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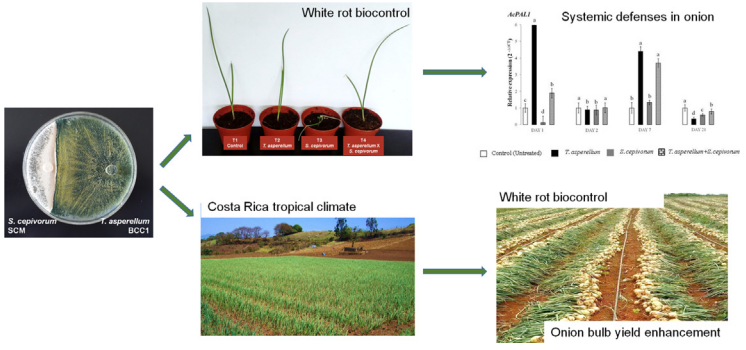
Trichoderma asperellum biocontrol activity and induction of systemic defenses against *Sclerotium cepivorum* in onion plants under tropical climate conditions

William Rivera-Méndez[a,b], Miguel Obregón[c], María E. Morán-Diez[b], Rosa Hermosa[b], Enrique Monte[b,*]

[a] Costa Rica Institute of Technology (ITCR), Calle 15, Avenida 14, Cartago, Costa Rica
[b] Spanish-Portuguese Institute for Agricultural Research (CIALE), Department of Microbiology and Genetics, University of Salamanca, C) Duero 12, 37185 Salamanca, Spain
[c] Laboratorios Dr. Obregón, 350m Oeste del Hospital nuevo de Heredia, Heredia, Costa Rica

GRAPHICAL ABSTRACT



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ABSTRACT

The biocontrol potential of three native Costa Rican *Trichoderma asperellum* strains has been evaluated against the necrotrophic ascomycete *Sclerotium cepivorum*, the causal agent of onion (*Allium cepa* L.) white rot. In Costa Rica, where climatic conditions enhance the development of this pathogen, white rot reduces onion yields up to 50% of total harvest. In our study, the three *T. asperellum* strains tested in *in vitro* assays showed their capacity to antagonize *S. cepivorum*, with BCC1 displaying percentages of colony growth inhibition of the pathogen of 81 and 90% in dual culture and cellophane membrane assays, respectively. In addition, this *Trichoderma* strain was able to reduce a 74% the plant mortality compared to untreated plants under greenhouse conditions. In field trials, carried out in two consecutive harvest years and in two different tropical locations, the two tested dosages of *T. asperellum* BCC1 reduced the incidence of white rot in a 3.41% for the lowest dose and 3.61% for the highest dose when compared to onion plants treated with chemical fungicides. Additionally, a significantly increase of 20.4% in onion bulb yield was recorded for the highest dose of BCC1. The potential of *T. asperellum* BCC1 to induce systemic defenses in onion plants against *S. cepivorum* was evaluated for four onion defense marker genes in a 21-day time course study by quantitative real-time PCR and using onion plants grown under greenhouse

* Corresponding author at: Spanish-Portuguese Institute for Agricultural Research (CIALE), Department of Microbiology and Genetics, University of Salamanca, C) Duero 12, 37185 Salamanca, Spain.
E-mail addresses: wirivera@itcr.ac.cr (W. Rivera-Méndez), m.obregon@doctor-obregon.com (M. Obregón), me.morandiez@usal.es (M.E. Morán-Diez), rhp@usal.es (R. Hermosa), emv@usal.es (E. Monte).

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conditions. The expression profile of *AcPR1* and *AcPAL1*, characterized by being undulating, indicates an initial activation of salicylic acid-dependent defense pathways (1 and 7 days) by *T. asperellum* BCC1 when either applied alone or in combination with the pathogen, while the up-regulation of *AcEIN3* observed in those same treatments at day 21 revealed the activation of ethylene-dependent defense pathways by this *Trichoderma* strain. *T. asperellum* BCC1 exerts efficient biocontrol against *S. cepivorum* and activates onion systemic defenses against this pathogen under greenhouse conditions, while it reduces onion white rot incidence and increases crop yield in field trials performed under Costa Rica's tropical climate conditions.

1. Introduction

Onion (*Allium cepa* L.) is a profitable vegetable crop grown for the appreciated attributes of its bulbs associated to flavor and health benefits. Onion is also considered among the most consumed vegetables worldwide, being exceed only by tomatoes (Havey, 2018). Its global area harvested reached more than 5 million ha and a production of near 100 million tons in 2017 (FAO, 2017), with a market estimated in 18 billion U.S. dollars in 2014 (Havey, 2018). In terms of cultivable land, Costa Rica's tropical climate allows for the successfully cultivation of different onion varieties. Most of the production in this country is concentrated in the northern regions of the province of Cartago at altitudes higher than 1700 m in a tropical mountain ecosystem, and onion is considered a valuable commodity for the local markets with a total production of 33,000 tons per year (Serrano and Morales, 2017).

The soil-borne ascomycete fungus *Sclerotium cepivorum* Berk (teleomorph: *Stromatinia cepivora*) is an economically important necrotroph pathogen that causes the white rot disease of several *Allium* species, this being a limiting factor to the onion production worldwide (Hanci, 2018; Havey, 2018; Hovius and McDonald, 2002). *S. cepivorum* has white mycelium without conidial development, but in absence of a host plant it persists as a survival structure, called sclerotia, which can survive in the soil for up to 20 years (Coley-Smith et al., 1990). These compact masses of black-hardened mycelia, with a spherical shape and range in size from 200 to 500 μm in diameter, are the primary source of infection of the pathogen (Elsherbiny et al., 2015). *Allium* root exudates play an active role in the release of volatile organic compounds which stimulate germination of the sclerotia (Coley-Smith and King, 1969). Once the germination occurs hyphae grow through the soil until encountering the onion plant, penetrating the roots and destroying their radicular system. In Costa Rica, especially in the province of Cartago, with soils rich in organic matter and very stable environmental conditions throughout the year, the white rot is the most relevant disease and has negative effects on crop yield with losses reaching the 50% of expected production (Aguilar-Ulloa et al., 2016).

Control of white rot disease is complicated as it has to face a number of biological and environmental challenges. The use of plant varieties with genetic resistance to the pathogen would be the preferred chosen method of controlling this disease, though these onion germplasms are not widely deployed in commercial cultivars (Havey, 2018). Additionally, the use of agrochemicals has been poorly efficient, due to the accumulated resistance by the broad application of a limited number of registered molecules, which has favored the use of other methods like the introduction of biological control agents, especially *Trichoderma* strains (McLean et al., 2012).

Biological control mechanisms displayed by *Trichoderma* species have been studied for decades (Harman et al., 2004; Lorito et al., 2010). These non-pathogenic soil-borne microorganisms have the ability to antagonize and mycoparasitize fungal pathogens, to colonize the plant radicular system and to induce plant defense responses (Hermosa et al., 2012). Phytohormone-mediated defenses are known as systemic acquired resistance (SAR) and induced systemic resistance (ISR) (Pieterse et al., 2009). As plant beneficial fungi, *Trichoderma* spp. are known by their capability to trigger ISR responses based on jasmonates (JA) and ethylene (ET)-dependent pathways (Hermosa et al., 2012). It has been also reported that some *Trichoderma* species trigger plant defenses mediated by salicylic acid (SA) (Martínez-Medina et al., 2013; Morán-Diez et al., 2009; Salas-Marina et al., 2011; Tucci et al., 2011). In early works, Metcalf et al. (2004) tested *Trichoderma koningii* as an antagonist to protect onion roots, and Clarkson et al. (2004) working with *Trichoderma viride* and *Trichoderma pseudokoningii,* achieved 80% of sclerotia parasitization. More recently Hussain et al. (2017) reported the use of *T. viride, Trichoderma hamatum* and *Trichoderma harzianum* to control white rot in onion plants with an efficiency that was close to 75% under greenhouse conditions. However, studies focusing on the mechanisms underlying the induction of onion defense responses mediated by *Trichoderma asperellum* against *S. cepivorum* in onion plants are not available. Furthermore, there are not evidences of the effect of *T. asperellum* on the control of onion white rot and crop yield in field trials in tropical agro-ecosystems.

The aim of this study was to evaluate the biocontrol potential of *T. asperellum* strains against *S. cepivorum* in onion plants. To achieve this goal, *in vitro* antagonism assays served to select the most promising *T. asperellum* strain, which was further tested in *in vivo* greenhouse assays, in terms of plant fitness, biocontrol of *S. cepivorum* and activation of defenses against this pathogen, and in field trials under Costa Rica's tropical climate conditions, in terms of biocontrol efficiency and crop yield. To our knowledge, neither biocontrol of white rot in onion bulb data nor studies with onion-defense marker genes have been reported under this environmental scenery what makes of this research study a significant contribution to this field.

2. Materials and methods

2.1. Fungi and onion seeds

Strains BCC1, BCF2 and BCF7 of *Trichoderma asperellum*, isolated from onion roots in production fields from the district of Llano Grande (Cartago, Costa Rica), were used as the antagonistic fungi. *Sclerotium cepivorum* SCM strain, previously characterized as a highly virulent pathogen to onion plants, was used as target in our experiments. Fungal strains were routinely grown on potato dextrose agar (PDA) (Difco, Franklin Lakes, NJ, USA) on Petri dishes for 8–14 days at 22–25 °C in the dark. *T. asperellum* strains were preserved in 30% glycerol conidia suspension at −80 °C, and *S. sclerotiorum* SCM strain was stored as PDA plugs suspended in sterile water at 4 °C. Fungi are deposited in the culture collection at the Biocontrol Laboratory in the Costa Rica Institute of Technology (ITCR) of Cartago.

Onion seeds (*Allium cepa* "Alvara") (Bejo Eurosemillas, Costa Rica) were surface-sterilized by vigorous sequential shaking in 70% ethanol and 2% sodium hypochlorite solutions for 10 min each, then washed thoroughly four times in sterile distilled water, and air-dried on a sterile gauze sheet. This onion variety was used throughout this study.

2.2. Fungal growth inhibition assays

Confrontation assays (dual cultures) between *T. asperellum* strains BCC1, BCF2 and BCF7, and *S. cepivorum* SCM strain were performed as previously described (Rubio et al., 2009) with some modifications. Briefly, a 5 mm-diameter agar plug colonized by *S. cepivorum* was grown on Petri dish at 23 °C in the dark for 3 days before placing a 5 mm-diameter agar plug colonized by a *T. asperellum* strain at 2 cm

from the border on the opposite side of the plate. Cultures of the SCM strain growing alone were used as controls. After 12 days of dual culture, the mycelial growth diameter of the pathogen was measured and the percentage of fungal inhibition (FI) was calculated according to Royce and Ries (1978). FI (%) = RGI × 100; RGI = (C-T)/C; where T is the average diameter of mycelial growth in the presence of a *T. asperellum* strain, and C is the average diameter of the mycelial growth in the control plates. The assays were performed in triplicate.

Membrane antifungal assays on cellophane sheets were carried out on PDA medium and in triplicate as previously described (Rubio et al., 2009). The diameters of the fungal colonies were measured after 5 days of incubation at 23 °C in the dark. Results were expressed as the growth inhibition percentage of *S. cepivorum* SCM strain by each *T. asperellum* strain tested, BCC1, BCF2 and BCF7, with respect to the mean colony diameters of the SCM strain grown alone.

2.3. In-planta assays

2.3.1. Plant growth promotion by T. asperellum

An *in vitro* assay was carried out to analyze the effect of strains BCC1, BCF2 and BCF7 of *T. asperellum* on onion seedlings growth. Onion seeds, previously surface-sterilized, were grown in Murashige and Skoog (MS) medium (Duchefa Biochemie, Haarlem, The Netherlands), supplemented with 1% sucrose and 0.8% agar (pH 5.7), for 3 days. Each biological replicate included one plate with four seeds per tested strain. Conidia from 7-day-old PDA plates were harvested by adding 5 mL of water to the plates and scraping the culture with a rubber spatula. These suspensions were filtered through a double layer of cheesecloth to separate large mycelial fragments from conidia. Conidia concentrations were calculated using a counting chamber and suspensions were used to inoculate the MS medium. An inoculum containing 1×10^6 conidia of each *Trichoderma* strain was placed at the opposite side of the plates containing 3-day-old onion seedlings. Plates were cultured in a growth chamber under conditions of 40% humidity, 24 °C, and a 16-h light/8-h dark photoperiod. Plates containing only onion seedlings, without *Trichoderma* conidia, and grown under similar conditions were used as control. Experiments were performed in triplicate, and measurements of onion seedlings roots and aerial sections were recorded 5 days after *Trichoderma* inoculation.

2.3.2. Biocontrol assays under greenhouse conditions

Biocontrol tests were carried out with *T. asperellum* BCC1 and *S. cepivorum* SCM in onion plants under greenhouse conditions. Two-hundred onion plants were grown in a mixture of peat:soil (3:1) for 50 days before being transplanted and used in this assay. The plants were planted individually in pots of 0.7 L capacity using a sterile mixture of peat:soil (3:1) supplemented with smart-release fertilizer (Osmocote®, dosage 4 kg/m^3 of substrate) (Agrotico, Cartago, Costa Rica). Four different treatments (T1 to T4) were considered, using 50 onion plants per treatment, in a completely randomized test: T1, control (untreated); T2, plants treated with *T. asperellum* BCC1; T3, plants infested with *S. cepivorum* SCM; and T4, plants treated with both fungal strains. For treatments T2 and T4, 10 mL of a conidial suspension of *T. asperellum* BCC1, with a concentration of 1×10^7 conidia/mL, was applied to the roots of 50-days-old onion plants. For treatments T3 and T4, 10 g of sand containing 100 *S. cepivorum* sclerotia/g were mixed with the sterile mixture peat:soil (3:1) per pot, and 57-days-old onion plants were transplanted. Thus, plants of T4 treatment were infested with the pathogen one week after *Trichoderma* application, and similar infection timing was followed for plants in T3, except no *T. asperellum* was applied to them before. The plants were maintained in a greenhouse at 23 ± 4 °C, and watered as needed. Fifteen days post-inoculation (dpi) with the pathogen, root and aerial part lengths as well as their respective dry weights were recorded for 10 plants in each treatment. Mortality was evaluated 21 dpi in 20 plants per treatment. For expression levels of defense marker genes, the central leaf of five onion plants was collected from each treatment and each considered time (1, 2, 7, and 21 days), and immediately frozen for further experiments of real-time quantitative PCR (RT-qPCR) and phenylalanine ammonia-lyase (PAL) activity measurements. The greenhouse assay was repeated twice.

2.3.3. Biocontrol assays under field conditions

Experimental plots were established in two farms (referred to here as “Tierra Blanca“ and ”Llano Grande“) in the province of Cartago in Costa Rica, under the climatic conditions of the tropical mountain. “Tierra Blanca” soil was characterized as silt loam (pH 5.9) while “Llano Grande” was clay loam with a pH of 5.7. The experiments were carried out in the rainy season of years 2017 and 2018. Onion plants mortality caused by *S. cepivorum* and total yield were evaluated under these conditions. The initial inoculum level of *S. cepivorum* was established according to the method of Papavizas (1972). Briefly, 50 g of soil were mixed with 200 mL of distilled water in a blender at 2000 rpm and sieved through 20-mesh and 80-mesh sieves. The residue on the 80-mesh sieve was washed with running tap water for 15 min and later dried in an oven for 1 h at 50 °C. Sclerotia were observed under the light microscopy and their number was expressed as sclerotia per g of soil. Fifty-days-old onion seedlings, grown in a mixture of peat:soil (3:1) under greenhouse conditions as described above, were used in these field assays. Three treatments were considered: T1, control; T2 and T3, *T. asperellum* used at two different concentrations. For control treatment (T1), onion plants were sprayed with a mixture (1:1 ratio) of the fungicides carboxin and captan (Vitamax 400®, Arysta Lifesciences, Saltillo, Mexico) in the field at the initial stage of the experiment, and with the fungicide tebuconazole (Folicur 25 EC®, Bayer CropScience, San José, Costa Rica) after 30, 45, 60, and 75 days of planting. Carboxin-captan was applied at a rate of 0.25% (w/v) of the product per ha of soil, and tebuconazole was used as 0.12% (v/v) of the product per ha of soil. Treatment T2, onion plants treated by root immersion in 4 L of a conidial suspension (1×10^6 conidia/mL) of *T. asperellum* BCC1 for 5 min, then planted in the field soil, and later an additional sprayed of 4 mL of 1×10^6 BCC1 conidia/mL were applied per plant at 30, 45, 60, and 75 days after planting; for T3, onion plants were treated similarly to those of T2 treatment but instead a concentration of 1×10^7 conidia/mL of BCC1 was used. Each experimental unit was a 1.1×1.1 m^2 with a density of 100 plants/m^2. For each treatment, 10 repetitions were made with 100 plants in each repetition. In addition to the treatments applied, each unit received the same general care with applications of granular NPK fertilizer of 10-30-10 formula at 30 days, of 20-20-20 at 60 days, and of 12-11-17 at 90 days. In addition, a solution of 400 mL/m^3 of calcium nitrate (20 g of active compound per kg of commercial product) (AugeCalcio 18®, AgroPro Centroamérica, San José, Costa Rica) was sprayed on the leaves, and 4 mL of an acaricide (Akaramic 1.8 EC®, based on abamectin B1a and B1b) (0.25% v/v) (Rotam CropSciences, Hong Kong, China) were applied per plant 70 days after planting. Total mortality and final yield were recorded at the last stage of the experiment, 5 months after initial planting.

2.4. Real-time quantitative PCR (RT-qPCR)

Expression analyses were performed with onion plant leaves sampled from the greenhouse assay described above. The central leaf of five plants was separately collected for each treatment at 1, 2, 7 and 21 dpi and considered as biological replicate. Total RNA was extracted using the GenElute Universal Total RNA Purification Kit® (Sigma-Aldrich, St. Louis, MO, USA) and then used to synthetize cDNA with the ReadyScript cDNA Synthesis Mix® (Sigma-Aldrich) following manufacturés recommendations. The PCR reaction was performed on a Light Cycler 480® (Roche Molecular Diagnostics, Pleasanton, CA, USA) and using the Light Cycler 480 SYBR Green I Master® (Roche Diagnostics GmbH, Mannheim, Germany). Reaction mixtures and amplification conditions were carried out as previously described

(Montero-Barrientos et al., 2011). The reaction was carried out in a total volume of 10 µL and three technical replicates for each tested condition. The Ct values were normalized with the values of the onion actin gene (*AcACT*) and the relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak, 2008). Marker genes of SA-, JA-, and ET-dependent defense pathways: *AcPR1* and *AcPAL1*, *AcLOX1*, and *AcEIN3* respectively were analyzed. The sequence of primers used in this study and the GenBank access numbers of each gene are listed in the Supplementary Material Table S1. The availability of any genomic database for *Allium* is limited to the Onion Genomic Database (Shukla et al., 2016) and was used as a source of references for these genes. The Primer3Plus® software (Free Software Foundation Inc., Boston, MA, USA) and the OligoAnalyzer 3.1 (Integrated DNA Technologies, Skokie, IL, USA) were used to design and test *in silico* the sequences.

2.5. Determination of L-phenylalanine ammonia-lyase (PAL) activity

The central leaf from five plants of the greenhouse assay per treatment and sampling time was processed as described above and used to determine the PAL activity. The plant material was homogenized in 3 mL of 0.1 M trisodium borate buffer (pH 8.5) containing 1.4 mM 2-mercaptoethanol and 0.1 g of insoluble polyvinylpyrrolidone. The extract was filtered through cheesecloth and centrifugation was carried out at full speed for 15 min. The determination of PAL activity was analyzed as the rate of conversion of L-phenylalanine into *trans*-cinnamic acid at 270 nm in a spectrophotometer. A 200-µL aliquot of the sample was added to 400 µL of borate buffer and 200 µL of 40 mM L-phenylalanine, and the reaction mixture was incubated at 37 °C for 15 min. Reaction was stopped with an equal volume of 10% (w/v) of trichloroacetic acid (TCA), and samples were centrifuged at full speed for 15 min. The supernatants were used to measure the absorbance at 270 nm, and *trans*-cinnamic acid was calculated using a standard curve according to Lee et al. (2015). Results are presented as µmol/min (U) per g of fresh tissue.

2.6. Statistical analyses

The data were analyzed using Minitab 18 Statistical Software (Minitab, Inc., State College, PA, USA). Normality and homoscedasticity were evaluated. All data were analyzed with ANOVA followed by Fisher test ($P < 0.05$), with the exception of gene expression levels that were analyzed by ANOVA and Tukey test ($P < 0.05$). Particularly, data recorded from field assays were analyzed using a generalized linear model (GLM) with a significance of 5% and a Fisher comparison.

3. Results

3.1. Antagonism of T. asperellum strains against S. cepivorum

In both dual culture and cellophane membrane tests the three *T. asperellum* strains reduced growth of *S. cepivorum*. Significant differences in percentages in FI of *S. cepivorum* were observed for the three different strains of *T. asperellum* evaluated (Table 1). In the dual culture antagonism test, the strains BCC1 and BCF7 showed the highest FI while strain BCF2 gave a significantly lower value. The antagonistic capacity of *Trichoderma* strains was also analyzed in cellophane assays that allowed to determine the effect of hydrolytic enzymes and metabolites secreted to the culture medium. The strain BCC1 showed a significant highest FI percentage, compared to the other two strains, BCF2 and BCF7, which did not present significant differences.

3.2. Effect of T. asperellum strains on onion seedlings growth

The *T. asperellum* effect on the growth of 3-days-old onion seedlings was evaluated in *in vitro* tests (Table 2). Onion roots treated with *Trichoderma* were significantly shorter than those of the control treatment, but there were not differences among the strains. Regarding the stem length, there were no differences between treatments, including the control condition.

3.3. Biocontrol of onion white rot by T. asperellum BCC1 under greenhouse conditions

The capacity of *T. asperellum* BCC1 to control onion white rot was evaluated in 50-days-old onion plants. The root length and dry weight, stem length, canopy dry weight and the mortality were evaluated in a greenhouse assay (Table 3). The analysis of root and stem growth parameters, evaluated 15 dpi with the pathogen, revealed that onion plants infested with *S. cepivorum* alone displayed the lowest values differing significantly from the other three treatments, while those corresponding to the control and *T. asperellum* + *S. cepivorum* treatments showed the highest for the four growing traits measured. Onion plants treated solely with BCC1 showed values of stem and root lengths smaller than those of the control, though no significant differences were observed for the corresponding dry weights. Mortality percentages were evaluated on 20 onion plants for each treatment 21 dpi with *S. cepivorum*. As expected, control and *T. asperellum* treatments presented 100% healthy plants indicating that this *Trichoderma* strain did not cause damage to any plant. Moreover, onion plants treated with BCC1 showed a positive effect on the onion protection against *S. cepivorum* as the ratio of mortality observed in onion plants treated with both fungal strains was 14% compared to the 88% of mortality recorded in plants solely infected with *S. cepivorum*.

3.4. Biocontrol of onion white rot by T. asperellum BCC1 under field conditions

The initial inoculum determined for “Tierra Blanca” farmland was 0.020 sclerotia/g of soil and for “Llano Grande” farmland it was 0.025 sclerotia/g of soil. Based on these values both soils were considered homogeneous and suitable for trials.

The field tests showed the effects of two doses of *T. asperellum* BCC1 on onion white rot biocontrol compared to those of agrochemicals commonly applied by farmers which were used as control (Tables 4 and 5). For mortality analysis (Table 4), the GLM test determined that only the type of treatment had a significant effect on this parameter. The comparison test (Fisher method) determined that there were no differences between the data from the two farms considered ($P < 0.05$), but there were differences among the treatments. The control condition presented the highest plant mortality (4.59 ± 0.39 plants/m^2) which is equivalent to 45,900 plants/ha or 4.59% of mortality. Onion plants treated with the lowest concentration of spores of BCC1 (treatment T2) showed the lowest mortality ratio (3.41%) while a higher dose increased the plant mortality (3.61%). For yield analysis (Table 5), the GLM test determined that both the year ($P = 0.03$) and the treatment ($P = 0.00$) had a significant effect on onion yield ($P < 0.05$). The lowest yield (2.45 ± 0.28/m^2, equivalent to 24.5 ton/ha) was obtained with the control (treatment T1). On the other hand, the highest dose of *T. asperellum* (treatment T3) produced the highest yield with

Table 1
Percentage of growth inhibition of *S. cepivorum* SCM by three *T. asperellum* strains (BCC1, BCF2, and BCF7) in dual culture (12 days) and cellophane membrane (5 days) assays. Values are means from three biological replicates ± standard deviations. Different letters indicate significant differences (Fisher test, $P < 0.05$).

T. asperellum	Dual culture	Cellophane membrane
BCC1	80.66 ± 2.76 a	89.86 ± 0.63 a
BCF2	69.39 ± 2.65b	83.70 ± 1.09b
BCF7	79.20 ± 1.09 a	81.52 ± 3.92b

Table 2
Effect of three *T. asperellum* strains (BCC1, BCF2, and BCF7) on the growth of 3-day-old onion seedlings. Measurements of root and stem lengths were recorded in 8-day-old seedlings, five days after *Trichoderma* treatments were applied. Values are means from three biological replicates ± standard deviations. Different letters indicate significant differences (Fisher test, $P < 0.05$).

Treatment	Root length (cm)	Stem length (cm)
Control	2.19 ± 0.64 a	2.78 ± 0.63 a
BCC1	1.56 ± 0.24b	2.66 ± 0.75 a
BCF2	1.54 ± 0.56b	2.82 ± 0.99 a
BCF7	1.76 ± 0.50b	3.06 ± 0.94 a

30.5 ton/ha, that is, an increase of 20.4% with respect to the control. The onion yield at the lowest dose of BCC1 (treatment T2) was also significantly higher than the control, with an average of 29.02 ton/ha and an increase of 18.4%.

3.5. Systemic defense triggered by *T. asperellum* BCC1 against *S. cepivorum* SCM in onion plants

The expression levels of defense marker genes in the central leaf of onion plants were analyzed for the four treatments (T1, T2, T3 and T4) compared in the greenhouse assay at four time points (1, 2, 7, and 21 dpi with the pathogen).

The expression of *AcPAL1* showed a different profile according to whether the plant was challenged by *T. asperellum* BCC1 or *S. cepivorum* (Fig. 1). This gene was strongly up-regulated by BCC1 at 1 and 7 dpi comparing to the control but it was down-regulated at day 2 and 21 showing an undulating expression pattern. The expression level of *AcPAL1* in treatment T2 was 49.7- and 3.3-fold higher than in plants receiving treatment T3 at days 1 and 7, respectively. When onion plants were treated with the pathogen, the expression levels of *AcPAL1* were significantly lower, compared to control treatment, at 1 (0.12-fold), 2 (0.87-fold) and 21 dpi (0.30-fold). The combination of biocontrol agent and pathogen caused a significant increase of expression of this gene when compared to plants treated solely with the pathogen at all times analyzed; however, the levels were significantly lower than those of plants treated with *T. asperellum* at day 1 (0.32-fold) but higher at 2 (1.13-fold) and 21 (2.32-fold) dpi.

AcPR1 also showed a different expression pattern based on the inoculated microbe. The highest levels of expression were observed at day 7 for those plants treated with either *T. asperellum* or the combination of biocontrol agent and pathogen (compared to control plants) (Fig. 1). The expression of *AcPR1* in treatment T3 was 0.33-fold (at day 1), 0.87-fold (at day 2) and 0.30-fold (at day 21) lower, where only *S. cepivorum* was applied to the plants, compared to untreated plants (T1), except at day 7 where no significant differences were observed. Though later in time than the expression observed for *AcPAL1*, *AcPR1* also showed an undulating expression pattern starting from day 2 for those samples treated only with BCC1.

Compared to control plants, *T. asperellum* caused a strong decrease in the expression of *AcLOX1* at day 1 (34.4-fold) (Fig. 1). Although this

Table 4
Generalized linear model for onioń s mortality in field experiments.

Main effect	d.f.	Probability*
Farm	1	0.06
Treatment	2	0.00*
Year	1	0.84
Farm*Treatment	2	0.76
Farm*Year	1	0.48
Treatment*Year	2	0.91
Farm*Treatment*Year	2	0.65
Error	108	
Terms for *Fisher* comparisons		Media (plants/m^2)
Treatment	T1 Control	4.59 ± 0.39 a
	T2	3.41 ± 0.34c
	T3	3.61 ± 0.34b
Model		
Mortality = 3.87 + 0.72 T1 Control − 0,46 T2 − 0,26 T3		

Data represents the average of the replicates (n = 10) ± standard deviations. Treatments tested were: T1 Control (untreated plants); T2 (plants treated with 1×10^6 conidia/mL of BCC1); and T3 (plants treated with 1×10^7 conidia/mL of BCC1).
*For each effect, asterisks denote significant differences ($P < 0.05$). Values followed by different letters are significantly different according to the Fisher test ($P < 0.05$).

Table 5
Generalized linear model for onioń s yield in field experiments.

Main effect	d.f.	Probability*
Farm	1	0.40
Treatment	2	0.00*
Year	1	0.03*
Farm*Treatment	2	1.00
Farm*Year	1	0.57
Treatment*Year	2	0.71
Farm*Treatment*Year	2	0.62
Error	108	
Terms for *Fisher* comparisons		Media (Kg/m^2)
Year	2017	2.86 ± 0.36 a
	2018	2.75 ± 0.37b
Treatment	T1 Control	2.45 ± 0.28c
	T2	2.90 ± 0.25b
	T3	3.05 ± 0.27 a
Model		
Yield = 2.80–0.35 T1 Control + 0.10 T2 + 0.25 T3 + 0.05 Year 2017–0.05 Year 2018		

Data represents the average of the replicates (n = 10) ± standard deviations. Treatments tested were: T1 Control (untreated plants); T2 (plants treated with 1×10^6 conidia/mL of BCC1); and T3 (plants treated with 1×10^6 conidia/mL of BCC1).
*For each effect, asterisks denote significant differences ($P < 0.05$). Values followed by different letters are significantly different according to the Fisher test ($P < 0.05$).

Table 3
Effect of *T. asperellum* BCC1 treatment on the fitness of onion plants and biocontrol against *S. cepivorum*.

Treatment	Root length (cm)	Root dry weight (g)	Stem length (cm)	Canopy dry weight (g)	Mortality percentage (%)
T1 Control	10.73 ± 1.61 a	0.10 ± 0.03 a	16.91 ± 1.78 a	0.17 ± 0.04 a	0
T2	9.18 ± 1.47b	0.12 ± 0.02 a	14.61 ± 1.85b	0.17 ± 0.02 a	0
T3	6.88 ± 0.99c	0.08 ± 0.01b	10.09 ± 2.34c	0.11 ± 0.03b	88
T4	9.32 ± 2.05 ab	0.11 ± 0.03 a	15.49 ± 1.73 ab	0.18 ± 0.03 a	14

Values are the mean of the replicates (n = 10, for growth parameters) and (n = 20, for mortality) with their corresponding standard deviation. Data were collected 15 dpi (for growth parameters) and 21 dpi (for mortality) with the pathogen. Treatments tested were: T1 control (untreated plants); T2 (plants treated with *T. asperellum* BCC1); T3 (plants infested with *S. cepivorum*); and T4 (plants treated with BCC1 and *S. cepivorum*).
Values followed by different letters are significantly different according to the Fisher test ($P < 0.05$).

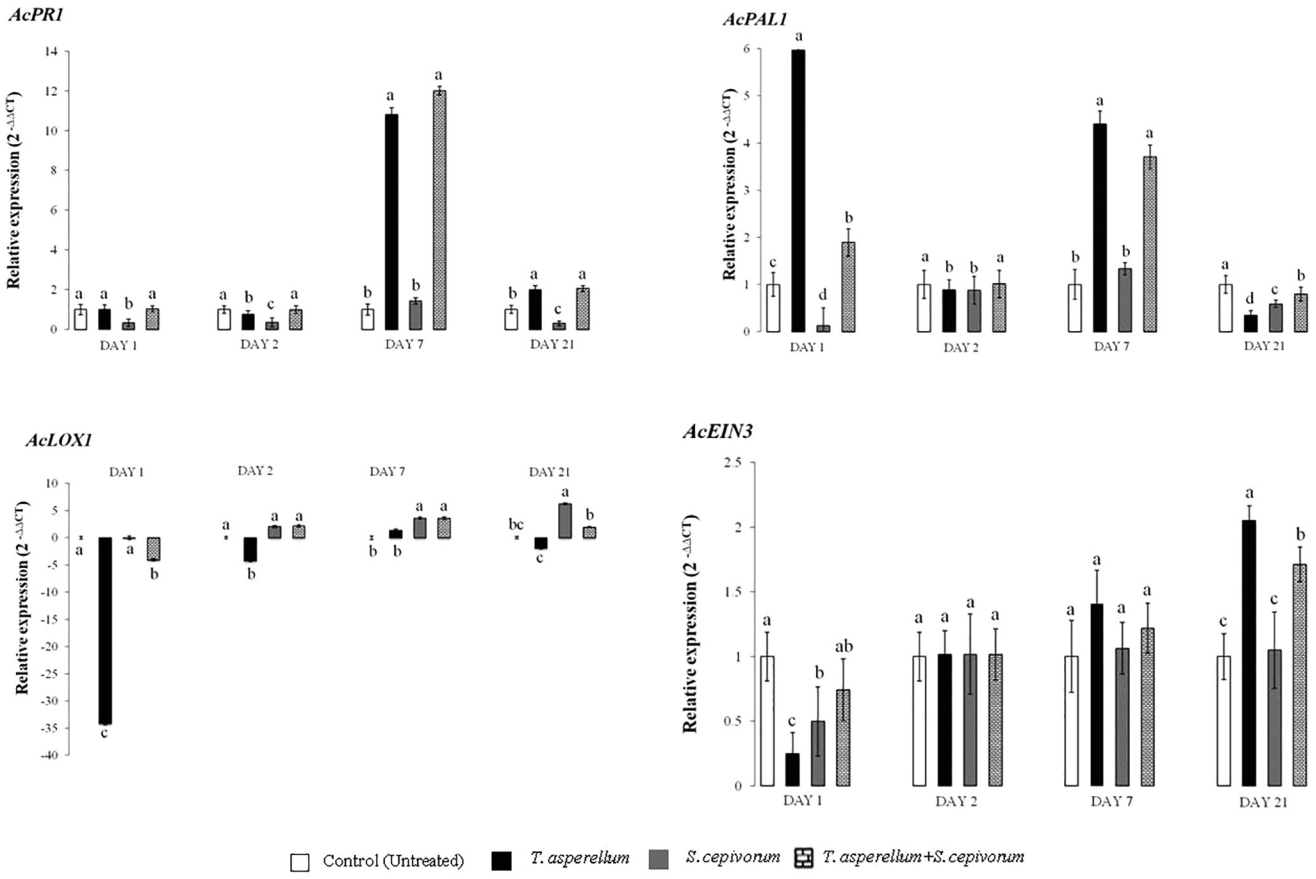

Fig. 1. Relative expression of *AcPR1*, *AcPAL1*, *AcLOX1*, and *AcEIN3* in onion plants treated with *T. asperellum*, *S. cepivorum* or the combination of both fungal strains (*T. asperellum* + *S. cepivorum*) in a 21-days time course study. Values correspond to relative measurements against the respective control condition (Untreated plants) ($2^{-\Delta\Delta CT} = 1$) for each time point. Error bars represent standard deviations for five biological replicates at each time point. The actin gene (*AcACT*) was used as a reference. Data was analyzed by one-way ANOVA and Tukey test. Different letters represent significant differences ($P < 0.05$).

down-regulation was moderately reduced from day 2, and the expression of this gene was never up-regulated at any of the other three time points analyzed. The expression of *AcLOX1* seems to be more induced by the pathogen, but only significantly up-regulation was detected at 7 (3.6-fold) and 21 (6.3-fold) dpi.

The expression levels of *AcEIN3* showed a continuing increment from day 1 to 21 in those plants treated with *T. asperellum* though only was down-regulated at 1 dpi (0.25-fold) and up-regulated at 21 dpi (2-fold) when compared to control plants. No significant differences were observed among treatments at day 2 and 7 and only combination of both fungal strains showed significant up-regulation of *AcEIN3* at 21 dpi when compared to control plants (1.7-fold).

The enzymatic PAL activity was calculated for all treatments (Table 6). Only data collected at day 2 showed no significant differences among treatments. The highest PAL activity was detected in plants from the *T. asperellum* treatment at 1 and 7 dpi (19.15 and 16.21 U/g, respectively), while plants infected with *S. cepivorum* showed the lowest activity (0.77 U/g and 6.40 U/g, respectively).

4. Discussion

The potential of *T. asperellum* BCC1 as effective biocontrol agent of *S. cepivorum* in onion plants was clearly proved in this study, as reductions in plant mortality due to white rot were observed in those plants treated with this strain in greenhouse and field experiments. We should point out here the fact that the three *T. asperellum* strains assessed in this work were isolated from onion roots in production fields from the district of Llano Grande in Cartago, Costa Rica (Alvarado-Marchena and Rivera-Méndez, 2016), where field experiments described in this study were taken place. This aspect should be considered a valuable asset for biocontrol applications as native isolates are better adapted to their local climate conditions and pathogenic targets than

Table 6
PAL activity (U/g of fresh tissue) measured in onion leaves in 4 treatments.

Treatment	*dpi 1	dpi 2	dpi 7	dpi 21
Control	4.68 ± 0.18c	4.50 ± 0.15 a	4.58 ± 0.26c	4.52 ± 0.12 a
T. asperellum	19.15 ± 0.12 a	4.42 ± 0.34 a	16.21 ± 0.54 a	1.61 ± 0.08c
S. cepivorum	0.77 ± 0.00 d	4.25 ± 0.09 a	6.40 ± 0.33 d	2.70 ± 0.20b
T. asperellum + *S. cepivorium*	9.03 ± 0.20b	4.76 ± 0.12 a	12.30 ± 0.94b	1.87 ± 0.07c

Values are the mean of the replicas with their corresponding standard deviation.
Values followed by different letters are significantly different according to the Fisher test ($P < 0.05$).
*Days post-inoculation.
Regression equation of trans-cinnamic acid' standard curve (y = 0.3836x − 0.6501).

foreign isolates (Debbi et al., 2018).

Based on the *in vitro* results, *T. asperellum* BCC1 was selected for biocontrol assays in onion plants in greenhouse and field trials. In fact, direct antagonism, to variable extent of performance among the three *T. asperellum* strains tested, was observed in both dual culture and cellophane membrane assays. In any case, BCC1 showed the best performance. The antagonism ability of *Trichoderma* species against *S. cepivorum* is well known (Hernández et al., 2011; Shalaby et al., 2013), but even though this pathogen is a main cause of important economic losses in onion cultivars, yet white rot control has not been fully explored with *Trichoderma* strains. Some studies describe strategies to biocontrol *S. cepivorum* in greenhouse or field trials in different climatic regions. Torrés-Barragán et al. (1996) inoculated onion plants with the arbuscular mycorrhizal consortium *Glomus* sp. Zac-19 achieving significant protection against *S. cepivorum* and 22% yield increment as compared with nonmycorrhizal controls in Mexico's field assays. Similarly, onion plants treated with *Rhizophagus irregularis* (formerly *Glomus intraradices*) showed less white rot incidence than those plants untreated in onion organic fields in Ontario region (Canada) (Jaime et al., 2008). Antagonist bacterial isolates have been also described to reduce white rot in onion plants compared with the untreated control in Egypt's field trials (Elshahawy et al., 2018). The use of *Trichoderma* species to biocontrol *S. cepivorum* in onion plants has been also described in field trials localized in Egypt, using *T. koningii* and *T. harzianum* (Shalaby et al., 2013), United Kingdom with *T. viride* (Clarkson et al., 2006; Coventry et al., 2006), or Australia with *T. koningii* (Metcalf et al., 2004). However, in countries like Costa Rica with soils rich in organic matter and where tropical climatic conditions easily promote the development of white rot, there are not relevant studies that can contribute to implement the use of *Trichoderma* spp. as biocontrol agents themselves or as a tool within the integrated management control of this onion disease.

Before field experiments were taken place, greenhouse assays were carried out with *T. asperellum* BCC1 to test its capabilities against *S. cepivorum* in onion plants. Several studies had shown the potential of *T. asperellum* to biocontrol pathogens with diverse life styles in different plants such as *Rhizoctonia solani* in cucumber (Trillas et al., 2006), *Pythium myriotylum* in cocoya (Mbarga et al., 2012), *Fusarium oxysporum* in tomato (Debbi et al., 2018), or *Verticillium dahliae* in olive cultivars (Carrero-Carrón et al., 2016). The amount of *S. cepivorum* sclerotia that was used as infective dose in the greenhouse assay, proved to be adequate for the outset of white rot development in onion seedlings because percentages of mortality of 88% were recorded in the treatment T3. As would be expected, onion plants infested with *S. cepivorum* (treatment T3) presented significantly less root and canopy dry biomass and smaller root and stem lengths. The mortality percentage obtained for the treatment T4 was 14%, showing the efficiency of strain BCC1 against *S. cepivorum*. Although lower root and stem length values were obtained when applying *T. asperellum* BCC1 to onion plants (treatment T2), no significant differences were observed in dry weight values between these plants and those from the control condition. It is important to point out the reduced root growth response of the onion seedlings inoculated with any of the three *T. asperellum* strains tested. In this respect, it is worth mentioning that the ability to promote plant growth is not a common characteristic extensible to all *Trichoderma* strains (Rubio et al., 2012). However, onion plants treated with the combination of strain BCC1 and *S. cepivorum* (treatment T4) did not show worse phenotypic parameters (root and stem lengths) compared with control plants but decreased the onion plants mortality compared to that from treatment T3. The absence of significant differences in plant fitness between treatment T4 and treatments T1 and T2 may be indicative that activation of plant defenses is occurring in response to the application of *T. asperellum* BCC1 alone. This outcome has been described earlier in *Trichoderma* (Alonso-Ramírez et al., 2014; Hermosa et al., 2013), and the activation of onion systemic defenses has been investigated in this study. In the greenhouse assay where environmental conditions were controlled, defense marker onion genes were analyzed in a 21-days time course study in order to know whether the reduction in onion plants mortality observed at 21 dpi with the pathogen in the treatment T4, mirrored in changes of gene expression levels.

Plant defense responses to the *T. asperullum* root inoculation has been described as ISR type (Shoresh et al., 2005), but an increasingly number of studies are reporting SA-dependent defense responses triggered by *Trichoderma* spp. (Salas-Marina et al., 2011; Tucci et al., 2011). Our data show an undulating expression pattern for *AcPAL1* and *AcPR1* genes, both SA-dependent defense markers, triggered by strain BCC1 when applied alone to onion plants, as observed in *Trichoderma parareesei*-tomato interactions (Rubio et al., 2014). At this point, as shown in Fig. 1, a down-regulation of JA/ET-dependent defense genes (*AcLOX1* and *AcEIN3*) was also observed at 1 day after application of strain BCC1 to onion plants. The lack of any relevant source of information regarding defenses regulation in onion plants against *S. cepivorum* leads us to speculate that the response might be JA/ET-dependent, similarly to that displayed against other necrotrophic pathogens (Glazebrook, 2005). In this sense, the expression of *AcLOX1* was up-regulated at 7 and 21 dpi while there was not any significant up-regulation of SA-dependent genes compared to control plants. The use of *S. cepivorum* sclerotia as inoculum could explain the delay in the JA-mediated systemic defense observed in plants at 1 dpi with the pathogen. As reported by Adams and Papavizas (1971), on autoclaved soil, hyphae emerge from the sclerotium within a period of about 1–2 days. Considering that *T. asperellum* was applied to onion plants 7 days before the pathogen in the combined treatment (T4), it is quite likely that there might be a SA-dependent priming based on the higher *AcPAL1* expression levels in this condition compared to what was observed in plants treated with BCC1 alone. Thus, at day 21 when onion plant mortality was recorded, the reduction observed in onion plants from the treatment T4 could be explained by the synergic activity of the direct antagonism of *T. asperellum* against *S. cepivorum* observed in the *in vitro* studies and the plant systemic defenses induced by the BCC1 strain. An additional observation that corroborates the expression pattern of *AcPAL1*, was the PAL enzymatic activity data (Table 6) which showed similar undulating behavior to those detected in the expression of this gene (Fig. 1). The transcription factor *EIN3* activates ET-responsive genes (Contreras-Cornejo et al., 2015). ET is a key factor in the regulation of root development processes, by inhibiting lateral root formation and elongation (Contreras-Cornejo et al., 2015; Hermosa et al., 2012). Then, the up-regulation of *AcEIN3* observed in onion plants for treatment T2 at 21 days, treated solely with *T. asperellum*, might explain the absence of growth promotion. However, when in combination with the pathogen, plant growth data were similar to those of the control plants as well, being the *AcEIN3* expression down-regulated in comparison to treatment T2 but up-regulated when compared to untreated plants.

Results from controlled environment assays like greenhouses, rarely translate to field evaluations due to fluctuating environmental conditions. However, assessment of *T. asperellum* BCC1 performance carried out in two independent experiments in two consecutive season trials, provided conclusive results in terms of usefulness of this strain for future commercial purposes. Our data indicate no differences in the "Farm" factor what lead us to conclude that soil features (pH and soil composition) are sufficiently similar to not be a key factor controlling *S. cepivorum* development or *T. asperellum* performance, and therefore onion bulb production. However, year of planting revealed to be a factor affecting yield, though not mortality, which may be related to climatic conditions (temperature or humidity), despite no relevant variations were recorded in the closest weather station to the farms (data not shown). There was, however, a clear dose-dependent effect related to the application of *T. asperellum* BCC1 in the field trials. When used to the highest concentration, white rot incidence and onion bulb yield parameters were improved, regardless of farm localization and harvesting seasonal variations. Likewise, higher concentrations of

conidia of *T. asperellum* seem to perform better against *R. solani* when applied to cucumber seedlings (Trillas et al., 2006). And in rice plants exposed to drought stress, increasing doses of *T. harzianum* applied to the roots enhanced plant drought tolerance (Pandey et al., 2016). In Costa Rica, the absence of tolerant or resistant onion cultivars to *S. cepivorum*, linked to its tropical climate and the high organic content typical in volcanic soils, facilitate fungal diseases. This fact has been consistently observed in previous results which indicate that, using a similar *S. cepivorum* inoculum without any chemical or biological treatment, the level of white rot disease reached 85–100% of total plants in less than 25 days (Del Milagro-Granados, 2005). This is the reason that led us to not include as a control in the field trials untreated plants. In our study, the highest white rot incidence was observed in treatment T1 where a mix of chemical fungicides was applied to onion plants, although the most significant result to be highlighted here is the fact that the white rot disease was reduced in plants inoculated with any of the *T. asperellum* BCC1 concentrations (treatments T2 and T3) which translated into increasing onion bulb yield for the later. These results seem to contradict other studies reporting the use of antagonistic bacteria (Elshahawy et al., 2018) or *T. viride* (Clarkson et al., 2006) to control white rot in onion field assays, and in which the use of these biocontrol agents, thought showing promising results, yet was less effective than chemical treatment. For decades, chemical control of white rot in onion plants in Costa Rica has had as a result in an increasingly number of *S. cepivorum* strains resistant to these treatments. Rather than being using higher doses of chemical pesticide to overcome the matter, as actually is established in the management of white rot, our results propose not only an environmental friendly alternative but an effective crop production method to be used by farmers in this region.

5. Conclusion

T. asperellum BCC1 application to onion seedlings before transplanting to soils infested with *S. cepivorum* sclerotia in Costa Rica under tropical climate, reduces white rot incidence and increases onion bulb yield. Our results might be considered a major breakthrough in this field as not only biocontrol is confirmed in greenhouse and field trials but evidences of activation of systemic defense by *T. asperellum* against *S. cepivorum* in onion plants based on the analysis of defense marker genes are presented here. These results indicate the potential for commercial *T. asperellum* BCC1 biocontrol products to drastically reduce the overuse of fungicides in Costa Rica's agriculture.

CRediT authorship contribution statement

William Rivera-Méndez: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Resources, Validation, Writing - original draft. **Miguel Obregón:** Conceptualization, Formal analysis, Investigation, Validation. **María E. Morán-Diez:** Data curation, Formal analysis, Investigation, Supervision, Validation, Writing - original draft, Writing - review & editing. **Rosa Hermosa:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Writing - original draft, Writing - review & editing. **Enrique Monte:** Conceptualization, Funding acquisition, Project administration, Resources, Writing - original draft.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biocontrol.2019.104145.

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