In vitro interaction between *Trichoderma harzianum* and plant growth promoter rhizosphere bacteria

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In order to assess the interaction between a strain of *Trichoderma harzianum*, TH-382, with different strains belonging to Azospirillum and Sinorhizobium, an *in vitro* experiment was conducted. The growth and culture of the strains were conducted according to the pre-established methodology. A completely randomized design was applied with five treatments: as control, Trichoderma in solid pure medium; Trichoderma vs. A2, Trichoderma vs. NRG34, Trichoderma vs. N7 and Trichoderma vs. DS2 and ten repetitions. The variables colonies diameter and morphology were assessed. It was proved that Trichoderma may grow in a solid medium specific for rhizobia. Although there was no inhibitor halo, lower diameter of the Trichoderma colony was proved in two treatments cultured with the strains A2 and NRG34 (*Sinorhizobium meliloti*). This was evident at 72 h and 96 h of culturing. The strains N7 (*Azospirillum zeae*) and DS2 (*A. canadense*) were colonized by that of Trichoderma. It is concluded that there was negative interaction between Trichoderma and Azospirillum, predominating Trichoderma, and neutral interaction between Trichoderma and Sinorhizobium, where Sinorhizobium inhibited partially the fungi growth. The results of the interaction of Trichoderma with the strains A2 and NRG34 may be considered as promising for agricultural practice. These results are suggested to be considered for the combined inoculations of Trichoderma and rhizosphere bacteria in further experiments with useful crops for cattle feeding.

Key words: *Azospirillum, Sinorhizobium, fungi, compatibility, antagonism*.

The main objective of agriculture is the production of high-quality, safe and cheap feeds, to supply the constant growing world population. There is a growing interest on the use of benefic microorganisms to improve plant health and crops productivity, and, at the same time, guarantee the feeding safety of human beings and the environment protection (Avis *et al*. 2008). On this context, many soil microorganisms have demonstrated their positive effect and they integrate in a great variety of agricultural systems, as part of the management practices of productivity and integrated pest control. An example of this is the fungus Trichoderma and certain rhizosphere bacteria with plant growth promoter properties.

The Trichoderma strains are imperfect filamentous fungi, with teleomorphos belonging to the order of Hypocreales, division of the Ascomyceta (Kredics *et al*. 2003). The agricultural importance of this genus consists of the antagonist capacity of some strains in front of the pathogen fungi of the plants. This allows the development of bio-control strategies to restore the beneficial balance of the natural ecosystems (Kredics *et al*. 2004).

Among the bacteria with plant growth promoter effect, Azospirillum is one of the most promising ones, as it colonizes the rhizosphere of several crops in tropical and sub-tropical areas. Its mechanisms may have positive effect on plants: phytohormones production, nitrates reduction and dinitrogen fixation (Steenhoudt and Vanderleyden 2000). Other bacteria, such as rhizobia, colonize not only the legumes roots but also those of the plants of other families. Besides, they produce different metabolites that may act as plant growth promoters (Dakora 2003).

Bécquer *et al*. (2004) informed about the advantages of the co-inoculation of plants with rhizobia and Trichoderma in *Vigna radiata*, a legume of great importance for cattle feeding. There are reports on the advantages of co-inoculation of other rhizophere bacteria and Trichoderma on diverse crops (Saber *et al*. 2009 and Shaban and El-Bramawy 2011).

The objective of this experiment was to assess the growth and morphology of a strain of the fungus Trichoderma, cultured in a medium with strains belonging to the bacteria genera Azospirillum and Sinorhizobium, to determine the type of interaction between them. In other research stages, experiments with plants to apply these studies could be carried out to increase the productivity of crops for cattle feeding.

Materials and Methods

Procedence of the Azospirillum strains. The N7 strains, belonging to *Azospirillum zeae* and the strain DS2, to *A. canadense*, donated by Agriculture and Agri-Food Canada (London) were used. They were selected considering their potential of producing indoloacetic acid (IAA) when applying it in wheat (Mehnaz *et al*. 2010) for their possible use in further experiments with other cereals.

Procedence of the Sinorhizobium strains. The A2 and NRG34 strains, belonging to *Sinorhizobium meliloti* and donated by Agriculture and Agri-Food Canada (Québec) were used. They were recommended

by Danielle Prévost (personal communication) as highly dinitrogen fixer and for their properties as PGPR in different cultures.

Procedence of the Trichoderma strain. The TH-382 (*Trichoderma harzianum*) strain, donated by Agriculture and Agri-Food Canada-London was used. This Trichoderma species showed promising results in inoculation experiments when combined with *Bradyrhizobium sp*. in *Vigna radiata* (Bécquer *et al*. 2004).

Experimental procedure. The growth and application of the bacteria strains were conducted according to the methodologies described by Sabry *et al*. (1997) and Webster *et al*. (1997). They grew in solid medium yeast-manitol and were suspended in liquid medium yeast-manitol until reaching concentrations of viable cells of 10^7-10^8 UFC/mL. A sterile cotton hyssop was introduced in the inoculum and was cultured in Petri plates with the solid medium yeast-manitol with congo red (0.25 mg/mL). For that, the whole surface of the medium was covered and incubated at 29 ºC until reaching the uniform growth of bacteria.

For culturing a strain of Trichoderma in PDA medium (papa dextrose agar) (Difco), previously conducted with a conidial suspension (100.00 µL) of 10^6 -10^7 conidios/mL, discs of Ø 0.5 cm were cut with the help of a sterile stainless punch, previously flamed. These discs were inserted in holes of equal diameter, conducted in solid medium yeast-manitol. This was cultured one hour before with the different bacterial strains. It was incubated at 29 ºC in total darkness for its assessment at 24 h, 48 h, 72 h and 96 h (Steayert *et al*. 2009).

Experimental design and statistical analysis. A completely random design with five treatments was used: Trichoderma in solid medium with congo red, control; Trichoderma cultured on medium with A2, Trichoderma sembrado sobre medio con NRG34, Trichoderma cultured on medium with N7 and Trichoderma cultured on medium with DS2. Ten repetitions were established. Analysis of variance (ANOVA) was conducted. The differences between means were determined with the test of Duncan (1955). The data were processed with the statistical software SPSS/PC, version 15.0 for Windows. The diameter was measured (Ø cm) in the colonies of Trichoderma, using a ruler of microbiological use of 25 cm of longitude (Bayer). In each of the growth stages assessed, the morphology of the *in vitro* cultures was visually evaluated with a stereoscopy. No calculations in function of time were conducted as the growth curve of each microorganism was not determined. However, there are previous (Zafari *et al*. 2008) measurements conducted similar to those of this experiment.

Results and Discussion

Figure 1 presents the performance of Trichoderma growth in the different treatments used on a solid medium yeast-manitol. Although there are no antecedents about the growth of this fungus on a pure medium for rhizobia, Bécquer *et al*. (2001) informed growth *Trichoderma harzianum* on a pure culture medium, specific for dinitrofixer bacteria of free life.

The colonies diameter of this fungus, cultured together with the rhizobia strains A2 and NRG34, as well as those of Azospirillum DS2 and N7, was significantly inferior ($P < 0.001$) to that of the control treatment (6.73 cm) at 72 h. Nevertheless, these treatments shared common superscripts ($P < 0.05$) with the control at 24 h: Trichoderma vs. A2 (1.5 cm) and Trichoderma vs. DS2 (1.5 cm). They also shared them at 48 h: Trichoderma vs. DS2 (4.07 cm), and at 96 h: Trichoderma vs. N7 (8.20 cm) and Trichoderma vs. DS2 (8.37 cm). These data show the opposed effect of the fungi on some bacterial strains, mainly on those of the Azospirillum, as time increased.

In the treatments where Trichoderma interacted with the strains of Azospirillum at 48 h, in the plates cultured with N7 (*A. zeae*) the diameter of the fungus' colony (3.78 cm) was statistically similar ($P < 0.05$) to that fungi colonies that coexisted with the Sinorhizobium strains. However, from 72 h on, the colony diameter of Trichoderma was evidently higher compared with the rest of the treatments ($P < 0.001$), showing the marked antagonism of the fungus in respect to Azospirillum, probably due to the competence by nutrients and parasitism (Zafari *et al*. 2008). These conditions imply the presence of fungus micelles in the colonies of this bacterium. The treatments with these characteristics were: Trichoderma vs. DS2 (6.20 cm) and Trichoderma vs. N7 (6.00 cm), at 72 h; as well as at 96 h, Trichoderma vs. DS2 (8.37 cm) and Trichoderma vs. N7 (8.20 cm) (plate completely covered with the fungus' colony).

Shakeri and Foster (2007) and Reino *et al*. (2008) assured that Trichoderma can develop its biocontrolling action through mechanisms comprising the antibiosis, mico-parasitism and nutrients competition, modifying the environmental conditions when promoting plants growth. According to these authors, most of the Trichoderma strains produce volatile and non-volatile toxic metabolites that impede the colonization by other antagonist microorganisms. These metabolites were found by Saber *et al*. (2009) in *T. harzianum*, as they were emitted in the presence of *Botrytis fabae* pathogen fungus.

From 48 h on, in two of the treatments where Trichoderma grew together with the Sinorhizobium strains (figure 1), the fungus colonies had values statistically inferior ($P < 0.001$) in respect to

the control and the treatments cultured with the Azospirillum strains (except Trichoderma vs. N7 at 48 h), with which they shared common superscripts. These treatments were: Trichoderma vs. A2 and Trichoderma vs. NRG34 (48 h), as well as Trichoderma vs. A2 and Trichoderma vs. NRG34 (96 h). In the treatment with NRG34, from 24 to 96 h, the fungus colony was statistically inferior to the control treatment. The exponential curves presented in figure 1 correspond to the fungus growth in the control and to that of the treatment co-inoculated with the strain Sinorhizobium NRG34. In this last, the Trichoderma colony had lower diameter.

The highest evidence of the statistical difference ($P < 0.001$) in the growth of Trichoderma colony with the bacteria was proved at 72 h in the treatments cultured with the strain NRG34 and with DS2 (figure 1). These results indicate certain inhibition of the fungus growth, probably due to the presence of metabolites segregated by the bacteria that counteract the competitive action of Trichoderma. If considering the existence of a type of amenasalism where the fungitasis occur, process that inhibits but does not eliminate the fungus; although it does eliminate its conidias, hifas, esclerots and ascopores (Kragelund and Nybroe, 1996), the results could be due to this type of interaction among microorganisms.

Figures 2 and 3 show more clearly the morpho-cultural characteristics of the Trichoderma colonies as they grow in pure solid medium at 24 h (figure 2). Their micelles (whitish coloring) covered totally the plates' surface at 96 h (figure 3). There was absorption of congo red (not-showed color), that was evident from 24 h on.

Like Trichoderma, the strains of Azospirillum absorbed congo red (not-showed color in the figures), although this characteristic was observed from 48 h on, because at 24 h the colonies of this bacterium were white, dull and rounded (figure 4 and 5).

In the treatments with Trichoderma and the strains DS2 and N7 (not-showed figures), the fungus covered completely the area cultured with the bacteria at 96 h. However, Bécquer *et al*. (2001) observed that in plates with a solid medium free of nitrogen, cultured with *Azospirillum brasilense*, there was significant inhibition of the *T. harzianum* colony after three days of the culture. These different results could have their cause on the nutritional differences of the culture media used, as the medium used is rich in nitrogen. This condition can be the beginning of a competition for nutrients (Couteaudier 1992), predominating the defense mechanism of Trichoderma on the bacteria.

In the treatments with Trichoderma and NRG34, as well as with Trichoderma and A2 (*Sinorhizobium meliloti*) (figure 6 and figure 7), sticky and white bacterial colonies covering part of the plate with solid pure medium at 24 h and 96 h were observed. These colonies covered almost the all the fungus and plate

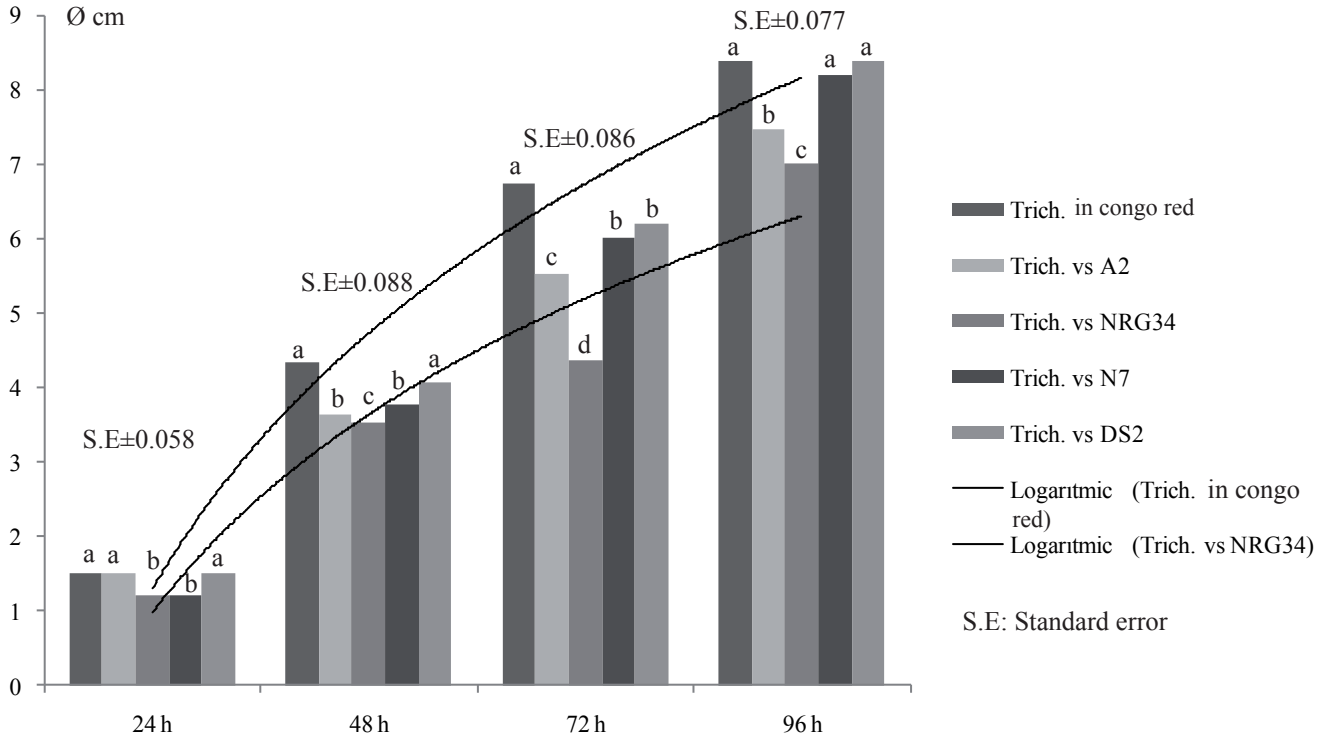

Figure 1. Growth of *Trichoderma harzianum*, strain TH-382, on solid medium + congo red, with cultures of *Sinorhizobium meliloti* (strains A2 and NRG39) or without them, *Azospirillum canadense* (strain DS2) and *A. zeae* (strain N7).

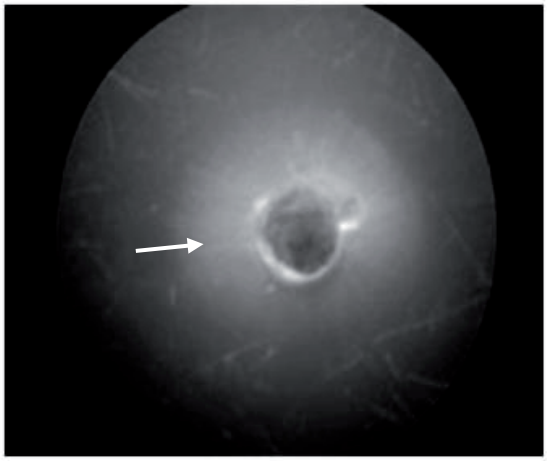

Figure 2. Colony of *Trichoderma harzianum* on a pure solid medium with congo red. Diameter at 24 h: 1.5 cm (white arrow). The white area corresponds to the fungus' micelles.

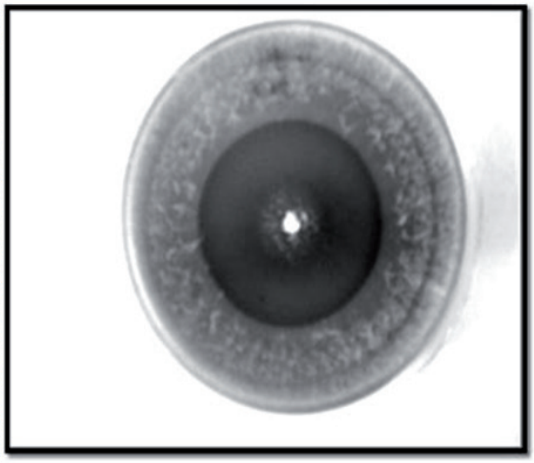

Figure 3. Colony of *Trichoderma harzianum* on a pure solid medium with congo red. Diameter at 96 h: 8.4 cm. The white area corresponds to the fungus' micelles. Notice the decadence area (black color)

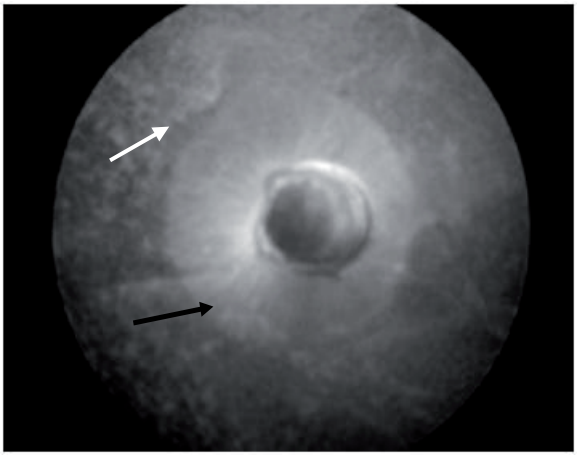

Figure 4. Colony of *Trichoderma harzianum* on a solid medium with congo red, cultured with a bacterial suspension of *Azospirillum canadense*, strain DS2. Diameter at 24 h: 1.5 cm. White arrow: bacterial colony. Black arrow: colony of Trichoderma.

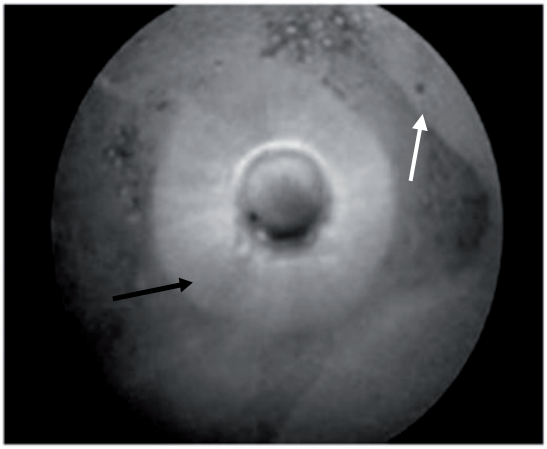

Figure 5. Colony of *Trichoderma harzianum* on a solid medium with congo red, cultured with a bacterial suspension of *Azospirillum zeae*, strain N7. Diameter at 24 h: 1.2 cm. White arrow: bacterial colony. Black arrow: colony of Trichoderma.

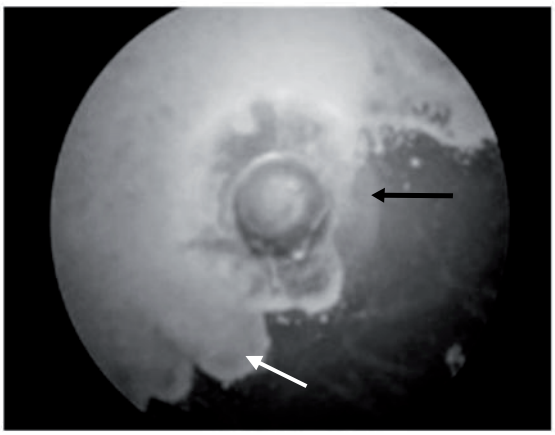

Figure 6. Colony of *Trichoderma harzianum* on a solid medium with congo red, cultured with a bacterial suspension of *Sinorhizobium meliloti* NRG34. Diameter at 24 h: 1.2 cm. White arrow: bacterial colony. Black arrow: colony of Trichoderma.

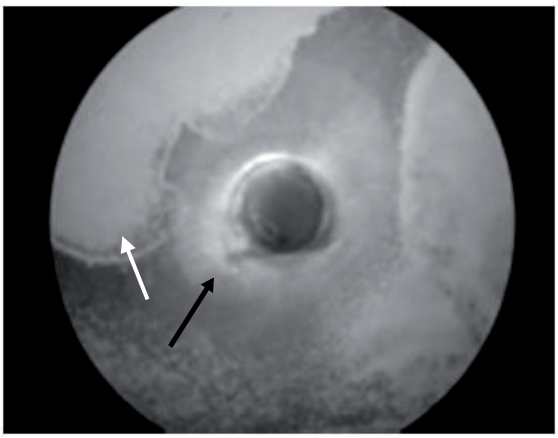

Figure 7. Colony of *Trichoderma harzianum* on a solid medium with congo red, cultured with a bacterial suspension of *Sinorhizobium meliloti* A2. Diameter at 24 h: 1.5 cm. White arrow: bacterial colony. Black arrow: colony of Trichoderma.

colony (nont-showed figures).

Breil *et al*. (1996) considered that the Rhizobium species have the potential needed to produce extracelular compounds (such as trifolitoxin) with direct antimicrobial activities. This indicates that antibiosis can be part of its efficacy like bio-control. According to Twelker *et al*. (1999), there are molecules of high molecular weight that segregate some rhizobia strains, called rhizobiocins (or middle bacteriocins). These strains, due to their biocide characteristics, play an important role in the competition at rhizosphere level. It should not be discarded that such substances were segregated by the studied rhizobia strains and inhibited the growth of Trichoderma, although it cannot as a marked antagonism, as there was no presence of inhibition halo. It should be more considered as a neutral interaction, where no microorganism was eliminated. Saber *et al*. (2009) observed in an *in vitro* experiment that there was no antagonism between *R. leguminosarum* and several strains of *T. viride* and *T. harzianum*.

It is concluded that there was negative interaction between the Trichoderma strain and those of Azospirillu, predominating Trichoderma. However, there was neutral interaction between the Trichoderma strain and those of Sinorhizobium where that of Sinorhizobium inhibited partially the fungus growth.

The data offered by this experiment cold have positive effect on further applications, combining these microorganisms in the plants as a synergy effect is expected that would favor the plant development and pathogens control in the cultures (Avis *et al*. 2008). Considering the present results for the combined inoculations of Trichoderma and rhizosphere bacteria is recommended in further experiments with plants, mainly in natural, forage and grain species, useful for cattle feeding.

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