Eur J Plant Pathol (2021) 160:825–840
https://doi.org/10.1007/s10658-021-02286-9

Biological control of *Fusarium oxysporum* f. sp. *ciceri* and *Ascochyta rabiei* infecting protected geographical indication Fuentesaúco-Chickpea by *Trichoderma* species

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Accepted: 15 April 2021 / Published online: 29 April 2021

Abstract The Protected Geographical Indication Fuentesaúco-Chickpea (F-C) is a legume crop that was developed in the south of the province of Zamora (Spain). It has great agronomic, economic, and cultural importance worldwide. Its cultivation is threatened mainly by the fungal diseases Fusarium wilt (causal agent *Fusarium oxysporum* f. sp. c*iceri*) and Ascochyta blight (causal agent *Ascochyta rabiei*). By conducting an in vitro antagonism study, we determined that strains belonging to the species *Trichoderma atroviride*, *T. hamatum*, *T. harzianum,* and *T. koningii* are the most effective against both pathogens. The mechanisms of action of these strains are as follows: mycoparasitism, antibiosis, and competition for space and/or nutrients. Subsequently, these four *Trichoderma* spp. strains were used for an infection test in F-C plants to test their activity against both fungal pathogens by plant root inoculation. We reported that *T. atroviride* and *T. koningii* were capable of controlling *F. oxysporum* f. sp. *ciceri* in F-C plants directly, and *T. koningii* activated the production of salicylic acid as a plant defense response. In the case of *A. rabiei* foliar infection, plant roots inoculated with *T. hazianum* and *T. koningii* species were able to significantly decrease the occurrence of disease through systemic activation of jasmonic acid as a defense response. Regarding the effects of the *Trichoderma* species on F-C productivity, the species *T. hamatum* and *T. koningii* were able to significantly increase (by 50% and 100%, respectively) the formation of grains per plant. Therefore, we propose that *T. koningii* could be used to control *F. oxysporum* f. sp. *ciceri* and *A. rabiei* in F-C plants under greenhouse conditions through direct mechanisms as well as the induction of local and systemic defense responses and thereby promoting crop productivity.

Keywords Chickpea · *Fusarium oxysporum* f. sp. *ciceri* · *Ascochyta rabiei* · *Trichoderma* · Antagonism · Plant systemic resistance · *Trichoderma koningii*

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Introduction

Chickpea (*Cicer arietinum* L.) is a highly nutritious, climate-resilient, and nitrogen-fixing crop, capable to provide nutrients for the expanding world population (Fikre et al. 2020). This crop is a good source of energy, protein, minerals, vitamins, and fiber, as well as containing potentially health-beneficial phytochemicals. Thus, it plays a leading role in food safety accounting for the protein deficit in the diets of Indian and Sub-Saharan African populations (Merga and Haji 2019).

According to the European Union Council Regulation (EC) No 510/2006, the Fuentesaúco-Chickpea (F-C) ("Garbanzo de Fuentesaúco", in Spanish), which was granted a Protected Geographical Indication in 1996, is produced in the southeast of Zamora province (Castilla y León, Spain). Although, it is not a very productive legume, it has great culinary quality due to its large size, texture, and flavor. The F-C not only represents a product of agronomic interest in the south of the province of Zamora, but it also represents an important cultural and social asset, as it has been declared as one of the most recognized legumes worldwide in terms of quality (EC 510/2006 2006).

The registry of plant diseases suffered by the F-C from 2003 to 2020 indicates that, according to the information provided by the Regional Diagnostic Center of the Regional Government of Catilla y León (Spain), in 80% of cases, the pathogens found are the fungi *Fusarium oxysporum* f. sp. *ciceri* and *Ascochyta rabiei*. *Fusarium oxysporum* f. sp. *ciceri* causes Fusarium wilt disease. The fungus penetrates through the roots, the germ tube penetrates the epidermal cells of plants, and later, the hyphae extend to the root cortical region and colonize the xylem vessels, thus preventing the upward translocation of water and essential solutes, resulting in wilt. In addition, *F. oxysporum* f. sp. *ciceri* is a saprophytic fungus that may survive in soil or debris for up to 6 years, causing large yield losses in years of severe disease outbreak (Caballo et al. 2019). On the other hand, *Ascochyta rabiei* is the most devastating disease that affects chickpea crops, causing Ascochyta blight. As the pathogen is seed-borne in nature, the disease might have spread from its site of origin to distant continents through chickpea germplasm exchanges. Stem breakage along with girdling and collapse of twigs and pod infection are the two most damaging symptoms of this disease. Disease is more severe in areas where cool temperatures and humid conditions prevail during the chickpea growing season (Rani et al. 2020). The main control strategy for both pathogens is based on the use of chemical fungicides, whose massive use causes serious damage to the environment and health (Benzohra et al. 2020; Jamil and Ashraf 2020).

The filamentous fungi genus *Trichoderma* includes various species that are widely used as biological control agents (BCAs) in agriculture due to their different mechanisms of action and their ability to grow naturally in very diverse habitats and climatic zones (Harman 2006; Vinale et al. 2008). Some species are able to mycoparasitize pathogenic fungi in crops such as *Trichoderma harzianum* on *Rhizoctonia solani* (Kullnig et al. 2000); produce chemical compounds that inhibit pathogenic growth and development, a mechanism known as antibiosis (Patil et al. 2016); or compete efficiently for space and nutrients in the rhizosphere (Segarra et al. 2010). In addition, *Trichoderma* spp. promote plant growth, increase crop productivity (Poveda et al. 2019a), and increase the tolerance of plants against abiotic stresses such as drought and salinity (Poveda 2020a).

Trichoderma root colonization involves the ability to recognize and adhere to roots, penetrate the plant, and withstand toxic metabolites produced by the plant in response to invasion. In this sense, the plant may perceive *Trichoderma* spp. as hostile, and activation of the plant's defences may limit penetration by the fungus into the root's outermost cell layers (Alonso-Ramírez et al. 2014; Poveda et al. 2020a). In this interaction, successful *Trichoderma* root colonization supposes the activation of a systemic plant resistance against possible attack from pathogens or pests. This defense occurs via induced systemic resistance (ISR), systemic acquired resistance (SAR), or both (Poveda et al. 2020b, 2020c).

The aims of this study are based on identifying different *Trichoderma* species that are effective BCAs that act against the pathogenic F-C fungi (P.G.I.) *F. oxysporum* f. sp. *ciceri* and *A. rabiei* through direct mechanisms of action as well as the activation of plant defenses. In order to develop the aims, antagonistic in vitro assays and inoculations and infections were performed *in planta*, and different molecular and biochemical analyses were performed.

Materials and methods

Fungal materials

Trichoderma asperellum CECT 2941 (referred from here as Tas), *T. atroviride* CECT 20512 (Tav), *T. hamatum* CECT 20103 (Thm), *T. harzianum* CECT 2424 (Thr), *T. koningii* CECT 2936 (Tk), *T. parareesei* CECT 20106 (Tp), *T. reesei* CECT 2416 (Tr), *T. virens* CECT 2460 (Tv) (Spanish Type Culture Collection, Valencia, Spain), *T. brevicompactum* CECT IBT 40841 (Tb) (IBT Culture Collection, Kongens Lyngby, Denmark), and the fungal pathogens *Fusarium oxysporum* f. sp. *ciceri* (Foc) and *Ascochyta rabiei*

(Ar) were facilitated by the Regional Diagnostic Center of the Regional Government of Castilla y León (Salamanca, Spain), isolated from F-C plants in the field and identified by microbiological and molecular techniques. Strains were routinely grown on potato-dextrose-agar (PDA, Sigma-Aldrich, Madrid, Spain) in the dark at 28 °C, and the spores were stored at −80 °C in 1.2 ml of 20% glycerol solution. Spores were harvested from 7-day-old PDA dishes, as previously described by Poveda (2021).

The choice of these specific *Trichoderma* species was made based on their effectiveness against other pathogenic fungi: Tas against *Fusarium oxysporum* f. sp. *cucumerinum* and *Fusarium graminearum* (Wu et al. 2017), Tav against *Guignardia citricarpa* (de Lima et al. 2016), Thm against *Sclerotinia sclerotiorum* (Shaw et al. 2016), Thr against *Fusarium graminearum* (Saravanakumar et al. 2018), Tk against *Rhizoctonia solani* (Gajera et al. 2016), Tp against *R. solani*, *F. oxysporum* and *Botrytis cinerea* (Pérez et al. 2015), Tv against *F. oxysporum* (Li et al. 2018), and Tb against *F. oxysporum* and *R. solani* (Das et al. 2019).

In vitro antagonism assay

To evaluate the antagonistic activity of *Trichoderma* species against Foc and Ar, the dual culture technique was used. Mycelial disks (8 mm diameter) of each fungus obtained from the edge of the colonies developed on PDA medium were placed on the opposite side (50 mm apart) in 90 mm Petri dishes containing PDA medium. In the controls, only disks of each fungus were placed on PDA medium. Plates were incubated at 25 °C in the dark, and photographs and measurements of growth were taken at 72, 144, and 192 h. For each condition, eight plates were used, and the assay was conducted twice.

Plant growth and fungal–chickpea inoculation

F-C seeds were surface-sterilized by vigorous sequential shaking in 70% ethanol and 5% sodium hypochlorite solutions for 10 min each and then washed thoroughly four times in sterile, distilled water. Thirty F-C seedlings (per condition and assay) were individually transferred to 3.5 L pots, containing a mixture of soil/vermiculite (3:1) sterilized by autoclave (twice, 24 h apart) and maintained in a greenhouse at 22 ± 2 °C. The soil was collected from cultivation fields of the F-C Protected Geographical Indication.

Two weeks after seeding, for the inoculation of chickpea plants with *Trichoderma* species, each plant was inoculated with 1 mL of a conidial suspension containing 2×10^7 spores mL^{-1}. This concentration was determined using a haemocytometer, as described by Poveda (2020a).

In the Foc infection test, F-C was inoculated with fungal spores one week after having been root inoculated with *Trichoderma*, according to the methodology described by Lyons et al. (2015). The roots of each plant were immersed in a solution with 1×10^6 spores mL^{-1} for 2 min. Two weeks after Foc infection, plants were collected (30 plants per condition).

For the infection of F-C plants by Ar, the methodology described by Leo et al. (2016) was followed. One week after having root inoculation with *Trichoderma* spp., each plant was sprayed with 5 ml of water with a pathogen spore suspension of 1×10^5 spores mL^{-1}. After 10 days of Ar infection, the plants were collected (30 plants per condition).

Direct pathogen effect analysis

Dry weight

Two weeks after Foc infection and 10 days after Ar infection, the aerial part (Foc and Ar infection) and roots (Foc infection) of 21 plants were collected. The dry weight was determined by keeping the fresh plants at 65 °C for 48 h, as reported by Poveda (2020b).

Damage of the vascular bundle by Foc

In all Foc-infected F-C plants, the affectation of the vascular bundle (30 plants per condition) was analyzed. For this, a neck cut was made between the roots and the stem of each plant, and a vascular damage value was assigned: 1—healthy plants, 2—slightly damaged plants, 3—moderately damaged plant, 4—damaged plants, and 5—very damaged plants. The disease severity (%) was calculated by the formula (Number of vascular bundles x Damage value)/(Total number of vascular bundles x Maximum damage grade)×100.

Percentage of leaves affected by Ar

For all Ar-infected F-C plants, the percentage of leaves affected by the pathogenic fungus per plant was calculated.

Vitality test and indirect quantification of reactive oxygen species (ROS) in tissues

To analyse and quantify the damage produced by both pathogens on F-C roots and leaves, the viability test and ROS quantification were assayed, as reported by Poveda (2020b).

The reduction of triphenyltetrazolium chloride (TTC) by tissue to red-colored insoluble triphenylformazan (TF) is directly linked to the activity of the mitochondrial respiratory chain. Thus, only living tissues should reduce TTC to TF. Pools were formed from nine plants per condition containing roots (Foc-infected plants) or three leaves per plant (Ar-infected plants). From these pools, 100 mg was transferred to 1 mL of 1% TTC in triplicate and incubated for 48 h at 37 °C. After incubation, 200 mg of Ballotini Glass Balls (0.15–0.25 mm and 1 mm diameter) was added to each sample in 1.5 mL Eppendorf tubes and shaken vigorously with a pulse of 20 s in Silamat S6 (Ivoclar Vivadent, Madrid, Spain). After centrifuging the samples for 15 min at 10,000 rpm, the supernatant was removed, and 1 mL of isopropanol was added to each tube. The samples were again agitated in Silamat and centrifuged in the same way, and the supernatant was used to quantify the absorbance at 620 nm; this acted as an indirect measure of the vitality of the F-C tissues.

The indirect quantification of reactive oxygen species (ROS) in F-C tissues was carried out by measuring electrolyte leakage, which really measures cellular oxidative damage related to the production of ROS. From each pool formed from roots (in Foc-infected plants) or 3 leaves (in Ar-infected plants) per plant and nine plants per condition, 1 cm^2 of fresh tissue was briefly mixed with water and floated on 5 mL of double-distilled water at room temperature for 6 h. The conductivity of the water was measured using a Crison™ Conductimeter GLP31 (Crison, Barcelona, Spain). This represented the electrolyte leakage from the tissues (Reading 1). Then, samples were boiled for 20 min at 90 °C. After the liquid cooled down, the conductivity of the water was measured again. This represented the total concentration of ions present in the tissues (Reading 2). Electrolyte leakage, an indirect measurement of ROS, was represented as the percentage of total ions released [(Reading 1/Reading 2) × 100].

Tissue-colonization analysis

In order to determine the differences in F-C tissue colonization by Foc and Ar, they were quantified by qPCR. For the analysis of Foc-root and Ar-leaf colonization, pools formed from roots (in Foc-infected plants) or three leaves (in Ar-infected plants) per plant and nine plants per condition were used. All material was immediately frozen with liquid nitrogen and pulverized with a mortar.

Following the methodology described by Poveda et al. (2019b), DNA was extracted using the cetyltrimethyl-ammonium bromide (CTAB) extraction method. A mixture was prepared in a 10 μl volume using 5 μl of Brilliant SYBR Green QPCR Master Mix (Roche), 10 ng of DNA, forward and reverse primers at a final concentration of 100 nM, and nuclease-free PCR-grade water to adjust the final volume. *Actin* genes from Foc and chickpea and the *Ubiquitin* gene from Ar were used as reference genes to calculate host plant and fungus DNA, and their corresponding primer pairs are indicated in Table 1. Amplifications were performed in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA), programmed for 40 cycles under the following conditions: denaturation at 95 °C for 15 s; annealing at 60 °C for 1 min; and extension at 72 °C for 1 min. Each round of PCR was performed in triplicate by using the DNA extracted from the roots collected. Cycle threshold values were used to calculate the amount of fungal DNA using standard curves. Values of pathogen-fungal DNArefer to the amount of chickpea DNA in every corresponding sample with the value of F-C + Foc or + Ar normalized to 1.

Defense gene expression analysis

To analyze the defense responses identified following F-C infection with Foc and Ar, an expression analysis of defense genes was performed by RT-qPCR, following the methodology described by

Poveda et al. (2019b). For gene expression studies, the pools formed from roots (in Foc-infected plants) or 3 leaves (in Ar-infected plants) per plant and 9 plants per condition were used for RNA extraction with the TRI reagent (Ambion, Austin, TX, USA), following the manufacturer's instructions. cDNA was synthesized from 2 μg of RNA, which was treated with DNase RQ1 (Promega Biotech Ibérica, Alcobendas, Spain), and then used for reverse transcription with an oligo(dT) primer with the Transcriptor First Strand cDNA Synthesis Kit (Takara Bio, Inc., Tokyo, Japan), following the manufacturer's protocol. Gene expression was analyzed by RT-qPCR using an ABI PRISM 7000 Sequence Detection System with Brilliant SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA, USA). All PCR reactions were performed in triplicate in a total volume of 10 μL for 40 cycles under the following conditions: denaturation at 95 °C for 30 s; annealing at 60 °C for 1 min; and extension at 72 °C for 1 min. Threshold cycles (CT) were determined using 7000 SDS System Software (Applied Biosystems, Foster City, CA, USA), and CT values were calculated using the chickpea *Actin* gene as an endogenous control. The primers used are shown in Table 1, as follows: the isochorismate synthase 1 (*ICS1*), pathogenesis-related protein 1 (*PR-1*), lipoxygenase 2 (*LOX2*), and SNAKIN2 antimicrobial peptide precursor (*SN2*) genes. Data are expressed using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Productivity assay

In order to quantify the possible effect of *Trichoderma* species root inoculation on F-C productivity, 20 plants per condition were used, and the grains produced per plant were collected at the end of the plant life cycle and counted (15 weeks).

Statistical analysis

Statistical analysis of the data was carried out with Statistix 8.0 software. The Student's t test was used for a comparison of means at $P < 0.05$; significant differences are denoted using an asterisk. One-way ANOVA using Tukey's multiple range test at $P < 0.05$ was used for pairwise comparisons; the different letters indicate significant differences.

Results

In vitro antagonism

The in vitro antagonism test between the different *Trichoderma* species and the pathogens *F. oxysporum* f. sp. *ciceri* and *A. rabiei* (Fig. S1) reported a significant decrease in pathogenic growth (50%) when exposed to the species *T. atroviride*, *T. hamatum*, *T. harzianum* and *T. koningii*. In the case of the *T. koningii*–*A. rabiei* interaction, a 75% reduction was reached (Fig. 1).

Foc-chickpea-*Trichoderma in planta* assay

The quantification of the dry weight of the F-C plants radicularly infected with Foc showed that inoculation with *Trichoderma* species was associated with a significant increase in plant biomass compared to plants without inoculation (283 mg), and this increase was significantly greater with *T. atroviride* (471 mg) and *T. koningii* (509 mg) (Fig. 2a, b). On the other hand, cross-sections made in the neck of the F-C plants showed that plants treated with *Trichoderma* were significantly less affected by Foc than non-inoculated plants, with this affectation being significantly lower following root inoculation with *T. atroviride* and *T. koningii* (Fig. 2c, d).

The root viability analysis showed that significantly greater absorbance was obtained (absorbance 0.0585–0.065) in all F-C plants inoculated with *Trichoderma* compared to non-inoculated plants (absorbance 0.0212), which is indicative of greater vitality (Fig. 3a). In the same way, the indirect quantification of ROS showed that inoculation with the different *Trichoderma* species led to a decrease in pathogenic stress in the roots of F-C plants compared to non-inoculated plants (3.85 r.i.l.). The stress level was significantly decreased following inoculation with *T. atroviride* (1.72 r.i.l.) and *T. koningii* (1.53 r.i.l.) (Fig. 3b).

Ar-chickpea-*Trichoderma in planta* assay

Regarding the direct analysis of the effects of *A. rabiei* in F-C plants, no significant differences in the dry weight of the aerial part were reported following the root inoculation of *Trichoderma* species (Fig. 4b). On the other hand, the application of the different species of *Trichoderma* led to a significant decrease in the percentage of leaves affected by the pathogen compared to non-

Table 1 Oligonucleotides used in this work

Code	Sequence (5′-3′)	Use	Reference
Foc-Act-F	TACCACCAGACATGACAATGTTGCC	Endogenous *F. oxysporum* f. sp. *ciceri* gene	Calero-Nieto et al. 2007
Foc-Act-R	GGAGATCCAGACTGCCGCTCAG		
Ar-Ubq-F	AAACCAAGACCCCAGCTCGTA	Endogenous *A. rabiei* gene	Singh et al. 2012
Ar-Ubq-R	GGGCCGATGATGGTTTCTG		
Ca-Act-F	GCCCAGTCAAAGAGGGGTATCCTCA	Endogenous *Cicer arietinum* gene	Cho and Muehlbauer 2004
Ca-Act-R	CACACCATCACCAGAGTCGAGCA		
Ca-ICS1-F	GAATCTCGCTGTTAATAGAGCTT	Synthesis gene of SA in *C. arietinum*	Bhar et al. 2018
Ca-ICS1-R	CATGCTAACAATGTTAGAGGATCA		
Ca-PR-1-F	ATGGGTGTATTCACATTTGAGCAAG	Response gene of SA in *C. arietinum*	Bhar et al. 2018
Ca-PR-1-R	TCAGTTGTAATTAGGATTGGCCAAA		
Ca-LOX2-F	TGAAGCCAGTGGCCATCGAAT	Synthesis gene of JA in *C. arietinum*	Cho and Muehlbauer 2004
Ca-LOX2-R	CGAAGGCCGTGTGGGAAGAT		
Ca-SN2-F	CATGGCAACAAGACCAAGTGTC	Response gene of JA in *C. arietinum*	Leo et al. 2016
Ca-SN2-R	GTTGGGAACAAAGTAGGGACTG		

inoculated plants (26.7%), and this was even more significant with the application of *T. harzianum* (8.9%) and *T. koningii* (6.5%) (Fig. 4c).

In F-C leaves infected with *A. rabiei*, a significant increase in the vitality of the plants radicularly inoculated with the different *Trichoderma* species was reported, and this was

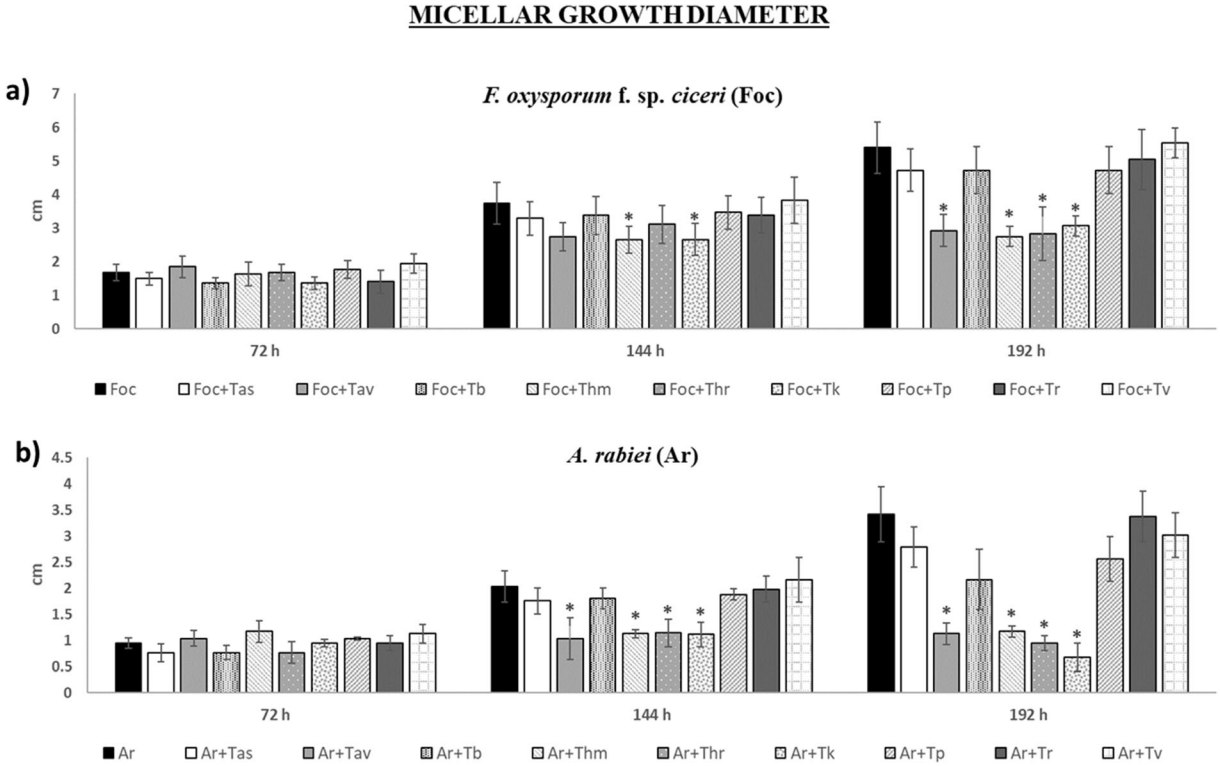

Fig. 1 Micellar growth diameter of *F. oxysporum* f. sp. *ciceri* (Foc) (a) and *A. rabiei* (Ar) (b) in their antagonistic confrontation in vitro against *T. asperellum* (+Tas), *T. atroviride* (+Tav), *T. brevicompactum* (+Tb), *T. hamatum* (+Thm), *T. harzianum* (+Thr), *T. koningii* (+Tk), *T. parareesei* (+Tp), *T. reesei* (+Tr), and *T. virens* (+Tv). Data are the mean of eight biological replicates for each condition with the corresponding standard deviation. Student's t test was performed. Asterisks denote significant differences at $P \leq 0.05$

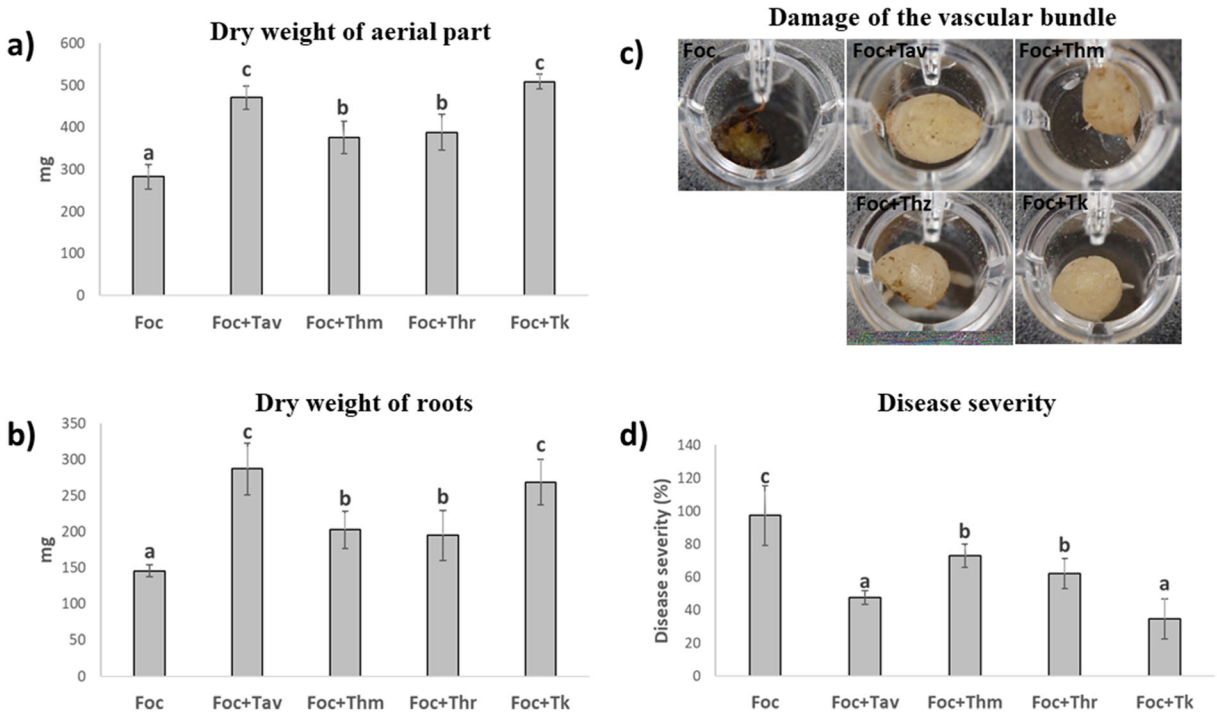

Fig. 2 Dry weight of aerial part (a) and roots (b), and damage of the vascular bundle (c) and disease severity (d) of Foc-infected F-C plants. Plants infected with *F. oxysporum* f. sp. *ciceri* (Foc) and root inoculated with *T. atroviride* (+Tav), *T. hamatum* (+Thm), *T. harzianum* (+Thr) or *T. koningii* (+Tk). Data are the mean of twenty-one (a and b) and thirty (d) plants for each condition. One-way analysis of variance (ANOVA) was performed, followed by the Tukey's test. Different letters represent significant differences ($P < 0.05$)

more significant following the application of *T. harzianum* and *T. koningii* (Fig. 5a). On the other hand, no significant differences in the indirect quantification of ROS in F-C leaves infected with *A. rabiei* were found (Fig. 5b).

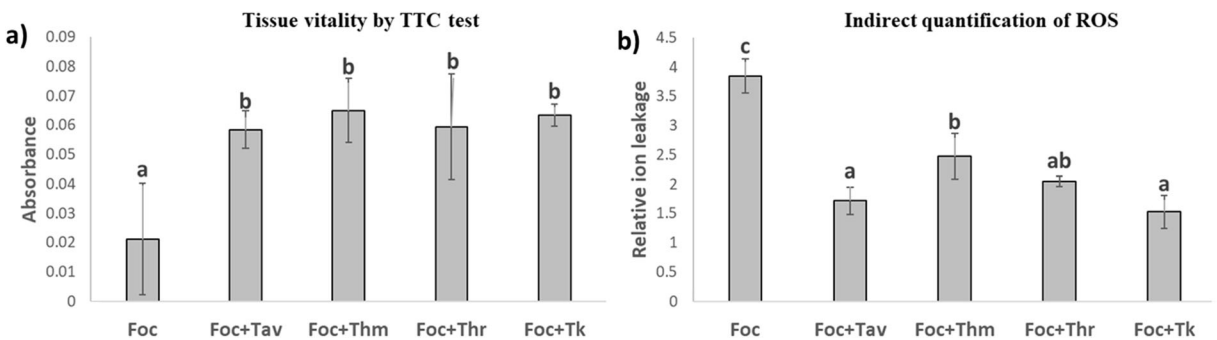

Fig. 3 Tissue vitality by TTC test (a) and indirect quantification of ROS (b) in F-C roots infected with *F. oxysporum* f. sp. *ciceri* (Foc) and root inoculated with *T. atroviride* (+Tav), *T. hamatum* (+Thm), *T. harzianum* (+Thr) or *T. koningii* (+Tk), where the absorbance at 620 nm (TTC test) and the relative ion leakage (indirect ROS measurement) were analyzed. Data are the mean of nine plants with the corresponding standard deviation. One-way analysis of variance (ANOVA) was performed, followed by the Tukey's test. Different letters represent significant differences ($P < 0.05$)

Quantification of chickpea tissue pathogen colonization and defense gene expression

The quantification of the colonization of F-C plant tissues by each pathogenic fungus showed that roots infected with Foc had significant decreases in F-C following root inoculation with the different *Trichoderma* species (0.07–0.53) compared to non-inoculated plants (1). The concentration was significantly lower with *T. atroviride* and *T. koningii* (Fig. 6a). On the other hand, in leaves infected with Ar, only roots inoculated with *T. harzianum* (0.23) and T. *koningii* (0.12) had significantly lower colonization of the leaf tissues by the pathogen (Fig. 6b).

In Foc-infected F-C roots, significant changes were only observed in the expression of the defense genes analyzed after inoculation with *T. koningii* compared to plants that did not undergo root inoculation with *Trichoderma* species. There were significant increases in the expression of genes related to the synthesis and response genes of salicylic acid (SA) (*ICS1* and *PR1*) and significant decreases in the expression of the synthesis and response genes of jasmonic acid (JA) (*LOX2* and *SN2*) (Fig. 7).

As far as Ar-infected F-C leaves are concerned, it was reported that only plants inoculated with *T. harzianum* and *T. koningii* had significantly reduced expression of SA-related genes and significantly increased expression of JA-related genes compared with plants not treated with *Trichoderma* (Fig. 8).

Chickpea productivity

At the productivity level, it was possible to verify that the application of *T. hamatum* and *T. koningii* significantly increased the productivity of F-C plants by 50% and 100%, respectively (Fig. 9).

Discussion

Through in vitro confrontation of the F-C Foc and Ar pathogens against different *Trichoderma* species, we were able to verify that the *T. hamatum*, *T. atroviride*, *T. harzianum,* and *T. koningii* species were capable of limiting the pathogen growth due to different mechanisms that can act in isolation or simultaneously: mycoparasitism, antibiosis, and/or competition for space and/or nutrients (Sánchez et al. 2019). It is important to note that the direct mechanisms of action possibly used by each of the *Trichoderma* species have not been studied in detail and, therefore, the exact mechanism cannot be identified. Therefore, mechanisms of action such as mycaparasitism require specific in-depth study to be confirmed in future studies using methodologies such as scanning electron microscopy (SEM). In this sense, the possible direct mechanisms of action observed in the in vitro results are indicated, based on other published studies.

It has been proven that *T. hamatum* is capable of parasitizing different pathogenic plant fungi, such as *Botrytis cinerea* (Fekete et al. 2001) and *Sclerotinia sclerotiorum* (Steyaert et al. 2004; Carpenter et al. 2005), in addition to producing specific secondary metabolites that are possibly involved in the biocontrol of plant pathogens (Studholme et al. 2013). Some are already known to be present in other *Trichoderma* species, such as viridin (Sakuno et al. 2000) and dermadin (Fujiwara et al. 1982). In the specific cases of *F. oxysporum* and *A. rabiei*, this work is the first to describe how *T. hamatum* acts as an effective BCA against them. There has only been one study on the direct biocontrol capacity of *T. hamatum* against *F. oxysporum* f. sp. *lentis*, a lentil pathogen (El-Hassan et al. 2013).

On the other hand, *T. atroviride* has been widely studied due to its ability to act as a mycoparasite, for example, on *Rhizoctonia solani* (Atanasova et al. 2018; Gómez-Rodríguez et al. 2018), *B. cinerea*, *Sclerotium cepivorum*, and *Colletotrichum lindemuthianum* (Romero-Contreras et al. 2019), due to its antibiosis capacity, which involves the production of secondary metabolites such as atrichodermones A-C (Zhou et al. 2017) or different volatiles (Stoppacher et al. 2010), or due to its rapid growth in the rhizosphere (Cripps-Guazzone et al. 2016). With regard to *Fusarium*, there are numerous examples of its control in various crops thanks to the use of *T. atroviride*, for example, on *F. avenaceum* and *F. culmorum* in corn (Coninck et al. 2020), or the use of its secondary metabolites against *F. graminearum* (Li et al. 2020). This work is also the first to describe *T. atroviride* as being an effective biocontrol agent against *F. oxysporum* f. sp. *ciceri* and *A. rabiei*. There are numerous examples of the capacity of *T. harzianum* to act as a mycoparasite of plant pathogens, such as *R. solani* (dos Reis Almeida et al. 2007), *Gibberella zeae* and *Aspergillus ustus* (Küçük and Kývanç 2008), *S. slcerotiorum* (Troian et al. 2014),

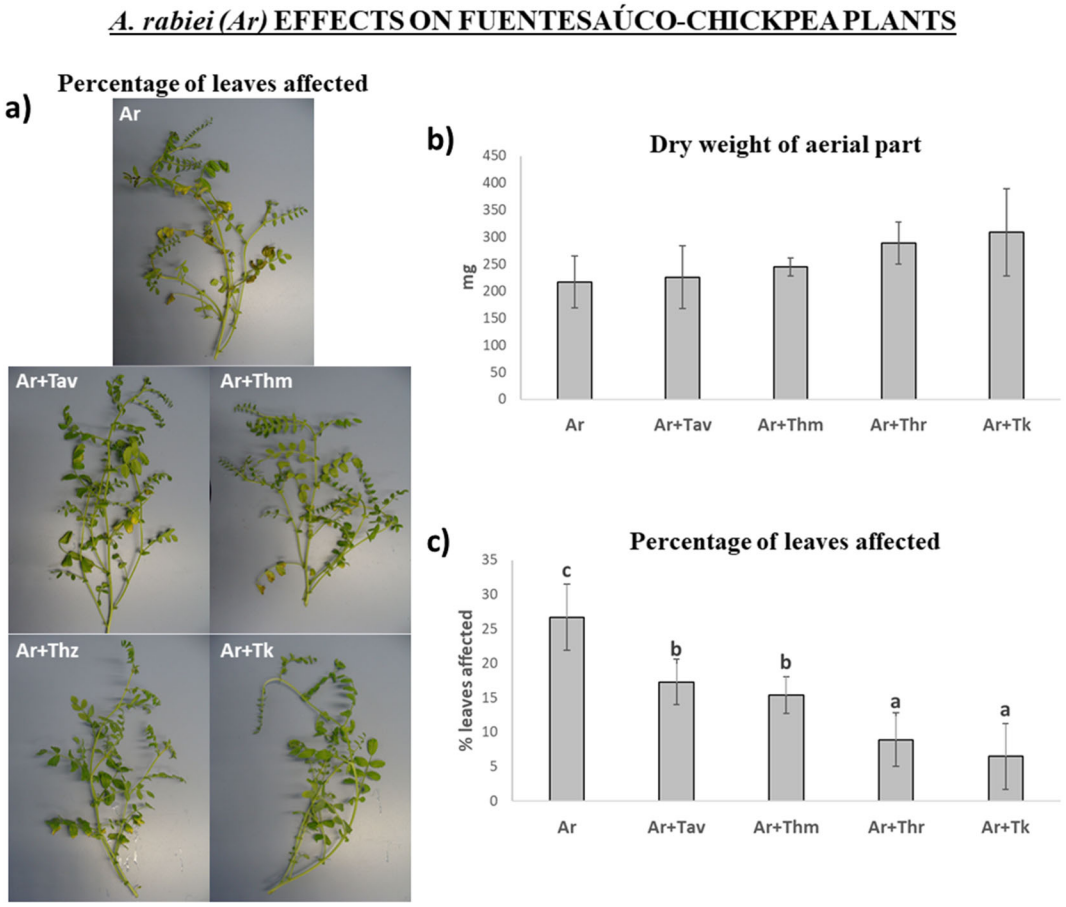

Fig. 4 Percentage of leaves affected (a and c) and dry weight of aerial part (b) of Ar-infected F-C plants. Plants infected with *A. rabiei* (Ar) and root inoculated with *T. atroviride* (+Tav), *T. hamatum* (+Thm), *T. harzianum* (+Thr) or *T. koningii* (+Tk). Data are the mean of twenty-one (b) and thirty (c) plants for each condition. One-way analysis of variance (ANOVA) was performed, followed by the Tukey's test. Different letters represent significant differences ($P < 0.05$)

and *Bipolaris oryzae* (Abdel-Fattah et al. 2007), among many others. In addition, *T. harzianum* has wide metabolic diversity related to its ability to inhibit the growth of pathogenic plant fungi, such as harzianic acid (Vinale et al. 2009), viridiofungin A (El-Hasan et al. 2009), and many others (Ahluwalia et al. 2015). In the specific case

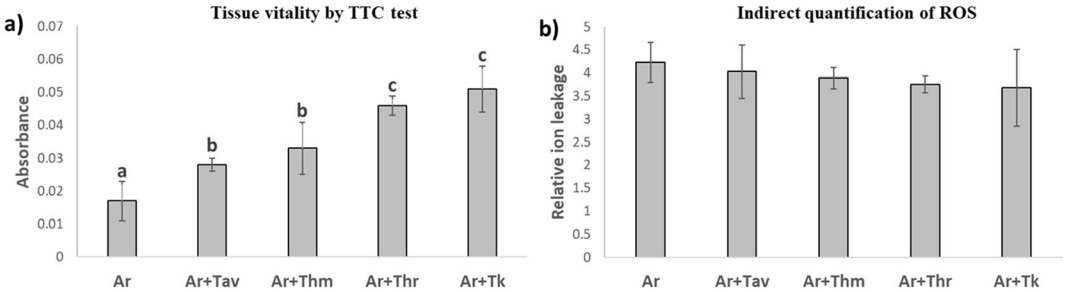

Fig. 5 Tissue vitality by TTC test (a) and indirect quantification of ROS (b) in F-C leaves infected with *A. rabiei* (Ar) and root inoculated with *T. atroviride* (+Tav), *T. hamatum* (+Thm), *T. harzianum* (+Thr) or *T. koningii* (+Tk), where the absorbance at 620 nm (TTC test) and the relative ion leakage (indirect ROS measurement) were analyzed. Data are the mean of nine plants with the corresponding standard deviation. One-way analysis of variance (ANOVA) was performed, followed by the Tukey's test. Different letters represent significant differences ($P < 0.05$)

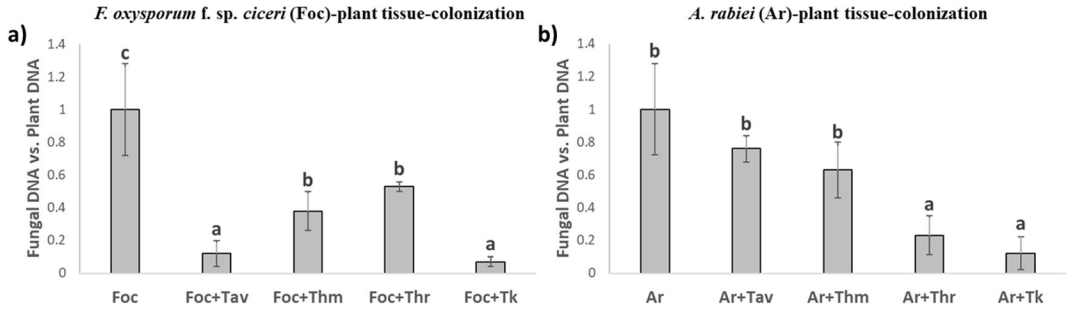

Fig. 6 Quantification of fungal-plant tissue-colonization in F-C roots infected with *F. oxysporum* f. sp. *ciceri* (Foc) (a) or leaves infected with *A. rabiei* (Ar) (b), and root inoculated with *T. atroviride* (+Tav), *T. hamatum* (+Thm), *T. harzianum* (+Thr) or *T. koningii* (+Tk). Data are the mean of nine plants with the corresponding standard deviation. One-way analysis of variance (ANOVA) was performed, followed by the Tukey's test. Different letters represent significant differences ($P < 0.05$)

of *Fusarium*, there are examples of mycoparasitism and antibiosis against *F. oxysporum* (Thangavelu et al. 2004; López-Mondéjar et al. 2011) and of antibiosis against *F. moniliforme* (El-Hasan et al. 2008). It also acts specifically against *F. oxysporum* f. sp. *ciceri* (Dubey et al. 2007; Rawat et al. 2013). Moreover, the ability of *T. harzianum* to inhibit the growth of *A. rabiei* through antibiosis and mycoparasitism has already been described (KüÇük et al. 2007). Finally, *T. koningii* has been described as a mycoparasite that is capable of antibiosis on pathogens such as *R. solani* (Gajera et al. 2016) and *Sclerotium rolfsii* (Rabinal and Bhat 2020) by secondary metabolites such as 6-pentyl-α-pirone (Ismaiel and Ali 2017) or by directly competing for

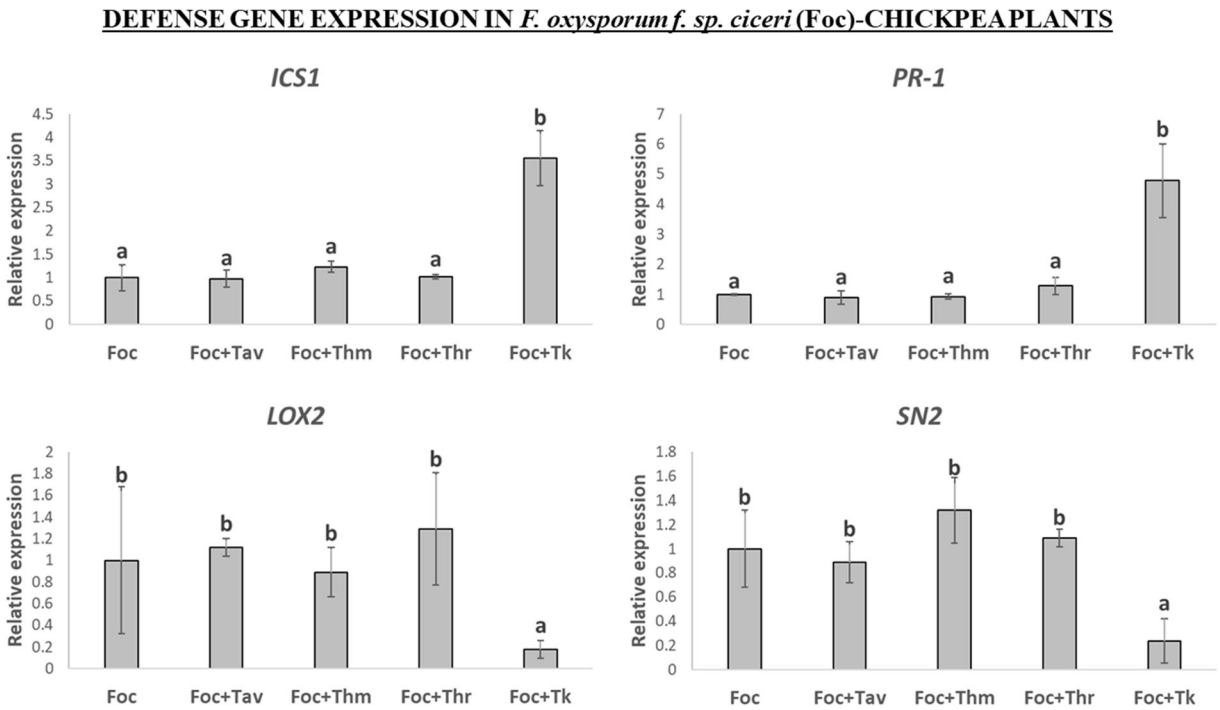

Fig. 7 Quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis of the expression of some defense genes in F-C roots infected with *F. oxysporum* f. sp. *ciceri* (Foc) and root inoculated with *T. atroviride* (+Tav), *T. hamatum* (+Thm), *T. harzianum* (+Thr) or *T. koningii* (+Tk). Genes of the isochorismate synthase 1 (*ICS1*), pathogenesis-related protein 1 (*PR-1*), lipoxygenase 2 (*LOX2*), and SNAKIN2 antimicrobial peptide precursor (*SN2*). Values correspond to relative measurements against plants without *Trichoderma*-roots inoculation ($2^{-\Delta\Delta Ct} = 1$). The chickpea *Actin* gene was used as an internal reference gene. Data are the mean of nine plants with the corresponding standard deviation. One-way analysis of variance (ANOVA) was performed, followed by the Tukey's test. Different letters represent significant differences ($P < 0.05$)

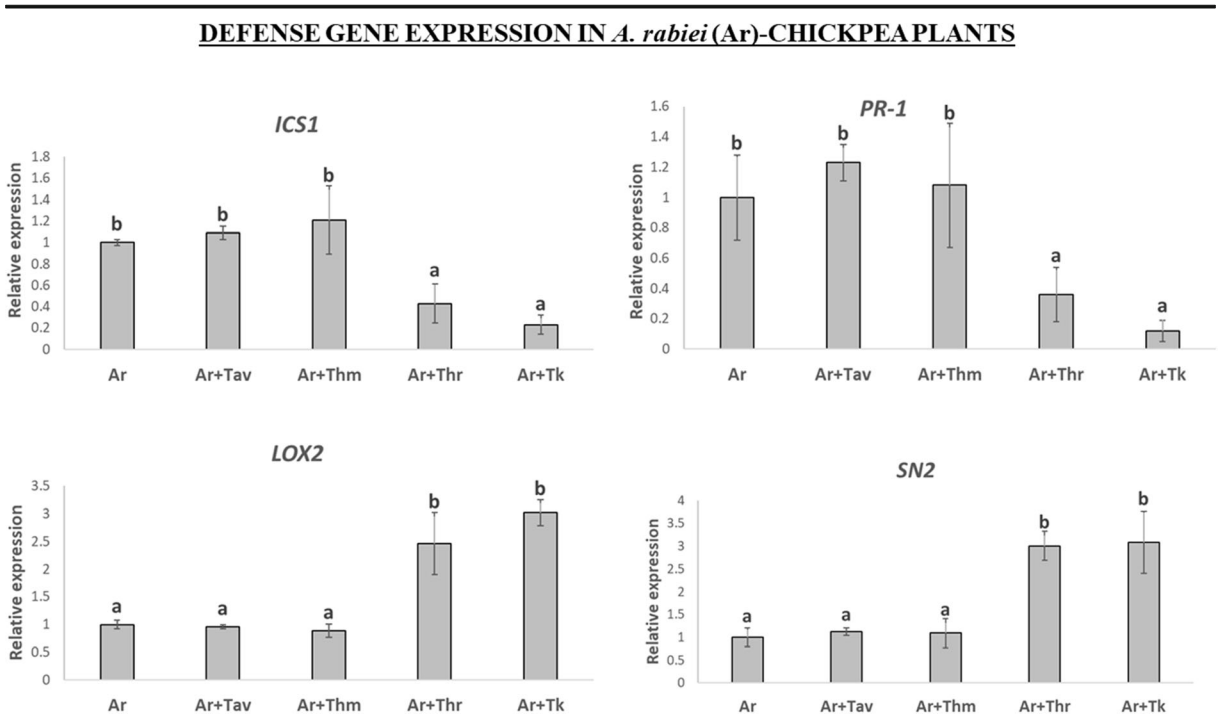

Fig. 8 Quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis of the expression of some defense genes in F-C leaves infected with *A. rabiei* (Ar) and root inoculated with *T. atroviride* (+Tav), *T. hamatum* (+Thm), *T. harzianum* (+Thr) or *T. koningii* (+Tk). Genes of the isochorismate synthase 1 (*ICS1*), pathogenesis-related protein 1 (*PR-1*), lipoxygenase 2 (*LOX2*), and SNAKIN2 antimicrobial peptide precursor (*SN2*). Values correspond to relative measurements against plants without *Trichoderma*-roots inoculation ($2^{-\Delta\Delta Ct} = 1$). The chickpea *Actin* gene was used as an internal reference gene. Data are the mean of nine plants with the corresponding standard deviation. One-way analysis of variance (ANOVA) was performed, followed by the Tukey's test. Different letters represent significant differences ($P < 0.05$)

space and nutrients in the rhizosphere (Tsahouridou and Thanassoulopoulos 2002). Despite this, no successful cases have been previously described regarding the control of *Fusarium* or *Ascochyta* with *T. koningii*.

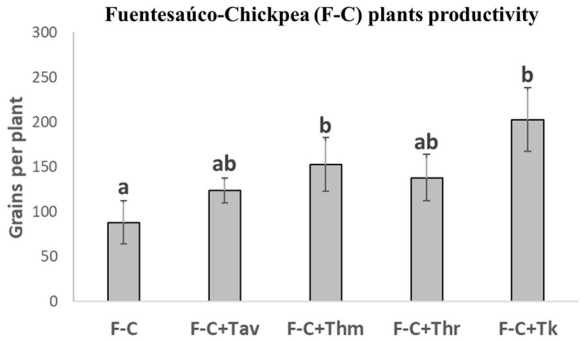

Fig. 9 F-C plants productivity (grains per plant) root inoculated with *T. atroviride* (+Tav), *T. hamatum* (+Thm), *T. harzianum* (+Thr) or *T. koningii* (+Tk). Data are the mean of twenty plants with the corresponding standard deviation. One-way analysis of variance (ANOVA) was performed, followed by the Tukey's test. Different letters represent significant differences ($P < 0.05$)

The results obtained *in planta* for *F. oxysporum* f. sp. *ciceri* showed how the application of the four *Trichoderma* species decreased the occurrence of disease caused by the pathogen at the plant biomass level, as well as the affectation of the vascular bundles, vitality, and stress. In the case of the application of the species *T. koningii*, this is a direct consequence of the decrease in the colonization of roots by the pathogen thanks to the antagonism already observed in vitro and the activation of the specific defenses of the plant at the root level. In the case of *T. atroviride*, a lesser affectation of plants was observed, but a specific defensive plant response was not measured, so it would be directly due to its ability to directly antagonize the pathogen. Regarding the differences observed in the root defensive response of F-C plants against the pathogen, we only report significant differences compared to plants that were not inoculated with *Trichoderma* for those that were inoculated with *T. koningii*. However, in the case of *T. harzianum*, results showing its ability to activate plant defense responses against pathogens, and even

against *F. oxysporum* f. sp. *ciceri* in chickpea plants (Sreeramulu et al. 2009), and contrary to those observed in our study. On the other hand, we verified that *T. koningii* is capable of activating plant defense responses mediated by SA, specifically against biotrophic pathogens such as *F. oxysporum* (Yang et al. 2015), an observation that has previously been identified in other plants such as corn against *F. moniliforme* thanks to the activation of plant defenses through recognition of the fungal compound 6-pentyl-α-pyrone (El-Hasan and Buchenauer 2009).

Regarding the results obtained *in planta* with the pathogen *A. rabiei*, it was reported that the application of different *Trichoderma* species was associated with a decrease in the occurrence of disease caused by the pathogen at the level of affectation as well as the vitality of the foliar tissues, and this was related to the lower level of plant tissue colonization by the pathogen. Following the application of *T. hamatum* and *T. atroviride*, an increase in the expression of plant defense genes at the systemic level was not observed, so the observed decrease in the occurrence of disease would have only been due to a strengthening of the plants thanks to the interaction with these *Trichoderma* species by other mechanisms, such as increased root development (Pelagio-Flores et al. 2017). The activation of systemic defensive responses associated with *T. harzianum* has also been shown in many other studies (Poveda et al. 2019b; Poveda 2021). In the case of *T. koningii*, the activation of plant responses had also been previously observed in maize plants against the pathogen *F. moniliforme* (El-Hasan and Buchenauer 2009). In our study, we verified the activation of a specific defensive response mediated by JA by both fungal species, and this is the most effective response against necrotrophic pathogens such as *A. rabiei* (Antico et al. 2012).

Regarding the productivity of F-C plants, it was possible to verify that the application of *T. hamatum* and *T. koningii* significantly increased the formation of grains per plant. This would have been a consequence of *Trichoderma*'s mechanisms for promoting plant growth and productivity. Similar results have been observed with *T. hamatum* in *Theobroma cacao* plants (Bae et al. 2009) and with *T. koningii* in *Diffenbachia* sp. (Cutler et al. 1989), the present work being the first to describe this process in chickpea plants.

T. koningii is the best choice for preventing diseases caused by *F. oxysporum* f. sp. *ciceri* and *A. rabiei* F-C. *T. hamatum*, *T. atroviride* and *T. harzianum* are also associated with reductions in disease or increases in the productivity of plants, but only *T. koningii* is associated with both an increase in productivity and a decrease in both diseases through direct antagonism and through the activation of SA- and JA-mediated defense plant responses against *F. oxysporum* f. sp. *ciceri* and *A. rabiei*, respectively. Despite having obtained these results using soil from Fuentesaúco under greenhouse conditions, the application and efficiency of the treatment in field conditions can give very different results due to the effects of other variables, such as the microbiota already present in the soil and adverse climatic conditions. Therefore, field assays with *T. koningii* and F-C should be carried out, and the effectiveness of the use of this method in the local agricultural system should be determined.

Author contributions JP is the Principal Researcher of the research project. JP thought and designed the work. JP performed the assays and analyzed the results. JP wrote the manuscript and made the corresponding revisions.

Funding This work was supported by the Instituto de Estudios Zamoranos "Florián de Ocampo" (IEZFO) as part of the XXVII Call for Research Grants in 2019.

Data availability Not applicable.

Code availability Not applicable.

Declarations

Research involving human participants and/or animals Not applicable.

Informed consent Not applicable.

Disclosure of potential conflicts of interest The author declare that he has no conflict of interest.

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