated)	100.0 a	100.0 b	100.0 b
<i>T. lignorum</i>	100.0 a	100.0 b	100.0 b
<i>T. harzianum</i>	75.0 ab	100.0 b	75.0 b
Tr001	75.0 ab	75.0 b	100.0 b
Tr003	50.0 ab	100.0 b	100.0 b
Tr002	25.0 ab	75.0 b	100.0 b
Blank control	0.0 b	0.0 a	0.0 a
Chemical control	0.0 b	75.0 b	50.0 ab

\* Values with same letters in column do not have statistical differences according to the Tukey's test ( $P < 0.05$ ).

**Table 3.** Percentages of *Fusarium* sp affected plants four months after the pathogen and the isolates of *Trichoderma* spp., *Azospirillum* spp. and *Azotobacter* were applied on pre-germinated seeds of passion fruit.

Treatments	Application moments	
	Seeds treated with antagonistic microorganisms 8 days before the <i>Fusarium</i> sp. inoculation (% infection)	Seeds treated with <i>Fusarium</i> sp. 48 hours before the application of antagonistic microorganismss (% infection)
Positive control	100.0 a*	100.0 a
<i>Azospirillum</i> 023	50.0 ab	87.5 ab
<i>Azospirillum</i> 028	50.0 ab	75.0 abc
<i>Azospirillum</i> 015	37.5 b	50.0 abc
Chemical control	25.0 b	50.0 abc
<i>Azospirillum</i> 002	12.5 b	62.5 abc
Blank	0.0 b	0.0 d
Tr003	0.0 b	12.5 cd
<i>Trichoderma lignorum</i>	0.0 b	25.0 bcd

\* Values with same letters in column do not have statistical differences according to the Tukey's test ( $P < 0.05$ ).

## Conclusions

- Interactions happening in the rhizosphere are very complex, it is required to deepen their knowledge to establish strategies allowing a more efficient management of agricultural systems.

- Results of this work indicate the importance of previous colonization of rhizosphere by beneficial organisms as a strategy for integral management of plant root diseases, when evidencing the effect of the different *Trichoderma* spp. strains in reducing the percentage of infection in plants between 75 and 50%.



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