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Abstract

The Alto Valle of Rio Negro is the main exporter and producer region of pear in Argentina, 'Bartlett' being the most important cultivar. Phytophthora cactorum and Phytophthora spp. cause significant economic losses in commercial pear production from tree death and weakening and fruit rot. The harmful effect of fungicides and market regulations have created the need to search for promising natural biocontrol agents in integrated crop management programmes. As regional isolates of Trichoderma spp. can be effective biological controllers, Trichoderma was selectively isolated from healthy trees next to trees with collar rot, using Rose Bengal selective medium. All Trichoderma isolates (n = 88) were evaluated against four Phytophthora spp., pathogens of pear by inhibition of mycelia growth (MG) and mycoparasitism. Eighteen isolates reduced the MG of at least two species of Phytophthora by more than 45% and showed mycoparasitism (2 to 4 scale degrees). These isolates were molecularly identified and evaluated in vitro (growth and metabolite production) and in vivo (growth promotion) against P. cactorum. From six isolates selected by PCA, three regional T. harzianum strains with the best antagonistic attributes and PHI K tolerant were evaluated against P. cactorum in a semi commercial bioassay in young pear trees. During the first year of our two-year study, all regional isolates of preventively evaluated Trichoderma spp. decreased the severity of collar rot on pear to a large extent, but without significant differences with the commercial T. atroviride strain and PHI K. Trichoderma harzianum 1330 and 1377 strains preventively reduced pear collar rot by 97% with respect to the diseased control. In the second year, the regional isolates again reached higher biocontrol percentages against P. cactorum. In the curative experiment, regional Trichoderma strains showed no significant differences from PHI K and the commercial isolate. Among all curative and preventive treatments, the regional T. harzianum 1367 strain controlled the rot area caused by P. cactorum by 97%, with the lowest average lesion area (0.11 cm2).

Keywords	Soil-borne pathogen; biocontrol; fungal antagonist; P. cactorum; Pyrus communis

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We submit the manuscript “Biological control of *Phytophthora* collar rot of pear using regional *Trichoderma* strains with multiple mechanisms”, with authors Aixa Daihana Sanchez, María Julia Ousset, María Cristina Sosa.

The manuscript has neither been published nor is it currently under consideration for publication either in whole in part, by any other journal.

We warrant that the manuscript is part of a study from which other manuscript may be generate and that all of the authors have contributed substantially to it and approved the final submission. We confirm compliance with the requirements given under Ethics and Conflict of Interest.

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Sincerely yours,
Dra. Maria Cristina Sosa

HIGHLIGHTS

- Regional *Trichoderma* strains potentially antagonist against *Phytophthora cactorum* were isolated from healthy trees and roots/soil in weakening pear orchards.
- Fifteen isolates (from 88) showed highest mycoparasitism and inhibition activity on mycelial growth of *P. cactorum* and roots growth promotion.
- Six regional isolates demonstrated in vitro compatibility with fungicide PHI K.
- Three *Trichoderma harzianum* strains showed highest antagonistic activity against *Phytophthora cactorum* in pear bioassays
- *Trichoderma harzianum* 1367 controlled *Phytophthora* rot in 97% in pear.

Biological control of *Phytophthora* collar rot of pear using regional *Trichoderma* strains with multiple mechanisms

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Abstract

The Alto Valle of Rio Negro is the main exporter and producer region of pear in Argentina, Bartlett being the most important cultivar. *Phytophthora cactorum* and *Phytophthora* spp. cause significant economic losses in commercial pear production from tree death and weakening and fruit rot. The harmful effect of fungicides and market regulations have created the need to search for promising natural biocontrol agents in integrated crop management programmes. As regional isolates of *Trichoderma* spp. can be effective biological controllers, *Trichoderma* was selectively isolated from healthy trees next to trees with collar rot, using Rose Bengal selective medium. All *Trichoderma* isolates (n = 88) were evaluated against four *Phytophthora* spp., pathogens of pear by inhibition of mycelia growth (MG) and mycoparasitism. Eighteen isolates reduced the MG of at least two species of *Phytophthora* by more than 45% and showed mycoparasitism (2 to 4 scale degrees). These isolates were molecularly identified and evaluated *in vitro* (growth and metabolite production) and *in vivo* (growth promotion) against *P. cactorum.* From six isolates selected by PCA, three regional *T. harzianum* strains with the best antagonistic attributes and PHI K tolerant were evaluated against *P. cactorum* in a semi commercial bioassay in young pear trees. During the first year of our two-year study, all regional isolates of preventively evaluated *Trichoderma* spp. decreased the severity of collar rot on pear to a large extent, but without significant differences with the commercial *T. atroviride* strain and PHI K. *Trichoderma harzianum* 1330 and 1377 strains preventively reduced pear collar rot by 97% with respect to the diseased control. In the second year, the regional isolates again reached higher biocontrol percentages against *P. cactorum.* In the curative experiment, regional *Trichoderma* strains showed no significant differences from PHI K and the commercial isolate. Among all curative and preventive treatments, the regional *T. harzianum* 1367 strain controlled the rot area caused by *P. cactorum* by 97%, with the lowest average lesion area (0.11 cm^2).

1. Introduction

Argentina is the largest pear producer and exporter country in the Southern Hemisphere. The main growing area of ‘Bartlett’ pear cultivar is situated in the irrigated valleys of Rio Negro, Argentina (Bruzone, 2010). The pathogens affecting the root system and collar of pome trees are widespread among countries producing fruit trees. The genus *Phytophthora* is considered one of the most destructive soil-borne pathogens, causing economic losses in fruit tree production (Erwin and Ribeiro, 1996).

Phytophthora root and crown rots are important and widely distributed diseases (Erwin and Ribeiro, 1996), which cause progressive weakening of the affected plant, reduction in its productivity and tree death. Collar rot on ‘Bartlett’ pear trees by *Phytophthora cactorum* is widely known in the irrigated valleys of Northern Patagonia (Rivero, 2010; Rossini, 2013). The infections occur mainly through the graft wounds between rootstock and scion (Rossini, 2013).

In recent research, we demonstrated that *P. cactorum* is not the only species weakening commercial pear orchards in our region, since *P. inundata, P. lacustris, P. rosacearum* and *P. termophila* have also caused root and fruit rot (Sanchez et al., 2019). Previous studies also showed that *Phytophthora lacustris* (ex. *Phytophthora* taxon *salixsoil*) caused pear fruit rot in orchard (Sosa et al., 2015) and during cold storage (Dobra et al., 2011). The effect of the *Phytophthora* species complex on fruit yield and quality have turned the disease into a limiting factor for sustainable production in our region.

Numerous studies have examined the use of fungicides to control *P. cactorum* in apples (Erwin and Ribeiro, 1996; Boughalleb et al., 2006; Rebollar-Alviter et al., 2007). Although fungicides are effective to some degree when applied as a preventative measure, they require repeated applications for controlling the disease. Utkhede (1984) reported that soil drenches with metalaxyl, followed by metalaxyl+mancozeb, prevented the growth of *P. cactorum* on the infected bark and infection in apple trees under orchard conditions. In pear and apple commercial orchards in our region, *Phytophthora* rot is controlled by soil drenching with metalaxyl or fosetyl-aluminium fungicides around naturally infected trees and foliar spraying with fosetyl-aluminium or potassium phosphite fungicides (Dobra et al., 2007). However, synthetic fungicides have a harmful effect on human and environmental health. Besides, repeated applications could lead to fungicide resistance by oomycete pathogens. Resistance to fungicides has been studied in field populations of *P. infestans* (Grünwald et al., 2006) and in strawberry populations of *P. cactorum* (Jeffers et al., 2004). These concerns have created the need to investigate other management options, including biological control. Moreover, the combined use of biocontrol agents and chemical fungicides has attracted much attention as it may result in more integrated and sustainable control of soil-borne diseases (Locke et al., 1985; Utkhede and Smith, 1993; Sharma et al., 2014).

Some biological control agents (BCA) have been registered and are available as commercial products (Whipps and Lumsden, 2001). Some of the most widely studied and promising fungi in biocontrol systems belong to the genus *Trichoderma* (Harman et al., 2004; Vinale et al., 2008;

Degenkolb et al., 2015). *Trichoderma* fungi are free-living microorganisms that are highly interactive in root, soil and foliar environments. The genus *Trichoderma* is one of the important groups, with its potential biological control ability and modes of action, including such mechanisms as competition with rhizosphere microorganisms for nutrients and/or space, antibiosis, mycoparasitism and induction of plant defenses (Harman et al., 2004; Shoresh et al., 2010; Druzhinina et al., 2011; Waghunde et al., 2016). The species can also impart some beneficial plant growth effects (Harman et al., 2004; Qi and Zhao, 2013; Waghunde et al., 2016).

The success of a biological control programme relies on the successful adaptation of a given BCA to the local environmental conditions in which it is supposed to work. One method to obtain effective BCA is to select the candidate *Trichoderma* strains from rhizosphere and soils where these agents are expected to control the disease. The best candidates for biocontrol will be those *Trichoderma* strains isolated and selected in the place where they grow under natural conditions of temperature, moisture, soil microbial composition and nutrient availability (Howell, 2003). Thus, BCA selection should consider the efficacy towards the target pathogen along with the conditions in which the BCA must develop (Brimner and Boland, 2003; Cordier and Alabouvette, 2009).

Populations of *Trichoderma* strains, which are often abundant in compost and compost-amended media, typically suppress *Pythium* and *Phytophthora* root rots within days after their formulation (De Ceuster and Hoitink, 1999). Species of *Trichoderma* were shown to suppress soil-borne diseases caused by *Phytophthora* spp. in containerised systems (Costa et al., 2000; Sharifi Tehrani and Nazari, 2004). *T. harzianum* isolated from the rhizosphere soil of rubber trees inhibited the mycelial growth of *P. palmivora*, the cause of leaf fall disease in rubber trees (Promwee et al., 2017). *T. koningii* and *T. harzianum* isolates, among other fungus genera, consistently reduced apple seedling mortality caused by *P. cactorum* in glasshouse trials (Alexander and Stewart, 2001).

Despite the extensive knowledge of biocontrol agents and although *Trichoderma* is the most widely registered genus of fungi for commercial use worldwide, no use of *Trichoderma* has, to date, been reported to biocontrol soil-borne pathogens in pear commercial orchards in our region. In addition, the current trend is to produce pear fruit in sustainable and environmentally friendly conditions, with reduced use of synthetic fungicides to control pathogens. This research was designed to (i) isolate and select regional *Trichoderma* spp. strains by their biocontrol ability against *Phytophthora cactorum,* (ii) characterise their attributes such as growth promotion and compatibility with fungicides, and (ii) compare their performance with commercial formulations in bioassays.

2. Materials and methods

2.1. Target pathogens

Isolates of *Phytophthora* spp. were obtained from declining □Bartlett□ pear orchards. For this research, *Phytophthora cactorum* 1378, *P. inundata* 1353, *P. rosacearum* 1315 and *P. lacustris* 1368 strains, which were isolated from soils and roots in a previous study, were selected due to their major aggressiveness in pear shoots, fruits and rootstock (Sanchez et al., 2019). All isolates were preserved in a sterile soil/water solution and stored at 15 °C in the culture collection of Laboratory of Phytopathology (Instituto de Biotecnologia Agropecuaria del Comahue, Facultad Ciencias Agrarias, CITAAC − UNCo). The isolates were grown and maintained on potato dextrose agar (PDA, Britania) to produce inoculum from the stored cultures.

2.2 Sample collection and *Trichoderma* isolation

To obtain highly effective antagonist fungus of the *Trichoderma* genera, a selective isolation method was employed. A directed sampling strategy was used in seven pear commercial orchards with root, crown and collar rot by *Phytophthora* in Alto Valle region, on trees with no *Phytophthora* symptoms that were near diseased trees. In the spring of 2015, trunk bark pieces (10 to 30) were collected, using a sterile scalpel, from trees in at least 15 rows and placed into polyethylene bags. Bark fragments were superficially disinfected by immersion in 70% ethylic alcohol (V V) for 30 s, in 5% sodium hypochlorite (V/V) for 1 min and then rinsed in sterile distilled water for 30 s (Ek-Ramos et al., 2013). The fragments were then transferred to plates with Rose Bengal Agar selective medium (RBA; Dhingra and Sinclair, 1985).

Besides, mixed soil samples from rhizosphere of pear tree were collected into polyethylene bags, transported to the laboratory and processed within 18-24 h. The samples were homogenised and spread on paper to remove the plant material, then air-dried, sifted with 2-mm mesh sieves, and stored at 4 °C in darkness until processing. *Trichoderma* strains were isolated using a serial dilution technique, and a 10^{-3} dilution of each sample was used. Aliquots of each soil suspension were transferred by duplicate to RBA plates and incubated at 25 ± 1 °C for 48 to 72 h. The culture plates were examined daily, and each colony was replicated on PDA.

2.3. Assays for selecting *Trichoderma* strains

In the first assays (inhibition of mycelial growth and mycoparasitism), the *in vitro* antagonistic activity of *Trichoderma* isolates was tested against four *Phytophthora* pathogenic isolates: *P. cactorum* 1378, *P. inundata* 1353, *P. rosacearum* 1315 and *P. lacustris* 1368. The subsequent assays (production and assessment of antimicrobial metabolites and semi-commercial biocontrol efficacy) were made against *P. cactorum* 1378.

2.3.1. Inhibition of mycelial growth

The inhibition of mycelial growth of the pathogen was evaluated for all *Trichoderma spp.* regional isolates using the modified dual culture method. Plates containing 15 mL of PDA were divided into four quadrants and in each of them 3 mm-diameter disks of mycelia of every *Phytophthora* isolate were seeded at 10-mm of plate edge. A 3 mm-diameter disk with the test antagonist was placed in the center of the plate. The assays were replicated three times. The plates were incubated in the dark at 20 ± 2 °C for 72 h. The pathogen alone on the plate was used as control. After incubation, the diameter of the pathogen colony was measured, and the percentage of mycelial growth inhibition (%MGI= larger diameter – smaller diameter/larger diameter * 100) was calculated (Guigón-López et al., 2010). Regional *Trichoderma* isolates exhibiting a reduction in MG higher than 45% against two *Phytophthora* species were selected as possible antagonists.

2.3.2. Mycoparasitism

To evaluate if *Trichoderma* spp. exert direct biocontrol by parasitising *Phytophthora* spp. pathogen, microscopic studies were performed.

Stereoscopic and optic microscopy. The antagonistic capacity by mycoparasitism on plate was evaluated using the method of Elías et al., (1983). The growth of the pathogen and *Trichoderma* isolates was observed in dual culture, and their antagonistic activities were classified into four classes based on an antagonism visual scale (0= no invasion of the pathogen colony, 1= invasion of ¼ of the pathogen colony, 2= invasion of ½ of the pathogen colony, 3= total invasion of the pathogen colony, 4= total invasion and sporulation).

With those *Trichoderma* isolates that presented a high degree of mycoparasitism, according to the scale above, preparations were made from the interaction zone between the antagonist fungus and the pathogen isolate. Observations were performed under an optic microscope (40 x objective, Leica)

Scanning electron microscopy. The antagonist-pathogen interaction zone was also characterised by scanning electron microscopy using the modified protocol of Kexiang et al. (2002). Mycelial samples from interaction regions were fixed in 2.5% glutaraldehyde in 0.067 M phosphate buffer (pH 7.2). After incubation, the samples were washed 3 times in 0.1 M phosphate buffer and dehydrated in a graded ethanol/acetone series. Dehydrated samples were critical-point dried using liquid carbon dioxide (E3000, Polaron), mounted on stubs and coated with gold in a sputter coater. Electron micrographs were taken in a scanning electron microscope (LEO EVO 40, Cambridge 2003) operating at 7.0 kV.

2.4. *Trichoderma* identification

Previously selected isolates of *Trichoderma* were molecularly identified by sequencing of the TEF 1 -α gene (translation elongation factor 1 alpha). DNA was extracted using the modified protocol of Liu et al. (2000). To obtain the DNA pellets, a portion of mycelium in active growth was taken and transferred to 1.5 mL Eppendorf tubes.

A portion of the TEF-1α gene encoding the elongation factor of the protein translation was amplified using the primers: EF728 (5'- CAT YGA GAA GTT CGA GAA GG) and EF2 (5'- GGA RGT ACC AGT SAT CAT GTT). The programme used was the one described by Barrera (2012). The PCR products of gen were purified using the AccuPrep PCR kit (BIONEER, Daejeon, Republic of Korea). For all the PCR protocols, a reaction mixture without DNA sample was used as a negative control. PCR products were sent to MACROGEN (Seoul, Republic of Korea) to be sequenced. Sequences were aligned using MEGA (Version 7.0) and compared by BLAST with library of NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). The sequences were deposited in GenBank. Sixteen molecularly identified *Trichoderma* isolates were used in the following experiments.

2.5. In vitro growth of *Trichoderma* strains

The fungal growth of each *Trichoderma* isolate was evaluated on Petri plate. A 3 mm-diameter mycelia disk was seeded in the center of a 90-mm Petri plate with 15 ml of PDA and incubated at 22 ± 2 °C in the dark. After 72 h, the colony diameter was measured. Three replicates were used for each isolate.

In addition, the time of appearance and conidia number were evaluated by counting the conidia using a Thoma camera (Samuels et al., 2006). A quantitative scale was created to determine the number of conidia (little= between 1 and 10^2 conidia.mL-1, abundant= 10^4 to 10^6 conidia.mL-1, very abundant= 10^7 or more conidia.mL-1).

2.6. Production and assessment of antimicrobial metabolites

The antagonist effect of antimicrobial metabolites produced in liquid medium by the *Trichoderma* isolates was evaluated on plate. *Liquid culture. Trichoderma* isolates were grown on PDA at 22 ± 2 °C for 5 days. A spore suspension of each isolate was obtained and an equal number of spores (10^6) was used to inoculate 50 ml flasks containing 20 ml of natural potato dextrose broth. Each strain was cultured at 25 °C and 220 rpm for 7 days (Vizcaíno et al., 2005).

Trichoderma-methanol extracts were prepared by mixing 2 ml of liquid culture with 2 ml of 100% methanol, shaking for 15 min and then centrifuged at 1500 g for 15 min. Two-ml aliquots of the methanol extracts were evaporated to half their volume in a nitrogen flow in order to increase the concentration of the metabolites and reduce any toxic effect due to the solvent. To test the efficacy of the extract, the modified dual culture technique was used (Vizcaíno et al., 2005). On Petri plates with 15 ml of PDA, 10 µl of the solution of each isolate was placed into well, and a mycelia disk of *P. cactorum* 1378 was placed at the opposite end of the plate. The control consisted in confronting the pathogen with 10 µl of sterile distilled water. Each treatment was carried out in triplicate. The percentage of mycelial inhibition was evaluated after incubation at 22 ± 2 °C for 72 h, as described in subsection 2.3.1.

2.7. In vitro growth promotion

For this assay, the antagonist isolates showing sporulation in 72 h or less (subsection 2.5) and testing positive in the mycoparasitism test (subsection 2.3.2.) were evaluated.

The growth improvement by *Trichoderma* strains was evaluated by colonisation of the roots of tomato seedlings in a modified Jensen semi-solid medium (Ca (NO3)2 1g; K2HPO4 0,2g; MgSO4 7H20 0,2g; ClNa 0,2g; FeCl3 0,1g; agar agar 8 g; distilled water 1000 ml), according to Azarmi et al. (2011). Sterile tubes (2 cm in diameter) with culture medium were inoculated with a suspension of conidia of each selected isolate, reaching a final concentration of 10^6 conidia. mL^{-1}. Once solidified, three superficially sterilised seeds of "Elpida" tomato were sown in each tube. The lower area of each tube was wrapped in aluminum foil, where to the roots grew in darkness.

The assay was carried out in a culture chamber with 60% relative humidity, photoperiod of light-dark 8-16 h at 22 ± 1 °C, for 21 days. At the end of the experiment, the root length, number of leaves and fresh weight of the plants were measured. Colonisation of roots was determined by re-isolation of *Trichoderma* strains. The roots were sterilised using 70% ethylic alcohol (V/V) for 30 s and cut into 1-cm-long pieces using a sterile blade. The pieces were rinsed in sterile distilled water and dried using sterile filter paper. The pieces were then plated on PDA for 7 days at room temperature (22-23 °C). The content of chlorophyll in the leaves of the seedlings was also analysed at the end of the assay. It was determined through extraction with dimethyl sulfoxide and quantification with spectrophotometry.

2.8. Statistical analysis

The variables evaluated in this assay were statistically analysed by Tukey mean difference analysis and compared to the control (seedling without inoculation). The statistical programme used was R commander, plugin NMBU (R Core Team, 2013). A principal component analysis (PCA) was performed after the last assays to select *Trichoderma* strains with the best attributes.

2.9. Fungicide tolerance

Six antagonist isolates selected by PCA were evaluated for their tolerance to the commercial systemic fungicide frequently applied in pear orchards. This ability represents an advantage for a system of sustainable production with reduced use of chemical fungicides.

In this assay, antagonist 3 mm-diameter disks were taken from the edge of each colony with active growth and placed on a Petri plate with 15 ml of PDA amended with potassium phosphite (PHI K; 12.9% Phosphorus, 16.3% Potassium; ANDO & CO) (Holmes and Eckert, 1999). The doses evaluated were 0; 0.1; 1.0; 10.0; 50.0 and 100.0 ppm. Three plates were used per isolate, dose and treatment. The plates were incubated for 72 h and the growth diameter was measured. Tolerance percentage was calculated based on the mycelial diameter of the control (PDA without fungicide addition, 0 ppm).

2.10. Semi-commercial biocontrol efficacy

The biocontrol effect of regional *Trichoderma* strains against *P. cactorum* was evaluated on pear trees in the orchard experiments.

2.10.1. Orchard Treatments

Orchard experiments were conducted for two years of crop growth and from early spring to mid-fall (leaf fall) of the following year. A preventive efficacy experiment was performed during the first year and repeated in the second, while a curative efficacy experiment was done in the second year. We used three-year-old ‘Bartlett’ pear trees grafted on pear rootstocks planted in a 0.5 ha block in Cinco Saltos, Rio Negro. Irrigation and other cultural practices were conducted in the orchard.

Three regional *Trichoderma* strains, *Trichoderma harzianum* 1330, 1367 and 1371, previously selected for their biocontrol attributes, were evaluated for orchard biocontrol tests against *P. cactorum* 1378. The control treatments were (i) a chemical treatment, i.e., plants inoculated with the pathogen and treated with a commercial dose of PHI K as positive control; (ii) diseased control, i.e., plants inoculated with the pathogen alone; (iii) *Trichoderma* control, i.e., plants inoculated with each regional antagonist isolate and commercial *T. atroviride* isolate.

Each antagonist fungus was prepared as a conidial aqueous suspension adjusted at 10^6 conidia/mL. Mycelia disks from the pathogen colonies with active growth on CMA were used as inoculum.

2.10.1. Preventive effect

The antagonist suspension (1 mL, 10^6 conidia/mL) was applied on the graft wound. After 48 h, the same antagonist volume and concentration was applied, and 24 h after the second application, a pathogen disk (8 mm in diameter) was inoculated into the wound. After each step, the wound was covered with sterile cotton soaked in sterile distilled water and then covered with adhesive tape.

2.10.2. Curative effect

In this experiment, the pathogen was first inoculated into the graft wound. The antagonist (1mL, 10^6 conidia/mL) was applied on the wound 24 and 48 h after inoculation. After each step, the wound was covered with sterile cotton soaked in sterile distilled water and then covered with adhesive tape.

2.10.3. Evaluation of experiments

In both years, the experiments were incubated during a full growing season, from early spring to leaf fall. In each experiment, there were three repetitions per treatment in a completely randomised design. The biocontrol effect for each treatment was evaluated by measuring (cm^2) the necrotic lesion area on the wound treated and analysed through the Image J 1x programme (Schneider et al., 2012). Besides, the development of typical disease symptoms was observed and compared with the diseased control. To evaluate the recovery of pathogen and antagonist in each treatment, bark samples were taken from the graft area and isolates were made in selective medium CMA-PARP (for *P. cactorum*) and PDA (for *Trichoderma* sp.).

2.10.4. Statistical analysis

Given the quantitative nature of the response variable in both experiments, a Tukey mean difference analysis was performed with R commander statistical programme, NMBU plugin (R Core Team, 2013). A statistical analysis of means difference was made by separately comparing the preventive and curative treatments in each experiment. Besides, a comparative analysis of all treatments was performed for the second year of both experiments.

3. RESULTS

3.1. Antagonist isolation

By means of the isolation strategy, a total of 88 isolates of *Trichoderma* spp. were obtained from the pear orchards. The number of *Trichoderma* isolates varied according to their origin. The greatest number of fungal isolates was obtained from soil/rhizosphere (n= 75, 84%). The remaining isolates derived from tree wood (n=14, 16%).

3.2. Antimicrobial assays of Trichoderma strains

3.2.1. Inhibition of mycelial growth

The 88 isolates of *Trichoderma* sp. were evaluated against *Phytophthora cactorum* 1378, *P. inundata* 1353, *P. rosacearum* 1315 and *P. lacustris* 1368, using the modified dual culture technique. The total number of isolates showed some degree of antagonist activity (at least 18.4%

reduction in MG compared to the control). However, 25 *Trichoderma* sp. isolates inhibited the MG of only one *Phytophthora* species by 45% or more (data not shown). By contrast, only 18 isolates of *Trichoderma* spp. reached the criteria arbitrarily established for the selection as a potential antagonist, i.e., isolate able to reduce the MG by more than 45% against two species of *Phytophthora* (Table 1). Among these, only one isolate of *Trichoderma* inhibited the MG of all *Phytophthora* species evaluated. *Trichoderma* 1384 isolate controlled the MG on plate (the highest control percentage being 62.2%) in more than 50% of *Phytophthora cactorum* 1378, *P. inundata* 1353, *P. rosacearum* 1315 and *P. lacustris* 1368 strains. Among the species of *Phytophthora, P. inundata* reached the highest MGI values (more than 65%) while *P. cactorum* 1378 showed the lowest MGI values (Table 1).

3.2.2. Mycoparasitism

Stereoscopic and optic microscopy. Mycoparasitism by *Trichoderma* sp. on *Phytophthora* spp. was demonstrated through dual culture. Seventy *Trichoderma* isolates (81%) presented the highest grade (4) of the mycoparasitism scale on all four *Phytophthora* species isolates. This implies that *Trichoderma* fungus totally colonised the pathogen colony and sporulated on it. Thirteen isolates (13.3%) corresponded to 1-3 grades of the mycoparasitism scale, and five isolates (5.7%) showed no mycoparasitism on any of the four *Phytophthora* species evaluated.

Representative *Trichoderma* isolates belonging to degree 4 of the mycoparasitism scale were selected to study the interaction zone under the microscope. In all cases, "coiling" and adherent growth by *Trichoderma* sp. was observed on the hyphae of *Phytophthora*. Moreover, *P. cactorum* 1378, *P. inundata* 1353, *P. rosacearum* 1315 and *P. lacustris* 1368 strains showed the vacuolated hyphae (Figure 1).

Scanning electron microscopy. The interaction zone in dual culture of one *Trichoderma* strain by degree of mycoparasitism was evaluated by scanning electron microscopy. Electron micrographs showed that the hyphae of antagonist isolates were tightly appressed to the *P. cactorum* hyphae. The hyphae of *T. brevicompactum* 1377 strain grew forming parallel cords and grouped around the pathogen hyphae. In addition, the hyphae growth of *T. harzianum* 1322 and *T. atroviride* 1310 strains was observed around the pathogen hyphae and penetrating it (Figure 1).

At the end of the first assays, 18 isolates of *Trichoderma* sp. showed, on average, more than 45% MGI against at least two pathogen species (*P. cactorum, P. rosacearum, P. inundata* and *P. lacustris*) and some level of mycoparasitism (Table 1).

3.3. *Trichoderma* identification

Molecular identification of fifteen previously selected isolates of *Trichoderma* was performed by sequencing the Tef 1 -α gene. Six species were identified to *Trichoderma harzianum* (7*), T. guizhouense* (3), *T. deliquescens* (1), *T. longibrachiatum* (2), *T. brevicompactum* (1) and *T. atroviride* (1) (Table 2).

Trichoderma deliquescens and *T. guizhouense* are recently named species, derived from the *T. harzianum* species and now renamed as "harzianum" clade, while the *T. brevicompactum* species belongs to the "longibrachiatum" clade.

3.4. In vitro growth of *Trichoderma*

Sixteen *Trichoderma* isolates grew on PDA at 22 ± 2 °C for 72 h. The mycelial growth of eleven isolates stopped on the edge of the Petri plate (90 mm) at 72 h, while the colony diameter of five isolates reached more than 70 mm. Among these groups, *T. harzianum* 1351 and 1368 strains did not sporulate after 96 h of colony growth. On the other hand, *T. atroviride* 1310 strain showed the lowest colony growth (50 mm). The isolates with non-sporulating colonies or slow growth were discarded for the next selection stage (Table 3).

3.5. Production of antimicrobial metabolites and assessment of antagonism

The antifungal activity on plate of secondary metabolites produced in liquid medium by each of the sixteen *Trichoderma* spp. isolates was variable. The percentage of mycelial growth inhibition of *P. cactorum* 1378 ranged from 2.2 to 24.2%, depending on the *Trichoderma* isolate. *Trichoderma* sp. isolate 1367 reached the highest percentage of control (Table 3).

3.6. In vitro growth promotion

The effect of 16 *Trichoderma* spp. isolates on the seedling growth promotion and on root colonisation was studied in semi-solid medium. Eleven *Trichoderma* isolates were able to promote the growth of seedling roots with significant differences in root length in relation to the control. *Trichoderma harzianum* 1367, *T. harzianum* 1322 and *T. deliquescens* 1343 promoted plant growth and were able to colonise the roots of tomato seedlings. Particularly, *Trichoderma* 1367 strain promoted root length, which were 2 cm longer than roots of the control treatment. Colonies of *Trichoderma* were re-isolated from the tomato roots. In contrast, no colonies were re-isolated from the roots in the control treatment. Although the *Trichoderma* 1371 strain caused the highest increase in fresh weight, no statistical differences from the control treatment were found.

The total chlorophyll content in all treatments had statistical differences from the control. *Trichoderma harzianum* 1336 strain produced almost four times more chlorophyll in leaves than the other isolates evaluated.

3.7. Statistical analyses

At the end of all previous assays, six antagonist isolates were pre-selected using a principal component analysis with the best conditions evaluated up to this point (Figure 1). The highest percentage of mycelial inhibition was found in *Trichoderma* sp. 1349 against *P. cactorum, T. guizhouense* 1384 against *P. rosacearum* and *T. harzianum* 1330 against *P. lacustris. Trichoderma harzianum* 1336 strain generated the highest values in total content of chlorophyll in tomato, while *T. harzianum* 1371 strain generated the highest value in weight of the root system and *T. harzianum* 1367 strain in root length.

3.8. Fungicide tolerance

Tolerance to the commercial formulation of potassium phosphite was demonstrated for all six *Trichoderma* isolates selected in the preceding stage. Although all regional isolates had the capacity to grow on PDA amended with PHI K, only three regional strains, *T. harzianum* 1330, 1367 and 1371, grew at all concentrations of the PHI K evaluated.

3.9. Semi-commercial biocontrol efficacy

Three regional strains, *T. harzianum* 1330, 1367 and 1371, with multiple control mechanisms and tolerance to PHI K were evaluated against *P. cactorum* 1378 in orchard biocontrol assays.

During the first year, all regional isolates of *Trichoderma* sp. preventively evaluated decreased, to a large extent, the severity of collar rot on pear. Besides, the tested antagonists had no significant differences from *T. atroviride* commercial isolate and the chemical control (Table 5).

The application of the antagonist fungus 48 h before inoculation with the pathogen decreased the rotted area in relation to the diseased control without treatment. More than 96% biocontrol was obtained with the regional *Trichoderma harzianum* 1330 and 1371 strains. The necrotic lesion area was reduced to insignificant values in comparison with the diseased control area (Table 5).

In the second year, through replication of the preventive bioassay, the biocontrol capacity of the regional *Trichoderma* strains was again demonstrated. The lesion area with rot was significantly reduced (84-96.8%) when wounds were treated with the three regional *Trichoderma harzianum* isolates. *Trichoderma harzianum* 1367 strain stood out, controlling the rot by *P. cactorum* and reducing the necrotic area to only 0.15 cm^2. This treatment was better than the chemical control (0.33 cm^2) (Table 5).

In the curative experiment, regional *Trichoderma* strains applied 24 h after pathogen inoculation showed no significant differences from the chemical control and the *T. atroviride* commercial isolate. Moreover, these regional isolates reached higher biocontrol percentages against *P. cactorum* (Table 5).

When the biocontrol effect of all the treatments (curative and preventive) was statistically analysed, the regional *T. harzianum* 1367 strain stood out for controlling 97% of the rot area caused by *P. cactorum* and presenting the lowest average lesion area (0.11 cm^2) (Table 5). In all experiments, the treatments with *Trichoderma* were significantly different from the diseased control.

With PDA, *Trichoderma* sp. was re-isolated in all cases. With CMA-PARP, the medium specific for *Phytophthora, P. cactorum* was only isolated from the diseased control, while *Trichoderma* was isolated from the treated trees.

4. DISCUSSION

Evidence supporting the potential antagonism of *Trichoderma* regional strains against *Phytophthora* sp. causing pear tree rots was reported here. Eighty-eight isolates of the genus *Trichoderma,* potential antagonists against soil pathogens, were obtained by directed sampling in asymptomatic plants close to diseased plants in pear commercial orchards in Alto Valle of Rio Negro. The results obtained with the isolation strategy proposed agree with Baker and Cook's (1974) hypothesis that the best strategy to isolate potential antagonists against a specific pathogen is to search for healthy plants in sites favorable to the development of the pathogen. More recently, Promwee et al. (2017) demonstrated that indigenous strains of *T. harzianum* isolated from rhizosphere soil of rubber trees controlled *P. palmivora* better than the commercial *Trichoderma* strain, which could indicate environmental adaptation of the indigenous strains.

Trichoderma species are one of the potential fungal biocontrol agents against soil-borne pathogens. In this research, we evaluated the attributes of regional isolates of *Trichoderma* sp. against four pathogenic species of *Phytophthora*. All isolates in *in vitro* assays showed mycelial growth inhibition of at least one *Phytophthora* species. The antagonism of the genus *Trichoderma* as biocontrol microorganism on soil-borne pathogens, mainly *Phytophthora* sp., has been widely discussed in different plant species. In apple, *Trichoderma* sp. and *Gliocladium* sp. controlled *P. cactorum* causing root and crown rot in soil (Smith et al., 1990) and in seedlings (Roiger and Jeffers, 1991). Apple fruit rot caused by *Phytophthora* was also controlled in *in vitro* tests by *T. harzianum, T. virens, T. viride* and *T. hamatum* (Bhaik, 2017). In cacao, *T. martiale* was used against *P. palmivora*, a cause of black pod disease (Hanada et al., 2009). Clay granules impregnated with *T. harzianum* were used to control *P. cinnamomi,* a cause of damping-off in pine seedlings (Kelley, 1976).

Only sixteen regional isolates satisfied the initial selection attributes, i.e. inhibited the mycelial growth, overgrew and sporulated on *Phytophthora* pathogen, and were molecularly identified as six species of the genus *Trichoderma*. These were *Trichoderma harzianum, T. longibrachiatum, T. atroviride, T. deliquescens, T. guizhouense* (ex: *T. harzianum*) and *T. brevicompactum*. Several species identified here corresponded to biocontrol microorganisms reported long ago. *Trichoderma harzianum* was the species most abundantly found in this study, including *T. guizhouense*, recently renamed as such. This demonstrates the cosmopolitan character of the species (Chaverri and Samuels, 2003; Chaverri et al., 2015). The species *T. deliquescens* was not reported in Argentina, possibly due to its recent name change (ex: *Gliocladium deliquescens*), which currently belongs to the clade *Deliquescens,* formed by this and two other species (Bissett et al., 2015; Jaklitsch and Voglmayr, 2015). *T. brevicompactum* belongs to the clade denominated with the same name (Jaklitsch and Voglmayr, 2015). This clade is not closely related to the species that have biological application but is cited for its ability to produce toxins such as "trichodermin", an antibiotic used at low concentrations that limits the growth of several fungal species (Degenkolb et al., 2008). *T. atroviride* (Clade: Viride) (Jaklitsch and Voglmayr, 2015) is one of the best-known species for its ability to generate glucanase enzymes for mycoparasitising hyphae of other fungal species (Benítez et al., 2004). It is also one of the few species recognised as endophytes, which agrees with the fact that it was isolated from pear bark (Ming et al., 2013). Finally, *T. longibrachiatum* (clade: *Longibrachiatum*) (Jaklitsch and Voglmayr, 2015) is one of the species most intensively studied by industrial producers of cellulase or as facultative opportunistic human pathogens (Druzhinina et al., 2012).

Mycoparasitism is one of the attributes most widely investigated for its direct benefits in the application of a biocontrol agent against the pathogen. This study demonstrated the ability of regional *Trichoderma* strains to mycoparasite *Phytophthora* species. *Trichoderma* regional isolates affected mycelial growth and sporulated over all pathogen colonies, *P. cactorum, P. rosacearum* and *P. lacustris.* Curls and vacuolisation of *Phytophthora* hyphae was also observed. By means of scanning electron microscopy, we demonstrated that *T. brevicompactum* 1377, *T.*

harzianum 1322 and *T. atroviride* 1310 strains parasitise the mycelia of *P. cactorum,* through colonisation of *Phytophthora* hypha, envelope and penetration of *Phytophthora* hypha and conidia reproduction on mycelia. Mycoparasitism was described for various species of *Trichoderma* with antagonism on the vegetative phase of *Phytophthora* (Harman, 2000; Promwee et al., 2017). It was also demonstrated that *T. harzianum* grows rapidly at the outset and then invades the colony of *P. capsici* by a marked process of hyperparasitism (Ezziyyani et al., 2007).

The production of antimicrobial metabolites is another biocontrol mechanism that can be associated with mycoparasitism. In this study, the *Trichoderma* isolates produced metabolites in liquid medium. This extract inhibited pathogen growth on plate in low percentages. The highest percentage of inhibition (24%) was observed with *Trichoderma guizhouense* 1367 (*T. harzianum* complex). Vizcaíno et al. (2005) obtained better results from isolates of the *Pachybasium* clade also in solid medium, with values of antifungal activity of 33%. The same species may not always produce the same compounds or respond in the same way, as this will depend on the microorganism, the environment (pH and temperature) and the substrate. Any species of *Trichoderma* can produce several antibiotic compounds and, similarly, different species of *Trichoderma* can produce the same antibiotic (Sivasithamparam and Ghisalberti, 1998). BCA are living organisms whose activities depend mainly on the different physicochemical environmental conditions to which they are subjected. Understanding the biocontrol mechanisms of *Trichoderma* strains will lead to improved application of the different strains as BCA. The biocontrol mechanisms are complex, and their synergic action will result in the disease biocontrol (Benítez et al., 2004).

The capacity of isolates of *Trichoderma* sp. to promote growth in plants has been well documented (Harman, 2006; Harman et al., 2004; Hermosa et al., 2013; Vinale et al., 2008). In this work, significant differences were observed from the control without antagonist in the chlorophyll content, the biggest difference being from *T. harzianum* 1336 strain. The increase in chlorophyll content in tomato leaves inoculated with *T. harzianum* was reported by Azarmi et al. (2011). In addition, in our study, other two isolates, *T. atroviridae* 1310 and *T. harzianum* 1322 stood out. This result would indicate that the effects of *Trichoderma* on the growth and vigor of the seedlings depend on the *Trichoderma* isolate used and not on the species.

The potassium phosphite fungicide applied on roots was reported as fungicide against *Phytophthora* sp. in various crops (Smillie et al., 1989). The tolerance of *Trichoderma* isolates to potassium phosphite is considered a positive attribute and, for this reason, it was evaluated by the amended plate technique. Obtaining regional isolates of *T. harzianum* tolerant to this chemical fungicide represents a great advantage of BCA, since they could be applied together into integrated management programmes. By contrast, Paredes Angulo (2016) evaluated the combination (*Trichoderma*-PHI K) and concluded that potassium phosphite decreased the conidial germination and growth rate of the antagonist. In our region as well as in other countries, PHI K is currently used in a wide variety of crops to control diseases caused by *Phytophthora* sp. both directly (fungicide) and indirectly (defense inducer) (Jackson et al., 2000; Hardy et al., 2001; Machinandiarena et al., 2012). In this work, the possibility of combined use with *Trichoderma* sp. and PHI K in pear was established.

In the semi-commercial bioassays of preventive or curative effects, regional *Trichoderma* strains significantly decreased collar rot by *P. cactorum* in pear trees. Disease biocontrol values greater than 90% were obtained. The high effectiveness of regional *Trichoderma* strains could promote the use of biological control agents and diminish the use of chemical fungicides, thus significantly changing the system of pear production in our exporter region. Selected regional *T. harzianum* strains could be preventively used by immersion of pear rootstocks including the graft wound at implantation and on active disease lesions on tree collar of □Bartlett□ cv. The results obtained in this study are coincident with numerous works that demonstrate the preventive effectiveness of regional isolates of *Trichoderma* sp. against different species of *Phytophthor*a sp., although in other species of woody plants. Alexander & Stewart (2001), Roiger & Jeffers (1991) and Smith et al. (1990) reached similar results in the biocontrol of root rot in apple seedlings. McLeod et al. (1995) reduced the incidence of root rot in avocado plants, caused by *P. cinnamomi*. Mpika et al. (2009) evaluated regional isolates of *Trichoderma* sp. against *P. palmivora*, with a sharp decrease in "black pod" disease in cocoa. To our knowledge, this is the first study on selection of effective regional *Trichoderma* strains with multiple antagonist mechanisms against *P. cactorum* in pear. The promising BCA will be incorporated in future studies in commercial orchard assays, due to their high efficiency in the biocontrol of the disease demonstrated in this research.

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Disclosure of potential conflicts of interest

Ethical approval: This article does not contain any studies with human participants or animals.

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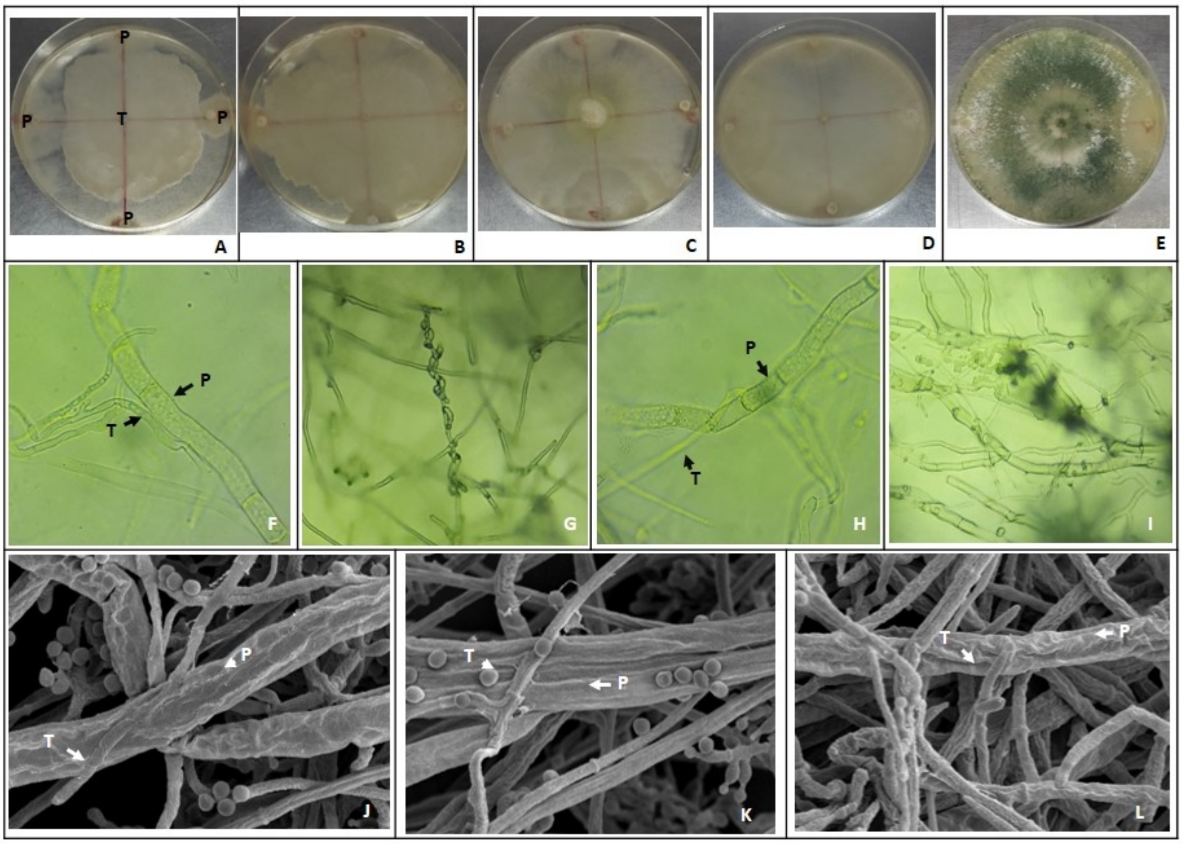

Figure 1. Mycoparasitism of *Trichoderma* strains to *P. cactorum* in dual cultures. (A-E) Scale for mycopasitism according to Elías et al., (1983) (A = no invasion on the pathogen colony, B = invasion on ¼ of the pathogen colony, C = invasion on ½ of the pathogen colony, D= total invasion on the pathogen colony, E= total invasion and sporulation on the pathogen colony; (F-I) optical microscope (40 X objective) of the interaction zone, F= Adhered hyphae growth. G-H= Hyphae coiling. I= Vacuolated hyphae; (J-L) Scanning electron microscope of interaction zone, J= Isolate of *T. atroviride*, K= *T. brevicompactum* and L= *T. harzianum* growing closely on hyphae of the *Phytophthora* pathogen.

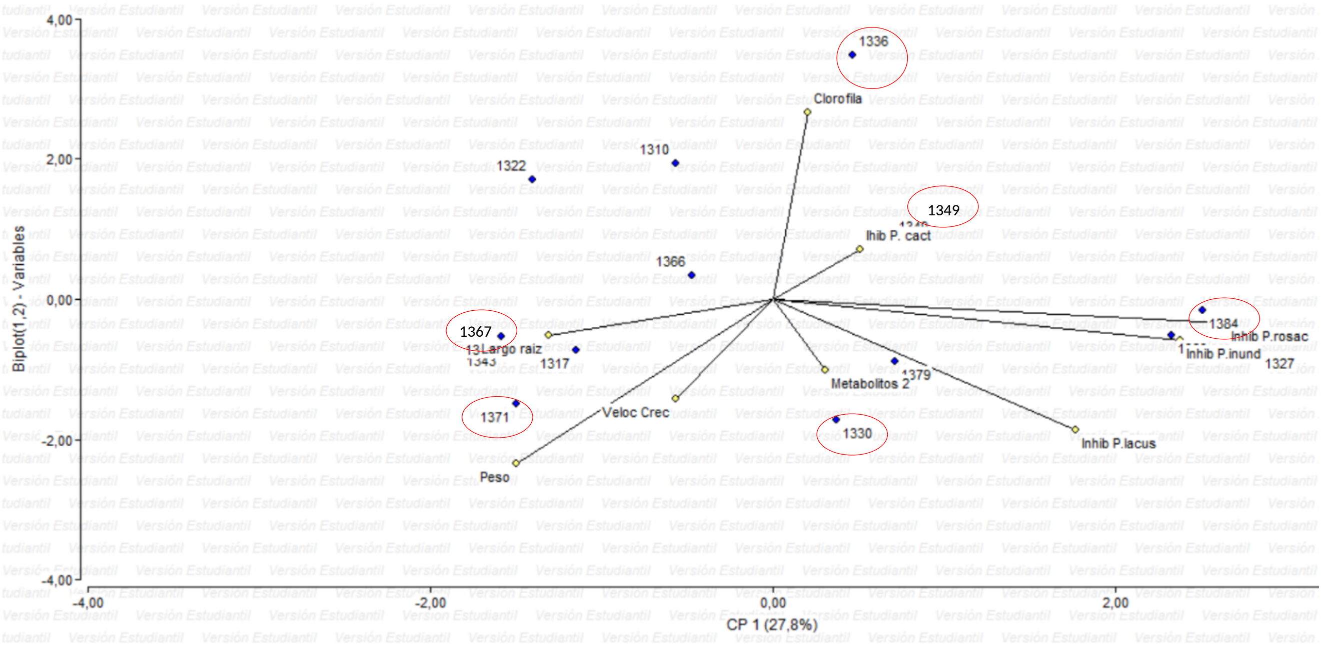

Figure 2. Analysis of the main components of 16 *Trichoderma* sp. strains selected. In circles, the isolates selected for fungicide tolerance assay are shown. The principal component 1 (CP1) are formed by the variables mycelial growth inhibition of *P. rosacearum* and *P. inundata* positively. And by the other hand, the CP2 is formed negatively by the variable roots weight of growth promotion assays.

Table 1. Percentage of inhibition and mycoparasitism scale of selected *Trichoderma* strains against *Phytophthora* spp.

Isolate	Isolation Strategy	Site	Percentages of mycelial inhibition[1]				Mycoparasitism
			P. rosacearum (1315)	*P. inundata* (1353)	*P. lacustris* (1368)	*P. cactorum* (1378)	Scale [2]
1310	Wood	General Roca	45,45	48,78	38,23	42,1	4
1317	Soil	Cipolletti	47,27	53,65	41,17	42,1	4
1322	Soil	Cipolletti	49,09	51,21	26,47	44,73	1
1327	Soil	Cipolletti	61,81	65,85	50	36,84	4
1330	Soil	Cipolletti	52,72	52,43	55,88	44,73	4
1336	Soil	Cinco Saltos	50	49,39	35,29	44,73	4
1343	Soil	Cinco Saltos	46,36	47,56	38,23	42,1	4
1349	Soil	Cmte. Cordero	49,09	54,87	41,17	47,36	4
1351	Soil	Cmte. Cordero	47,27	52,43	35,29	42,1	4
1366	Soil	Allen	49,09	47,56	38,23	44,73	0
1367	Soil	Allen	45,45	52,43	32,35	42,1	4
1368	Soil	Allen	45,45	51,21	20,58	31,57	4
1371	Soil	Allen	44,54	46,95	45,11	42,1	4
1375	Wood	Cipolletti	50,9	51,21	47,05	34,21	4
1377	Soil	Cinco Saltos	45,45	50	41,17	42,1	2
1379	Soil	Cinco Saltos	52,72	50	50	43,42	4
1383	Soil	Cinco Saltos	54,54	64,63	45,58	42,1	4
1384	Soil	Cinco Saltos	58,18	62,19	50	52,63	4

[1] % of mycelial inhibition calculated as larger diameter – smaller diameter/ larger diameter * 100. Values are the average of 3 replicates.

[2] Colonisation scale of the antagonist on the pathogen colony (Elías & Arcos, 1984).

Table 2. Molecular identification of pre-selected regional isolates of *Trichoderma* associated with pear orchard.

Isolate number	GENBank TEF 1-α [1]				Species
	Homology (%)	Coverage (%)	Reference strain (NCBI)	Accession [2]	
1310	99	100	AF456886.1	MK577774	*Trichoderma atroviride*
1317	93	87	KJ871186.1	-	*Trichoderma harzianum*
1322	100	100	KJ871174.1	MK577764	*Trichoderma harzianum*
1327	99	100	AY865640.1	MK577765	*Trichoderma longibrachiatum*
1330	99	99	AY605764.1	MK577775	*Trichoderma harzianum*
1336	98	99	AY605764.1	MK577773	*Trichoderma harzianum*
1343	93	100	AB856691.1	MK577766	*Trichoderma deliquescens*
1351	99	100	AY605764.1	MK577767	*Trichoderma guizhouense*
1367	99	100	AY605764.1	MK577776	*Trichoderma harzianum*
1368	99	100	KX463434.1	MK577768	*Trichoderma harzianum*
1371	98	99	AY605764.1	MK577777	*Trichoderma harzianum*
1375	99	100	KP008877.1	MK577769	*Trichoderma longibrachiatum*
1377	97	100	AY857297.1	MK577770	*Trichoderma brevicompactum*
1379	99	100	KJ665506.1	MK577771	*Trichoderma guizhouense*
1384	99	100	KJ665506.1	MK577772	*Trichoderma guizhouense*

[1] Isolates identified by TEF 1-α (translation elongation factor 1 alpha)

[2] Accession number of amplified sequences of TEF 1- α deposited in GenBank

Table 3. *In vitro* characterisation of regional, potentially antagonist, *Trichoderma* sp. strains

***Trichoderma* strain**	**Mycelial Growth[1]**	**Conidia**		**Inhibition Percentage by Secondary Metabolites[4]**
		Quantity[2]	**Time[3]**	
T. atroviride 1310	50	Very abundant	48	9,66
T. harzianum 1317	85	Little	72	4,6
T. harzianum 1322	85	Little -Abundant	72	2,22
T. longibrachiatum 1327	77,5	Very abundant	48-72	11,43
T. harzianum 1330	85	Abundant	48	12,35
T. harzianum 1336	73,5	Abundant	48-72	12,89
T. deliquescens 1343	85	Very abundant	48	22,24
Trichoderma sp. 1349	71	Very abundant	48	15,17
T. guizhouense 1351	76	Little	> 96	10,66
Trichoderma sp. 1366	85	Abundant	72	15,71
T. harzianum 1367	85	Very abundant	48	24,21
T. harzianum 1368	78,5	Abundant	> 96	20,99
T. harzianum 1371	85	Abundant	48	18,12
T. longibrachiatum 1375	85	Abundant	48-72	-
T. brevicompactum 1377	85	Abundant	72	6,23
T. guizhouense 1379	85	Very abundant	48	15,94
Trichoderma sp. 1383	85	Very abundant	48	16,87
T. guizhouense 1384	58	Very abundant	48-72	17,66

[1]Growth rate: diameter of colony (mm) on PDA after 72 h. [2]Quantity Scale: little: 1 - 10^2 conidia.mL^{-1}; abundant: 10^4 - 10^6 conidia.mL^{-1}; very abundant: 10^7 or more conidia.mL^{-1}. [3]Time: time of appearance of conidia in hours. [4]Percentage of mycelial growth inhibition of *P. cactorum* by production of secondary metabolites of *Trichoderma* strains.

Table 4. Growth promotion of *Trichoderma* strains in tomato seedlings

***Trichoderma* strain**	**Root lenght (cm)**	**Fresh weight (g)**	**Clorophyl A+B (µg.g-1PF)**
T. atroviride 1310	5,41 abcd*	0,06 a	318,0 m
T. harzianum 1317	6,05 abcd	0,09 ab	220,1 h
T. harzianum 1322	6,43 cd	0,07 ab	354,4 n
T. longibrachiatum 1327	5,42 abcd	0,06 ab	243,9 j
T. harzianum 1330	5,74 abcd	0,09 ab	216,2 g
T. harzianum 1336	3,97 abc	0,05 a	631,1 o
T. deliquescens 1343	6,42 cd	0,08 ab	226,9 i
Trichoderma sp. 1349	4,68 abcd	0,07 ab	199,3 e
Trichoderma sp.1366	4,54 abcd	0,07 ab	249,9 k
T. harzianum 1367	6,78 d	0,08 ab	175,4 b
T. harzianum 1371	4,35 abcd	0,10 ab	203,0 f
T. longibrachiatum 1375	6,26 bcd	0,07 ab	191,7 d
T. longibrachiatum 1377	5,90 abcd	0,08 ab	167,7 h
T. guizhouense 1379	3,60 a	0,08 ab	222,0 h
Trichoderma sp. 1383	3,77 ab	0,06 ab	187,0 c
T. guizhouense 1384	5,62 abcd	0,07 ab	260,1 l
CONTROL	4, 56 abcd	0,10 ab	168,6 a

*Equal letters do not represent significant differences by Tukey mean difference analysis with 95% of confidence.

Table 5. Orchard experiments to evaluate the biocontrol effect of regional *Trichoderma* sp. strains against *P. cactorum* rot

Experiments	**First year**		**Second year**			
Treatments	**Preventive**		**Preventive**		**Curative**	
	Lesion area*	**Biocontrol %**	**Lesion area (cm²)**	**Biocontrol %**	**Lesion area (cm²)**	**Biocontrol %**
T. harzianum 1330	0.13[a**]	97.13	0.62[a**]	86.97 [F***]	0.55[a**]	82.65 [C***]
T. harzianum 1367	0.42[a]	90.74	0.15[a]	96.8 [H]	0.11[a]	96.53 [H]
T. harzianum 1371	0.16[a]	96.47	0.76[a]	84 [D]	0.46[a]	85.48 [E]
T. atroviridae com.	0.00[a]	100	0.00[a]	100 [I]	1.24[ab]	60.88 [A]
Chemical control	0.00[a]	100	0.33[a]	93 [G]	0.97[a]	69.40 [B]
Diseased control	4.54[b]		4.76[b]	-	3.17[b]	-

* Lesion area: Measurement (cm^2) of necrotic lesion area through the Image J 1x programme in the wound treated and inoculated with *P. cactorum* 1378. **Equal letters in the same column mean that they do not have significant differences by Tukey mean difference analysis, with 95% of significance. Average area of three repetitions calculated with the Image J 1x programme. ***Statistical analysis of biocontrol variable with all treatments.

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Ethical responsibilities

We confirm compliance with the requirements given under publishing ethics.

We warrant that the manuscript has not been submitted to more than one journal for simultaneous consideration.

- The manuscript has not been published previously (partly or in full), and it is not currently under consideration for publication either in whole in part, by any other journal.
- The study had not been split up into several parts to increase the quantity of submissions and submitted to various journals or to one journal over time.
- No data have been fabricated or manipulated to support our conclusions and no data or text, by others are presented as if they were own (“plagiarism”).
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- All authors whose names appear on the submission have consented the submission and they have accorded with the order gave in the manuscript.
- All authors have contributed sufficiently to the scientific work and therefore share collective responsibility and accountability for the results.

Compliance with ethical standards

Disclosure of potential conflicts of interest: All authors declare that they have no conflict of interest.

Disclosure of potential conflicts of interest

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

Sincerely yours,

María Cristina Sosa