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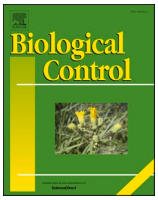

Effective biological control of chickpea rabies (*Ascochyta rabiei*) through systemic phytochemical defenses activation by *Trichoderma* roots colonization: From strain characterization to seed coating

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HIGHLIGHTS

- Chickpea rabies is a threat to the sustainability of global chickpea farming.
- *T. harzianum* is an effective direct/indirect biocontrol agent against *A. rabiei*.
- Gum arabic is the best coating to apply *T. harzianum* conidia on chickpea seeds.
- This *T. harzianum* coating induces effective systemic resistance against *A. rabiei*.
- The systemic resistance is ET- and Mel-mediated, causing nicotinic acid synthesis.

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ABSTRACT

Chickpea (*Cicer arietinum*) is a legume of great economic and agricultural importance worldwide, whose crop is severely affected by rust or Ascochyta blight, caused by the fungus *Ascochyta rabiei*. The fungal genus *Trichoderma* includes several species widely characterized as effective biological control agents against crop pathogens. First, this work characterized several species of the genus *Trichoderma* as potential biological control agents of *A. rabiei* directly (*in vitro* confrontation) or indirectly in chickpea plants (activation of systemic resistance), selecting *T. harzianum* EN1 as the most efficient strain. Subsequently, different materials were tested as coatings to apply the *T. harzianum* conidia on chickpea seeds, determining that gum arabic at 1 % concentration was the one that most promoted the germination of conidia and seeds. The third phase of the study was based on the application of the coating and *T. harzianum* conidia on chickpea seeds and to study plant survival after infection with the pathogen *A. rabiei*, characterizing root colonization by *Trichoderma* and systemic hormonal and metabolic changes related to the induction of systemic defenses. The treatment of chickpea seeds with gum arabic and *T. harzianum* conidia was found to increase *Trichoderma*-root colonization and to improve plant survival. The induction of systemic ethylene- and melatonin-mediated resistance, which led to the accumulation of nicotinic acid in plant tissues, was considered the cause of such a protection. Therefore, *T. harzianum* applied as seed coating with gum arabic could be a good biological control strategy against *A. rabiei* on chickpea, due to the induction of systemic resistance.

1. Introduction

Chickpea (*Cicer arietinum*) is a legume of great economic and agricultural importance worldwide, representing the second most cultivated legume crop in the world, only behind common bean (*Phaseolus vulgaris*) (Singh et al., 2021). In 2022, the total world harvested area of chickpea was 14.8 million hectares, producing 18.1 million tons. The most productive countries were India and Australia, with 13.5 and 1.1 million

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tons, respectively (FAOSTAT, 2024). Regarding their nutritional importance, chickpeas are a high-quality food with high protein, vitamins, fiber and minerals, providing important benefits to livestock and human health (Wang et al., 2021). In addition to the direct economic and food benefits of chickpea, its cultivation improves agricultural systems through biological nitrogen fixation (Singh et al., 2021). According to the European Union Council Regulation (EC) No 510/2006, the Fuentesaúco Chickpea (FC) ("Garbanzo de Fuentesaúco", in Spanish), which was granted Protected Geographical Indication in 1996, is produced in the southeast of the province of Zamora (Castilla y León, Spain). Although it is not a highly productive legume, it has an extraordinary culinary quality due to its large size, texture and flavor, being declared as one of the most recognized leguminous products in terms of quality (EC 510/2006).

One of the most widespread diseases worldwide in the chickpea crop is called rabies or Ascochyta blight, caused by the fungus *Ascochyta rabiei* (telomorph *Didymella rabiei*). This pathogen attacks the aerial part of chickpea (leaf, petiole, stem, pods and seeds). Symptoms are characterized by the appearance of necrotic spots with black concentric circles on leaves and pods, with more oblong lesions on stems. The appearance of the pathogen in pods leads to seed infection and dispersal to the next generation. However, the most damaging lesion is on stems, as it can girl and kill plants (Singh et al., 2022; Foresto et al., 2023).

At present, the management of Ascochyta blight is quite difficult, due to the absence of highly resistant cultivars and the ineffectiveness of chemical fungicides. However, several strategies can be implemented to mitigate the damages, such as the use of pathogen-free certified seed, rotation with non-host crops (such as cereals), burning chickpea stubble, use of moderately resistant varieties, application of systemic fungicides (demethylation inhibitors, succinate dehydrogenase inhibitors and quinone outside inhibitors), or the use of biological strategies. In recent years, different biological control agents have been developed against Ascochyta blight in chickpea, such as fungi of the genus *Trichoderma* or bacteria of the genera *Pseudomonas* or *Burkholderia* (Manjunatha et al., 2018; Foresto et al., 2023). However, a greater effort is needed in this area of research, specially on the characterization of the biological control agent and on its actual field application.

Trichoderma is a genus of filamentous fungi widely studied and used as biological control agents in agriculture, representing 50–60 % of the current biofungicides on the world market (Kubiak et al., 2023). Several species are used as plant growth promoting biofertilizers, due to their ability to provide ammonium, solubilize phosphorus, or produce and release siderophores and phytohormones (such as auxins) (Poveda et al., 2021). In addition, there are *Trichoderma* species capable of increasing plant tolerance under abiotic stresses, such as drought, cold or salinity (Abdullah et al., 2021) or by mycoremediation of anthropogenic pollutants (Poveda, 2022).

However, the main area of application and study of *Trichoderma* is as a biological control agent against biotic stresses that threaten crops. Against pathogens, such as bacteria, fungi, oomycetes and nematodes, the main direct mechanisms of action developed by various *Trichoderma* species include parasitism, antibiosis and competition for space and nutrients (TariqJaveed et al., 2021; Rodrigues et al., 2023); while against insects has been reported direct parasitism and/or the production and release of insecticidal/repellent compounds (Lana et al., 2023). On the other hand, *Trichoderma* stands out in recent years for the large amount of work focused on the study of the ability of various species to activate the plant defenses of its host plant against pathogens and pests. When *Trichoderma* colonizes the root cell apoplast it is recognized by plant cell receptors (recognize microorganism-associated molecular patterns, MAMPs), which causes the activation of local and systemic plant defenses, mediated by the salicylic acid (SA) or jasmonic acid/ethylene (JA/ET) hormonal pathways (Salwan et al., 2022). This activation of plant defenses involves the accumulation of defensive secondary metabolites in different organs, such as leaves (Velasco et al., 2021), which prepares the plant for possible future attack by the pathogen/pest.

Once the mechanisms of action of each *Trichoderma* species in interaction with the given crop have been characterized, it must be determined how to implement the fungus application in field conditions. In this sense, seed coating with conidia represents one of the most widespread strategies (Paravar et al., 2023). The most common compounds used for seed coating with *Trichoderma* include sodium alginate, carboxymethyl cellulose, chitin or gum arabic (Kumar et al., 2023; Martinez et al., 2023).

Therefore, the objective of this work was, first, to characterize different *Trichoderma* isolates with the ability to antagonize *in vitro* and to induce chickpea plant defenses against the pathogen *A. rabiei*. Secondly, to develop an effective chickpea seed coating strategy with *Trichoderma*. Finally, to determine the ability of the *Trichoderma*-coating combination to reduce Ascochyta blight in chickpea through systemic resistance, and to identify the hormonal pathways and metabolic changes involved.

2. Materials and methods

2.1. Biological material

The pathogen used in this work was the fungus *A. rabiei* (Ar) pathotype 1, (strain P1ENE/19), provided by Dr. Diego Rubiales, Institute of Sustainable Agriculture, CSIC (Córdoba, Spain), which was isolated from chickpea fields in Germany. *A. rabiei* was routinely grown on chickpea seed meal agar (CSMDA) medium (chickpea meal 40 g, dextrose 20 g, agar 20 g, distilled water 1 L) (Valetti et al., 2021).

Five different *Trichoderma* species were used in this work: *T. asperellum* (strain T20; GenBank: JF501661, KC47980), isolated from the rhizosphere of holm oak in the province of Viterbo (Italy); *T. atroviride* (strain HP151; GenBank: KT323352, PP763218), *T. citrinoviride* (strain EN2; GenBank: PP693577, PP701025), *T. harzianum* (strain EN1; GenBank: PP701026, PP763250), isolated from *Pinus* spp. bark in the province of Palencia (Spain); and *T. hamatum,* (strain H690; GenBank: MT641233, OL389793), isolated from kale roots in experimental plots in the province of Pontevedra (Spain). All strains were routinely grown on potato dextrose agar (PDA) medium (Sigma).

As plant material, chickpea seeds certified as FC-IGP were used, which were provided by the official producer center (Centro Legumbres de Calidad, Salamanca, Spain). The seeds were produced in the 2022 season and stored in a cool-dry place until use.

2.2. Trichoderma spp. screening: in vitro and in planta

The first phase of the study dealt with the screening five *Trichoderma* species in the control of *A. rabiei* by either a direct antagonism with the pathogen *in vitro* (direct mechanisms of action) or by activation of systemic defenses in chickpea plants (indirect mechanism). For the *in vitro* antagonism study, the Petri dish confrontation methodology as previously described by Poveda (2021a) was used, with modifications. Because both fungi routinely grow on different culture media, both PDA and CSMDA were used in the confrontations. First, a 5 mm diameter disc of *A. rabiei* mycelium, taken from the edge of actively growing colonies (3 weeks old), was placed on a Petri dish with PDA or CSMDA, two cm from the edge of the plate. The plates were incubated in the dark at 25 °C. After 48 h of pathogen growth, the plates were inoculated with a 5 mm diameter disk of mycelium of the different *Trichoderma* species, placed 2 cm from the opposite edge of the plate and 5 cm from *A. rabiei*. Again, the plates were incubated in darkness at 25 °C, taking photographs of all of them 48 h after *Trichoderma* inoculation and every 24 h until the fungal colonization of the plate was complete. The area colonized by the pathogen at every time was measured with the ImageJ software (NIH, USA) (Eugui et al., 2023). The control was based on plates with the pathogen and without *Trichoderma*. For each confrontation 5 plates were used, repeating the experiment 3 times.

In order to select the strain that had the greatest ability to induce systemic defensive responses of chickpea plants against *A. rabiei*, an *in planta* experiment was performed by root inoculating *Trichoderma* and infecting chickpea leaves with the pathogen. A substrate composed of a mixture of FC culture soil (physicochemical analysis provided in Table S1) and perlite (2:1) was used for chickpeas seeding. The substrate was sterilized in autoclave by two cycles of 30 min at 120 °C, spaced 48 h apart, in order to avoid the presence of other microorganisms. Chickpea 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, according to the methodology described by Rodriguez et al. (2023), with modifications. Chickpea seeds were sown individually in 0.5 L pots.

Inoculation with the different *Trichoderma* species was carried out one week after chickpea sowing, by means of a root application of 1 mL of conidia (2×10^7 conidia/mL; conidial quantification performed with a hemocytometer). Pathogen application was performed one week after root inoculation with *Trichoderma*, by spraying with sterile distilled water at a concentration of 5×10^5 conidia/mL (conidial quantification performed with a hemocytometer) of *A. rabiei*, until run-off, following the methodology described by Pande et al. (2011). Half an hour after pathogen application the plants were covered with transparent plastic to maintain high humidity to allow disease development, for one week. Starting two weeks after pathogen inoculation, plant mortality data were collected every 5–7 days until the death of all control plants (23–25 days after pathogen inoculation). For each treatment, 15 plants were used, repeating the experiment 3 times. During the experiments, the plants were kept in greenhouses, at 14 h photoperiod, environmental temperature (12–30 °C) and relative humidity above 80 %. The plants were watered 2–3 times a week, according to the observed needs, always with the same amount of water in all the plants.

2.3. Coating selection by compatibility

Based on the results obtained in the initial screening, *T. harzianum* EN1 was selected as the most promising strain to continue the rest of the study. Different materials were used to make coatings on *T. harzianum* spores and chickpea seeds: pregelatinized starch (PS) (Biosynth, UK), carboxymethyl cellulose (CMC) (SaporePuro, Italy), gum arabic (GA) (SaporePuro, Italy) and sodium alginate (NaA) (SaporePuro, Italy).

On the one hand, the ability of *T. harzianum* conidia to germinate in contact with the different coatings was analyzed. Coatings consisted of solutions of 1, 3, 5 and 7 % (w/v) in distilled water, all previously sterilized by autoclave. In Eppendorf tubes containing 1 mL of each of the different coatings and dilutions, 10^6 conidia of *T. harzianum* were added (conidial quantification performed with a hemocytometer). The control treatment was 1 mL of sterile distilled water. Subsequently, the tubes were kept under agitation (50 rpm) at 25 °C. After 12 and 24 h of incubation, the number of germinated and non-germinated conidia were counted in 100 μL samples. Each treatment consisted of 4 Eppendorf tubes and the whole experiment was performed in duplicate.

On the other hand, to study the effect of the different coatings on chickpea seed germination, their application was carried out following the methodology described by Dogaru et al. (2021), with modifications. Four dilutions of the different coatings in sterile distilled water (1, 3, 5 and 7 %, w/v) were used and applied to the seeds (superficially disinfected, as indicated in the previous section), in a ratio of 4 mL of treatment for each 50 g of seeds, mixing for 5 min. The control treatment was based on the same amount of sterile distilled water, without coating. After the application of the different treatments, the seeds were dried in a laminar flow hood for 2 h and sown in Petri dishes of 15 cm diameter, with filter paper moistened with sterile distilled water. Seeds were incubated in a growth chamber at 25/20 °C with a 16/8h light/dark photoperiod. The percentage of germination was determined twice (at 5 and 8 days) and the length of plumule and radicle was measured (at 8 days). For each treatment, 4 plates with 10 seeds each were used, repeating the experiment twice.

2.4. In planta study with Trichoderma-seed coating

Once the most effective *Trichoderma* species (*T. harzianum*) and the most efficient coating and concentration (GA 1 %) were selected, the activation of systemic resistance *in planta* by such a combination was studied. First, the application of the coating and *T. harzianum* on the seeds was carried out following the methodology described by Dogaru et al. (2021), with modifications. In 50 g of chickpea seeds, 2.5 mL of 1 % GA with a concentration of *T. harzianum* conidia (6×10^4 conidia/g of seeds) was applied. For this, 3×10^4 conidia were quantified (using a hemocytometer), which were suspended in the 2.5 mL of 1 % GA. Subsequently, the coating with *Trichoderma* conidia was applied to the chickpea seeds uniformly over their entire surface, by continuous mixing for 5 min (at which time the entire coating was covering the seeds).

Four treatments were carried out: (1) distilled water (control), (2) distilled water with *T. harzianum* conidia, (3) GA 1 % and (4) GA 1 % with *T. harzianum* conidia. After the application of the different treatments, the seeds were dried in a laminar flow hood for 2 h. Subsequently, the seeds were sown in 0.2 L pots, containing as substrate a mixture of FC culture soil (physicochemical analysis provided in Table S1) and perlite (2:1). In this trial, FC culture soil was not sterilized, in order to resemble as closely as possible, the real field conditions. For each treatment 56 seedlings were used: 36 plants (18 with pathogen and 18 without pathogen) that were removed at different times for the evaluation of *Trichoderma*-root colonization, hormone and metabolomic analyses and 20 plants to test the mortality until the end of the experiment.

Two weeks after sowing, 38 out of the 56 plants were infected in the aerial part with the pathogen *A. rabiei*, following the methodology already described in section 2.2. One week after pathogen-infection, the protective plastic was removed and 18 plants were collected from each treatment (9 with *A. rabiei*-infection and 9 without infection), whose aerial part was immediately frozen in liquid nitrogen (in 3 pools of 3 plants each) and stored at −80 °C for subsequent hormone and metabolomic analysis. In addition, the root part of the *T. harzianum*-inoculated plants was washed with sterile distilled water (until there was no substrate in contact with the roots), frozen in liquid nitrogen and stored at −80 °C for subsequent qPCR colonization analysis. This procedure was also repeated two weeks after *A. rabiei*-infection. Furthermore, from the remaining 20 plants per treatment infected with the pathogen, mortality was weekly recorded during four weeks. The experiment was repeated twice.

2.5. Quantification of Trichoderma-roots colonization by qPCR

The roots collected in section 2.4 were freeze-dried and pulverized. Subsequently, using the methodology described by Velasco et al. (2021), *Trichoderma*-root colonization was analyzed by qPCR. DNA was extracted from roots of *Trichoderma* inoculated plants, using the Phire Plant Direct PCR Kit (Termo Fisher Scientifc, Waltham, MA, USA). A mix was prepared in a 10 μL volume using 5 μL of Brilliant SYBR Green QPCR Master Mix (Roche, Penzberg, Germany), 10 ng of DNA, the forward and reverse primers at a final concentration of 100 nM and nuclease-free PCR-grade water to adjust the final volume. Actin was used as endogenous gene both for chickpea (Forward: GCCCAGTCAAAGAGGGGTATCCTCA; Reverse: CACACCATCACCAGAGTCGAGCA; Cho & Muehlbauer, 2004) and *Trichoderma* (Forward: ATGGTATGGGTCAGAAGGA; Reverse: ATGTCAACACGAGCAATGG; Velasco et al., 2021). 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, 95 °C for 15 s; annealing, 60 °C for 1 min; extension, 72 °C for 1 min. Each PCR was performed in triplicate by using the DNA extracted from 3 root pools of 3 plants each per treatment. Cycle threshold values served

to calculate the amount of fungal DNA using standard curves (elaborated with 10 known concentrations of fungal DNA and their amplification cycles for *Trichoderma actin*). Values of fungus DNA were referred to the amount of chickpea DNA in every corresponding sample.

2.6. Hormonal and metabolomic analysis

For the analysis of defense-related hormones, pools from the aerial part of chickpea collected one week after infection with *A. rabiei* were used. The plant pools were lyophilized and mechanically milled to a fine powder in a grinder (Janke and Kunkel A10 mill, IKA-Labortechnik, Staufen, Germany). These samples were used in the hormone and metabolomic analyses.

The main classes of plant defense-related hormones, SA, JA, ET precursor 1-aminocyclopropane-1-carboxylic acid (ACC) and melatonin (Mel) were extracted and analyzed as described previously in Albacete et al. (2008), with some modifications. Powdered plant material (0.05 g) was incubated in 1 mL of cold (−20 °C) extraction mixture of methanol/water (80/20, vol/vol) for 30 min at 4 °C. Solids were separated by centrifugation (20,000*g*, 15 min at 4 °C) and re-extracted for another 30 min at 4 °C with 1 mL of extraction solution. Pooled supernatants were passed through Sep-Pak Plus C18 cartridges (previously conditioned with 3 mL of extraction buffer) to remove interfering lipids and some plant pigments. The supernatant was collected and evaporated at 40 °C with a vacuum. The residue was dissolved in 1 mL methanol/water (20/80, vol/vol) solution using an ultrasonic bath. The dissolved samples were filtered through 13-mm diameter Millex filters with a 0.22-μm pore size nylon membrane (Millipore, Bedford, MA) and placed into opaque microcentrifuge tubes. Ten microlitres of the filtered extract was injected into an Accela Series UHPLC (ThermoFisher Scientific, Waltham, MA) instrument coupled to an Exactive mass spectrometer (ThermoFisher Scientific, Waltham, MA) using a heated electrospray ionisation (HESI) interface. Mass spectra were obtained using Xcalibur software version 2.2 (ThermoFisher Scientific, Waltham, MA). For the quantification of plant hormones, calibration curves were obtained for each analyzed component (1, 10, 50 and 100 μg/L) and corrected for 10 μg/L deuterated internal standards. Recovery rates ranged from 92 to 95 %.

Metabolite extraction and analysis were performed using the methodology previously described by Rodríguez et al. (2023). Lyophilized powder (50 mg) was dissolved in 500 mL of 80 % aqueous methanol and then sonicated for 15 min. After centrifugation for 10 min (16,000*g*, at room temperature), the extract was filtered through a 0.20-μm micropore PTFE membrane and placed in vials for further analysis. For metabolomic composition analysis, we used ultra–performance liquid chromatography (Thermo Dionex Ultimate 3000 LC; Thermo Fisher Scientific, Waltham, MA, USA) coupled to electrospray ionization time-of-flight mass spectrometry (UPLC-Q-TOF–MS/MS) (Bruker Compact™) with an electrospray ionization (ESI) source. Chromatographic separation was performed on an Intensity Solo 2 C18 column (2.1 × 100 mm 1.7 μm pore size; Bruker Daltonics, Billerica, MA, USA) using a binary gradient solvent mode consisting of 0.1 % formic acid in water (solvent A) and acetonitrile (solvent B). The following gradient was used: 3 % B (0–4 min), 3 to 25 % B (4–16 min), 25 to 80 % B (16–25 min), 80 to 100 % B (25–30 min), maintain 100 % B for 32 min, 100 to 3 % B (32–33 min), and maintain 3 % B for 36 min. The injection volume was 5 μL, the flow rate was set at 0.4 mL/min and the column temperature was controlled at 35 °C. MS analysis was performed in the spectrum acquisition range of 50 to 1200 m/z. Both polarities (±) of the ESI mode were used under the following specific conditions: gas flow 9 L/min, nebuliser pressure 38 psi, dry gas 9 L/min and dry temperature 220 °C. The capillary and end-plate displacements were set to 4500 and 500 V, respectively. The instrument was externally calibrated with a calibration solution of 1 mM formate/sodium acetate in 50/50 iPrOH/H_2O with 0.2 % formic acid infused directly into the source. Prior to sample injection, the stability of the LC-qTOF system was tested using three consecutive injections of chloramphenicol (ESI mode; $\Delta RT = 0.02$ min; $\Delta m/z = 0.002$) and triphenyl phosphate (ESI + mode; $\Delta RT = 0.02$ min; $\Delta m/z = 0.001$). Calibration solution was injected at the beginning of each run, and all spectra were calibrated prior to statistical analysis. MS/MS analysis was performed based on the previously determined exact mass and RT and was fragmented using different collision energy ramps to cover a range from 15 to 50 eV. The T-Rex 3D algorithm of MetaboScape 4.0 software (Bruker Daltonics, Billerica, MA, USA) was used for alignment and peak detection.

2.7. Statistical analysis

Chi-square tests (χ^2) were applied to determine the effects of type of coatings and dilution percentages on germination percentage. Yates' correction for continuity was applied in those cases where the expected frequencies were below 5. Analyses of variance (ANOVAs) and multiple comparison procedures were performed to test the effects of *Trichoderma* species on *A. rabiei* mycelium *in vitro* and the coating effects on radicle and plumule growth. As the data violated two of the ANOVA assumptions (normality and homogeneity of variances), robust statistical methods were applied (García-Pérez, 2010). In particular, heteroscedastic one-way ANOVAs were performed using the generalized Welch procedure and a 0.1 trimmed mean transformation. The ANOVAs were carried out using the "Wilcox' Robust Statistics (WRS2)" package (Mair and Wilcox, 2020). Survival analyses based on the nonparametric estimator Kaplan–Meier were carried out with the "Survival" package (Therneau, 2023). Survival curves were created with the "Survfit" function and the differences between the curves were tested with the "Survdiff" function. All analyses were performed using R software environment (R Foundation for Statistical Computing, Vienna, Austria).

Statistical analysis of metabolomic data was performed using the web-based software Metaboanalyst (Chong et al., 2019). In order to remove non-informative variables, data were filtered using the interquantile range filter (IQR). Moreover, Pareto variance scaling was used to remove the offsets and adjust the importance of high- and low-abundance ions to an equal level. The resulting three-dimensional matrix (peak indices, samples and variables) was further subjected to statistical analysis. Univariate analysis (one-way ANOVA) with a p value ≤ 0.05 was carried out to find differentially expressed metabolites. Using the Volcano Plot (VP) approach, which measure differentially accumulated metabolites based on *t*-statistics and fold changes simultaneously, we also highlighted the metabolites with a $|\log2(FC)| \geq 1$ and statistically significant difference (FDR ≤ 0.05) between plants with and without *T. harzianum* inoculation.

Root colonization and hormone data were analyzed using Statistix 8.0 software (Analytical Software, Tallahassee, USA). The combined effect of coating and presence/absence of foliar infection with *A. rabiei* was analyzed by two-way ANOVA followed by Sidak's multiple comparison test; different letters indicate significant differences ($p < 0.05$). One-way ANOVA using Tukey's multiple range test was used for pairwise comparisons of the hormone data.

3. Results

3.1. In vitro antagonism and activation of systemic defenses against *A. rabiei* in chickpea plants by *Trichoderma* spp.

The results obtained from the conformation between different *Trichoderma* species and the pathogen in PDA medium showed no significant differences between the growth of the pathogen without *Trichoderma* (Control) and the different *Trichoderma* confrontations at 48 and 72 h (Fig. 1a). However, at later times (96, 120 and 144 h) *T. asperellum*, *T. atroviride*, *T. hamatum* and *T. harzianum* significantly reduced the growth of *A. rabiei* compared to the control. Specifically, at 144 h, the growth of the pathogen without confrontations with *Trichoderma* was (62.6 cm^2), while in confrontations with the different *Trichoderma* strains it was 40.4 cm^2 (*T. asperellum*), 35.8 cm^2 (*T. atroviride*),

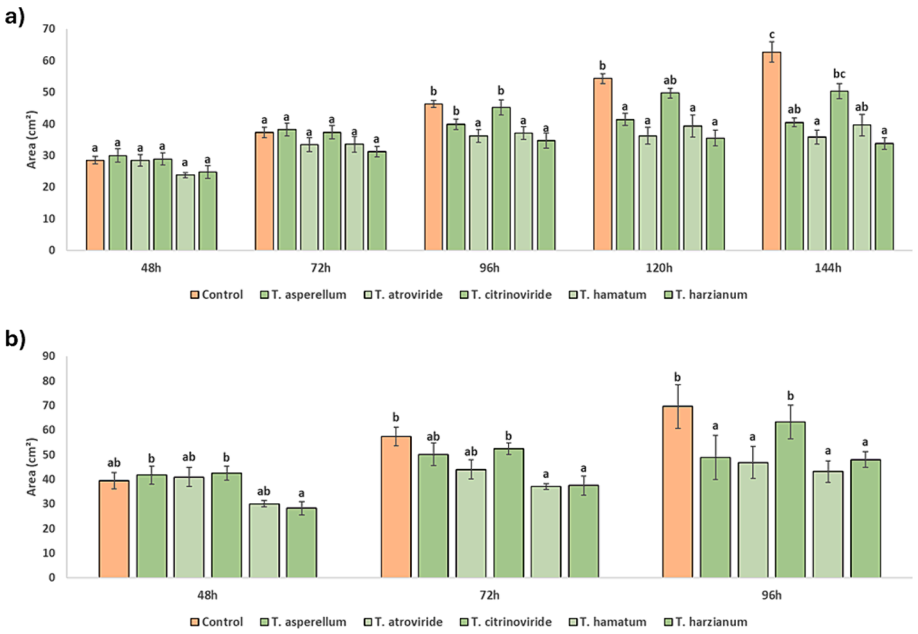

Fig. 1. Growth area (cm^2) of *A. rabiei* mycelium *in vitro* against *T. asperellum*, T. *atroviridae*, *T. citrinoviride*, *T. hamatum*, *T. harzianum* or without antagonist agent (Control), in PDA (a) or CSMDA (b) medium. Data are the mean of fifteen biological replicates for each condition with the corresponding standard error. Bars with different letters indicate significantly different means (Posthoc tests, $\alpha = 0.05$).

50.3 cm^2 (*T. citrinoviride*, without significant differences against the control), 39.6 cm^2 (*T. hamatum*) and 33.8 cm^2 (*T. harzianum*) (Fig. 1a).

With respect to the CSMDA medium, no significant differences were reported in the growth of *A. rabiei*, compared to the control, with the different confrontations with *Trichoderma* at 48 h (Fig. 1b). Subsequently, at 72 h, only the *T. hamatum* (37 cm^2) and *T. harzianum* (37.5 cm^2) confrontations caused a significant reduction in pathogen growth compared to no confrontation (57.4 cm^2) (Fig. 1b). Finally, at 96 h, when fungal colonization of the plate was complete in controls, confrontations with *T. asperellum* (48.9 cm^2), *T. atroviride* (46.8 cm^2), *T. hamatum* (43.1 cm^2) and *T. harzianum* (48 cm^2), significantly reduced the growth of *A. rabiei*, compared to the confrontation with *T. citrinoviride* (63.2 cm^2) or the control without *Trichoderma* (69.6 cm^2) (Fig. 1b).

On the other hand, the root inoculation of chickpea plants with the different *Trichoderma* species was carried out in order to determine what strain was able to activate more effectively the systemic resistance of the host plant against aerial infection by *A. rabiei*. Plants root inoculated with *T. asperellum*, *T. citrinoviride* and *T. hamatum* did not show a higher probability of survival than plants without root inoculation (control) (Fig. 2). Both plants inoculated with *T. atroviride* and those inoculated with *T. harzianum* were significantly more likely to survive than control plants. Furthermore, plants inoculated with *T. harzianum* were significantly more likely to survive than plants inoculated with *T. citrinoviride*. However, there were no significant differences between plants inoculated with *T. atroviride* and the other *Trichoderma*-inoculations (Fig. 2).

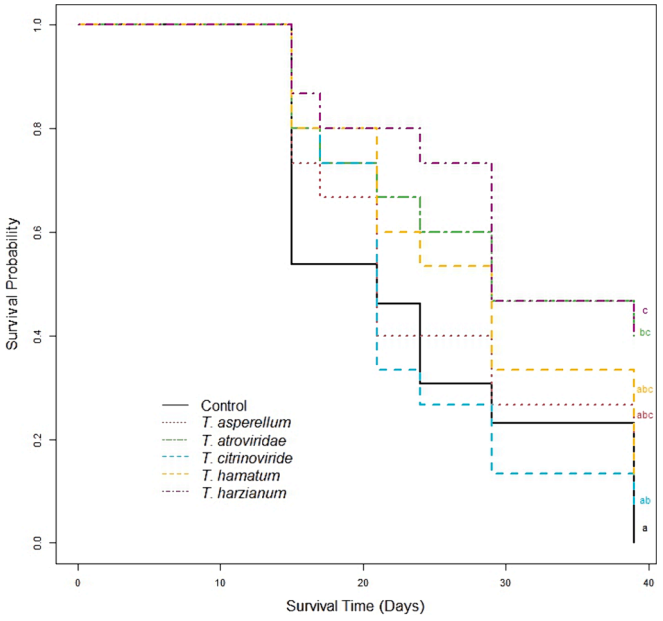

Fig. 2. Curves of survival probability of chickpea plants infected with *A. rabiei* and root-inoculated with *T. asperellum*, T. *atroviridae*, *T. citrinoviride*, *T. hamatum*, *T. harzianum* or without root-inoculation (Control). Curves with the same letter were not significantly different according to the Kaplan-Meier estimator ($\alpha \leq 0.05$).

3.2. *Effect of coatings on the germination of T. harzianum conidia and chickpea seeds*

The effect of coatings, at different concentrations, on conidial germination of *T. harzianum* showed, after 12 h of incubation, a significant increase in conidial germination, with respect to the water control, in the treatments PS 1, 3 and 5 %, GA 1 and 5 %, and NaA 3, 5 and 7 %; with PS 1 %, and toa lesser extent GA 5 %, being those that most increased conidial germination (Table 1). The CMC-based coating completely inhibited the germination of *Trichoderma* conidia (Tables 1 and 2). Subsequently, at 24 h, the coating based on PS (at concentrations 1, 3, 5, and 7 %), GA (1, 5 and 7 %) and NaA (3, 5 and 7 %) reported an increase in the germination of *T. harzianum* conidia, compared to the treatment with water (Table 2). Furthermore, PS 1, 3 and 5 % treatments resulted in a significant increase in conidial germination compared to the GA 3 % or NaA 3 % treatments. Similarly, GA treatments at concentrations 1, 5 and 7 % reported significantly higher conidial germination than GA 3 % (Table 2).

On the other hand, the effect of the different coatings and concentrations on chickpea seed germination was studied, eliminating CMC

Table 1
Chi-square comparative matrix comparing the number of germinated and non-germinated *T. harzianum* conidia between the different coatings and dilution percentages used, after 12 h of incubation·H_2O: control treatment with distilled water; PS 1 %, 3 %, 5 % and 7 %: pregelatinized starch at different dilutions (w/v) in distilled water; CMC 1 %, 3 %, 5 % and 7 %: carboxymethyl cellulose at different dilutions (w/v) in distilled water; GA 1 %, 3 %, 5 % and 7 %: gum arabic at different dilutions (w/v) in distilled water; NaA 1 %, 3 %, 5 % and 7 %: sodium alginate at different dilutions (w/v) in distilled water. The numbers under each treatment indicate the percentage of germinated conidia compared to the total number of conidia quantified.

	H_2O (0.8 %)	**PS 1 %** (15.1 %)	**PS 3 %** (6.1 %)	**PS 5 %** (4,3 %)	**PS 7 %** (2.9 %)	**CMC 1 %** (0 %)	**CMC 3 %** (0 %)	**CMC 5 %** (0 %)	**CMC 7 %** (0 %)	**GA 1 %** (4.6 %)	**GA 3 %** (3.6 %)	**GA 5 %** (7.5 %)	**GA 7 %** (3.4 %)	**NaA 1 %** (3.1 %)	**NaA 3 %** (5.4 %)	**NaA 5 %** (3.8 %)	**NaA 7 %** (6 %)
H_2O (0.8 %)		***	**	*	NS	X	X	X	X	*	NS	***	NS	NS	**	*	**
PS 1 % (15.1 %)	***		**	**	***	X	X	X	X	**	***	*	***	***	**	***	**
PS 3 % (6.1 %)	**	**		NS	NS	X	X	X	X	NS	NS	NS	NS	NS	NS	NS	NS
PS 5 % (4,3 %)	*	**	NS		NS	X	X	X	X	NS	NS	NS	NS	NS	NS	NS	NS
PS 7 % (2.9 %)	NS	***	NS	NS		X	X	X	X	NS	NS	NS	NS	NS	NS	NS	NS
CMC 1 % (0 %)	X	X	X	X	X		X	X	X	X	X	X	X	X	X	X	X
CMC 3 % (0 %)	X	X	X	X	X	X		X	X	X	X	X	X	X	X	X	X
CMC 5 % (0 %)	X	X	X	X	X	X	X		X	X	X	X	X	X	X	X	X
CMC 7 % (0 %)	X	X	X	X	X	X	X	X		X	X	X	X	X	X	X	X
GA 1 % (4.6 %)	*	**	NS	NS	NS	X	X	X	X		NS	NS	NS	NS	NS	NS	NS
GA 3 % (3.6 %)	NS	***	NS	NS	NS	X	X	X	X	NS		NS	NS	NS	NS	NS	NS
GA 5 % (7.5 %)	***	*	NS	NS	NS	X	X	X	X	NS	NS		NS	*	NS	NS	NS
GA 7 % (3.4 %)	NS	***	NS	NS	NS	X	X	X	X	NS	NS	NS		NS	NS	NS	NS
NaA 1 % (3.1 %)	NS	***	NS	NS	NS	X	X	X	X	NS	NS	*	NS		NS	NS	NS
NaA 3 % (5.4 %)	**	**	NS	NS	NS	X	X	X	X	NS	NS	NS	NS	NS		NS	NS
NaA 5 % (3.8 %)	*	***	NS	NS	NS	X	X	X	X	NS	NS	NS	NS	NS	NS		NS
NaA 7 % (6 %)	**	**	NS	NS	NS	X	X	X	X	NS	NS	NS	NS	NS	NS	NS	

NS: non-significant differences; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; X: Absence of germination in any of the treatments compared.

from the assays (because it totally inhibited *T. harzianum* conidial germination). After 5 days of seeds incubation, only the 5 % PS treatment showed significant differences with the water control, specifically by a significant reduction in germination (Table 3). In addition, the GA 1 % and NaA 3 % treatments caused a significant increase in the germination percentage, compared to all the different treatments with PS (Table 3). At 8 days of incubation, the PS 3 % treatment continued to cause significantly lower germination than the water control, with no significant differences with the other treatments (Table 4). The treatment with GA 1 % achieved 100 % germination, being significantly higher than the different treatments with PS or NaA (Table 4). In addition, with respect to radicle and plumule length of germinated seeds, all GA treatments reported a significant increase in plumule length, compared to the water control, with the GA 1 % treatment being the one that reported the greatest length (Fig. S1).

3.3. Effect of Trichoderma-seed coating on chickpea plants

The mortality of the plants resulting from the seeds inoculated with the different treatments was analyzed by means of a survival probability curve (Fig. 3). Only the treatment of seeds with GA and *T. harzianum* conidia significantly increased the probability of survival of chickpea plants infected with *A. rabiei*, compared to the control treatment. However, treatment with GA and *T. harzianum* conidia showed no

Table 2
Chi-square comparative matrix comparing the number of germinated and non-germinated *T. harzianum* conidia between the different coatings and dilution percentages used, after 24 h of incubation·H_2O: control treatment with distilled water; PS 1 %, 3 %, 5 % and 7 %: pregelatinized starch at different dilutions (w/v) in distilled water; CMC 1 %, 3 %, 5 % and 7 %: carboxymethyl cellulose at different dilutions (w/v) in distilled water; GA 1 %, 3 %, 5 % and 7 %: gum arabic at different dilutions (w/v) in distilled water; NaA 1 %, 3 %, 5 % and 7 %: sodium alginate at different dilutions (w/v) in distilled water. The numbers under each treatment indicate the percentage of germinated conidia compared to the total number of conidia quantified.

	H_2O (1.3 %)	**PS 1 %** (21.6 %)	**PS 3 %** (19.8 %)	**PS 5 %** (17 %)	**PS 7 %** (20.8 %)	**CMC 1 %** (0 %)	**CMC 3 %** (0 %)	**CMC 5 %** (0 %)	**CMC 7 %** (0 %)	**GA 1 %** (17 %)	**GA 3 %** (1.3 %)	**GA 5 %** (17.5 %)	**GA 7 %** (17.8 %)	**NaA 1 %** (4.7 %)	**NaA 3 %** (12.9 %)	**NaA 5 %** (12.7 %)	**NaA 7 %** (12.9 %)
H_2O (1.3 %)		***	***	***	***	X	X	X	X	***	NS	***	***	NS	***	***	***
PS 1 % (21.6 %)	***		NS	NS	NS	X	X	X	X	NS	***	NS	NS	***	NS	NS	NS
PS 3 % (19.8 %)	***	NS		NS	NS	X	X	X	X	NS	***	NS	NS	***	NS	NS	NS
PS 5 % (17 %)	***	NS	NS		***	X	X	X	X	***	NS	***	NS	***	NS	NS	NS
PS 7 % (20.8 %)	***	NS	NS	***		X	X	X	X	NS	***	NS	NS	***	NS	NS	NS
CMC 1 % (0 %)	X	X	X	X	X		X	X	X	X	X	X	X	X	X	X	X
CMC 3 % (0 %)	X	X	X	X	X	X		X	X	X	X	X	X	X	X	X	X
CMC 5 % (0 %)	X	X	X	X	X	X	X		X	X	X	X	X	X	X	X	X
CMC 7 % (0 %)	X	X	X	X	X	X	X	X		X	X	X	X	X	X	X	X
GA 1 % (17 %)	***	NS	NS	***	NS	X	X	X	X		***	NS	NS	***	NS	NS	NS
GA 3 % (1.3 %)	NS	***	***	NS	***	X	X	X	X	***		***	***	NS	***	***	***
GA 5 % (17.5 %)	***	NS	NS	***	NS	X	X	X	X	NS	***		NS	***	NS	NS	NS
GA 7 % (17.8 %)	***	NS	NS	NS	NS	X	X	X	X	NS	***	NS		***	NS	NS	NS
NaA 1 % (4.7 %)	NS	***	***	***	***	X	X	X	X	***	NS	***	***		**	**	**
NaA 3 % (12.9 %)	***	NS	NS	NS	NS	X	X	X	X	NS	***	NS	NS	**		NS	NS
NaA 5 % (12.7 %)	***	NS	NS	NS	NS	X	X	X	X	NS	***	NS	NS	**	NS		NS
NaA 7 % (12.9 %)	***	NS	NS	NS	NS	X	X	X	X	NS	***	NS	NS	**	NS	NS	

NS: non-significant differences; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; X: Absence of germination in any of the treatments compared.

significant difference in survival probability compared to GA alone or water and *T. harzianum* conidia treatments (Fig. 3).

In plants from seeds inoculated with *T. harzianum*, root colonization by the fungus was quantified by qPCR. Both in the absence and presence of foliar infection with *A. rabiei*, the application of *T. harzianum* conidia with GA 1 % as coating resulted in a significant increase in root colonization of chickpea plants, compared with the water and *T. harzianum* conidia treatment (Fig. 4). Similarly, increased root colonization by *T. harzianum* was reported in plants foliarly infected with *A. rabiei*, compared to uninfected plants; except in plants inoculated with water and *T. harzianum* conidia two weeks after pathogen infection (Fig. 4).

The analysis of defense-related hormones in *A. rabiei*-infected plants reported that there were no significant differences between the different seed treatments with respect to SA and JA hormones (Fig. 5). However, in the treatment of chickpea seeds with GA and *T. harzianum* conidia, a significant increase in the systemic accumulation of the ET precursor 1-

Table 3
Chi-square comparative matrix comparing the number of germinated and non-germinated chickpea seeds between the different coatings and dilution percentages used, after 5 days of incubation·H_2O: control treatment with distilled water; PS 1 %, 3 %, 5 % and 7 %: pregelatinized starch at different dilutions (w/v) in distilled water; GA 1 %, 3 %, 5 % and 7 %: gum arabic at different dilutions (w/v) in distilled water; NaA 1 %, 3 %, 5 % and 7 %: sodium alginate at different dilutions (w/v) in distilled water. The numbers under each treatment indicate the percentage of germinated seeds compared to the total number of seeds quantified.

	H_2O (80 %)	**PS 1 %** (65 %)	**PS 3 %** (52 %)	**PS 5 %** (25 %)	**PS 7 %** (62 %)	**GA 1 %** (90 %)	**GA 3 %** (80 %)	**GA 5 %** (67 %)	**GA 7 %** (72 %)	**NaA 1 %** (85 %)	**NaA 3 %** (87 %)	**NaA 5 %** (70 %)	**NaA 7 %** (82 %)
H_2O (80 %)		NS	NS	**	NS	NS	NS	NS	NS	NS	NS	NS	NS
PS 1 % (65 %)	NS		NS	NS	NS	**	NS	NS	NS	NS	*	NS	*
PS 3 % (52 %)	NS	NS		NS	NS	**	*	NS	NS	*	**	NS	**
PS 5 % (25 %)	**	NS	NS		NS	***	**	NS	*	**	**	NS	**
PS 7 % (62 %)	NS	NS	NS	NS		*	NS	NS	NS	NS	**	NS	NS
GA 1 % (90 %)	NS	**	**	***	*		NS	NS	NS	NS	NS	NS	NS
GA 3 % (80 %)	NS	NS	*	**	NS	NS		NS	NS	NS	NS	NS	NS
GA 5 % (67 %)	NS	NS	NS	NS	NS	NS	NS		NS	NS	NS	NS	NS
GA 7 % (72 %)	NS	NS	NS	*	NS	NS	NS	NS		NS	NS	NS	NS
NaA 1 % (85 %)	NS	NS	*	**	NS	NS	NS	NS	NS		NS	NS	NS
NaA 3 % (87 %)	NS	*	**	**	**	NS	NS	NS	NS	NS		NS	NS
NaA 5 % (70 %)	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS		NS
NaA 7 % (82 %)	NS	*	**	**	NS	NS	NS	NS	NS	NS	NS	NS	

NS: non-significant differences; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; X: Absence of germination in any of the treatments compared.

Table 4
Chi-square comparative matrix comparing the number of germinated and non-germinated chickpea seeds between the different coatings and dilution percentages used, after 8 days of incubation·H_2O: control treatment with distilled water; PS 1 %, 3 %, 5 % and 7 %: pregelatinized starch at different dilutions (w/v) in distilled water; GA 1 %, 3 %, 5 % and 7 %: gum arabic at different dilutions (w/v) in distilled water; NaA 1 %, 3 %, 5 % and 7 %: sodium alginate at different dilutions (w/v) in distilled water. The numbers under each treatment indicate the percentage of germinated seeds compared to the total number of seeds quantified.

	H_2O (90 %)	**PS 1 %** (75 %)	**PS 3 %** (57 %)	**PS 5 %** (72 %)	**PS 7 %** (85 %)	**GA 1 %** (100 %)	**GA 3 %** (82 %)	**GA 5 %** (73 %)	**GA 7 %** (82 %)	**NaA 1 %** (90 %)	**NaA 3 %** (80 %)	**NaA 5 %** (82 %)	**NaA 7 %** (82 %)
H_2O (90 %)		NS	**	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
PS 1 % (75 %)	NS		NS	NS	NS	**	NS	NS	NS	NS	NS	NS	NS
PS 3 % (57 %)	**	NS		NS	*	***	*	NS	*	*	NS	*	*
PS 5 % (72 %)	NS	NS	NS		NS	*	NS	NS	NS	NS	NS	NS	NS
PS 7 % (85 %)	NS	NS	*	NS		NS	NS	NS	NS	NS	NS	NS	NS
GA 1 % (100 %)	NS	**	***	*	NS		*	**	*	NS	*	*	*
GA 3 % (82 %)	NS	NS	*	NS	NS	*		NS	NS	NS	NS	NS	NS
GA 5 % (73 %)	NS	NS	NS	NS	NS	**	NS		NS	NS	NS	NS	NS
GA 7 % (82 %)	NS	NS	*	NS	NS	*	NS	NS		NS	NS	NS	NS
NaA 1 % (90 %)	NS	NS	*	NS	NS	NS	NS	NS	NS		NS	NS	NS
NaA 3 % (80 %)	NS	NS	NS	NS	NS	*	NS	NS	NS	NS		NS	NS
NaA 5 % (82 %)	NS	NS	*	NS	NS	*	NS	NS	NS	NS	NS		NS
NaA 7 % (82 %)	NS	NS	*	NS	NS	*	NS	NS	NS	NS	NS	NS	

NS: non-significant differences; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; X: Absence of germination in any of the treatments compared.

aminocyclopropane-1-carboxylic acid (ACC) was reported, an increase not found with the treatment with water and *T. harzianum* conidia. Moreover, the addition of *T. harzianum* conidia with the GA coating, and to a lesser extent with water, resulted in a significant increase in the systemic accumulation of melatonin (Fig. 5).

In order to characterize the main metabolites present in chickpea

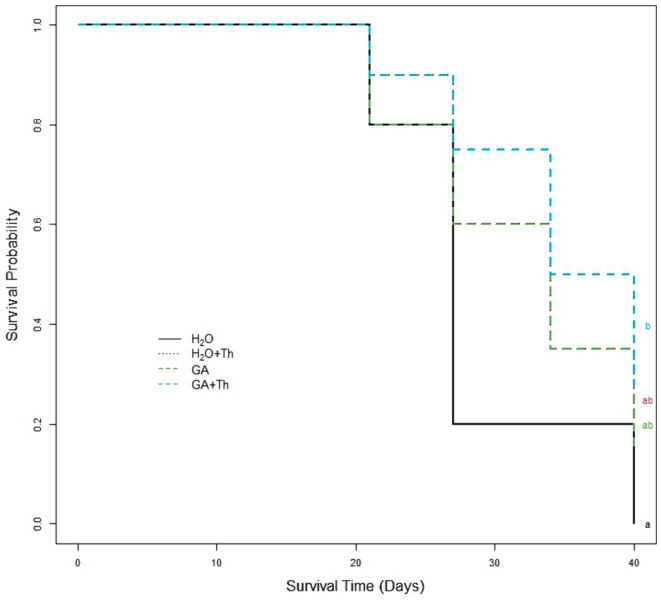

Fig. 3. Curves of survival probability of chickpea plants infected with *A. rabiei*, from seeds treated with water (H_2O), water and *T. harzianum* conidia (H_2O + Th), gum arabic 1 % (GA) or GA 1 % and *T. harzianum* conidia (GA 1 % + Th). Curves with the same letter were not significantly different according to the Kaplan-Meier estimator ($\alpha \leq 0.05$).

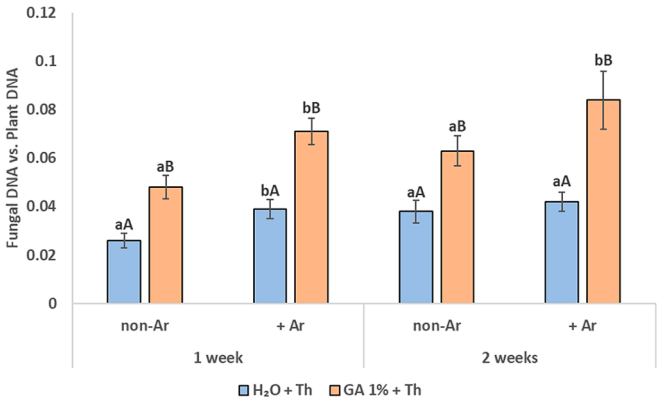

Fig. 4. Measurements of *Trichoderma*-root colonization by qPCR. The analysis was performed on roots of plants from seeds treated with H_2O and *T. harzianum* conidia (H_2O + Th) or gum arabic 1 % and *T. harzianum* conidia (GA 1 % + Th), and whose aerial part was infected with the pathogen *A. rabiei* (+Ar) or non-infected (non-Ar), having been collected one and two weeks after infection with the pathogen. Different letters represent significant differences ($p < 0.05$), between *A. rabiei*-infected and non-infected plants (identify by small letters) and between treatments with H_2O or GA 1 % (identify by capital letters).

plants previously infected by the pathogen *A. rabiei*, a non-targeted metabolomics analysis was performed comparing treatments with and without *T. harzianum* conidia. In the comparison between the control and the seeds treated with water and *T. harzianum* conidia, 671 different features were analyzed from the raw data matrix, of which 16 were significantly different between the two groups of plants analyzed. Only 2 metabolites were present in higher concentration in the plants whose seeds were treated with water and *T. harzianum* conidia (Fig. 6a). These metabolites were tentatively identified as L-tyrosine and deoxysphinganine (Table 5). On the other hand, in the comparison between the treatment of seeds with GA 1 % against GA 1 % with *T. harzianum* conidia, 25 were significantly different between both groups of plants. Four metabolites were found to be present in higher concentrations in the treatment with GA 1 % and *T. harzianum* conidia (Fig. 6b). Of these metabolites, only two could be tentatively identified as nicotinic acid and hexadienoic acid (Table 5).

4. Discussion

In the initial screening, different *Trichoderma* strains were tested as direct antagonists (*in vitro*) and inducers of systemic plant resistance against the pathogen *A. rabiei* in chickpea plants, finding that *T. atroviride* and *T. harzianum* were the most effective biocontrol agents. In the case of *T. atroviride*, its ability to inhibit the *in vitro* growth of *A. rabiei* had already been previously described, in addition to its ability to antagonize the chickpea pathogen *Fusarium oxysporum* f. sp. *ciceris* (Poveda, 2021a). The same work also described both antagonistic abilities for *T. harzianum* (Poveda, 2021a). This *Trichoderma* species has been described to have the ability to form a zone of inhibition against *A. rabiei* (Benzohra et al., 2011), possibly due to the production and release to the medium of lytic enzymes, such as chitinases and β-1,3-glucanases (KüÇük et al., 2007).

Regarding the interaction of *T. atroviride* and *T. harzianum* with chickpea plants, several studies have been conducted. *T. atroviride* had not been described so far as an effective inducer of systemic defenses against the pathogen *A. rabiei* in chickpea plants. However, this indirect biocontrol capacity has been described against the root pathogen *F. oxysporum* f. sp. *ciceris*, through the activation of SA-mediated defenses (Poveda, 2021a). In the case of *T. harzianum*, this fungal species has been described as a plant growth promoter in its interaction with chickpea plants (Kumar et al., 2014; Saxena et al., 2015), as well as an inducer of systemic resistance against the pathogen *Sclerotinia rolfsii* (Saxena et al., 2015). Regarding its use as a biological control agent by inducing systemic resistance in chickpea against *A. rabiei*, it has been described as an effective organism by inducing JA-mediated defenses (Poveda, 2021a). However, its effectiveness in the field is lower than commercial fungicides and different plant extracts (Ahmad et al., 2021).

Based on the results obtained from the initial screening, *T. harzianum* EN1 was selected as the strain with the greatest potential to directly and indirectly control *A. rabie* on chickpea plants. The next phase of the study focused on selecting the best coating to apply the *T. harzianum* conidia on chickpea seeds, obtaining that 1 % gum arabic was the best strategy. Our study evidenced that the coating with carboxymethyl cellulose completely inhibits the germination of *T. harzianum* conidia, conversely to what has been described by other authors for the same species (Brondi et al., 2022) or for *T. atroviride* (Coninck et al., 2020). In the case of pregelatinized starch, we obtained positive effects on the germination of *T. harzianum*, however, it inhibited the germination of chickpea seeds. In this sense, this coating has been previously described as compatible with the survival of *T. koningiopsis* conidia (Cortés-Rojas et al., 2021), although its application on seeds was still undescribed. In the case of sodium alginate, it was shown to be a safe coating for *T. harzianum* conidia and chickpea seeds, but without outstanding results of increased germination. Precisely, the encapsulation of *T. harzianum* conidia with this material has been widely described as one of the best strategies to favor the long-term viability of its conidia (Bhai, 2020; Lotfalinezhad et al., 2024), being used as an effective coating in the application of other biological control agents on chickpea seeds (Khan et al., 2011). Our work reported the best results on *T. harzianum* conidia and chickpea seeds with the use of gum arabic 1 % coating. This material has been described as a protector of *Trichoderma* conidia by microencapsulation, increasing the survival of *T. asperellum* and *T. harzianum* subjected to temperatures up to 90 °C (Muñoz-Celaya et al., 2012; Braga et al., 2019). In addition, this coating on chickpea seeds has been previously described as a germination promoter (Malviya et al., 2023) and as an effective fungal spore vehicle (Rocha et al., 2019).

After coating chickpea seeds with *T. harzianum* conidia, the use of gum arabic as a vehicle for the fungal inoculum was found to be the only strategy that significantly reduced the mortality of chickpea plants infected by *A. rabiei*. After analyzing root colonization by *Trichoderma*, it

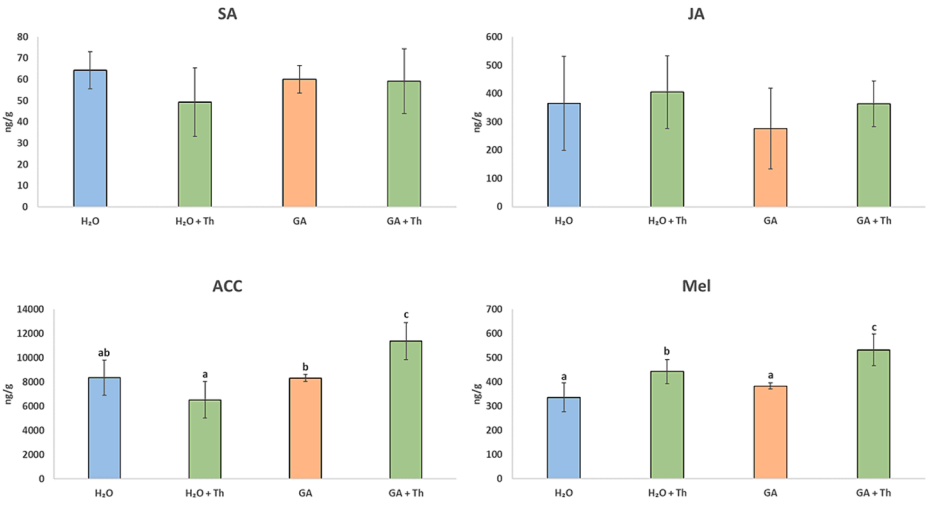

Fig. 5. Defense hormone content in chickpea plants, whose source seeds were treated with water (H_2O) and *T. harzianum* conidia (H_2O + Th) or with 1 % gum arabic (GA) and *T. harzianum* conidia (GA + Th), which were foliar infected with the pathogen *A. rabiei*. SA: salicylic acid; JA: jasmonic acid; ACC: 1-aminocyclopropane-1-carboxylic acid; Mel: melatonin. Data are the mean of three pools of three chickpea plants with the corresponding SE. Different letters represent significant differences ($p < 0.05$).

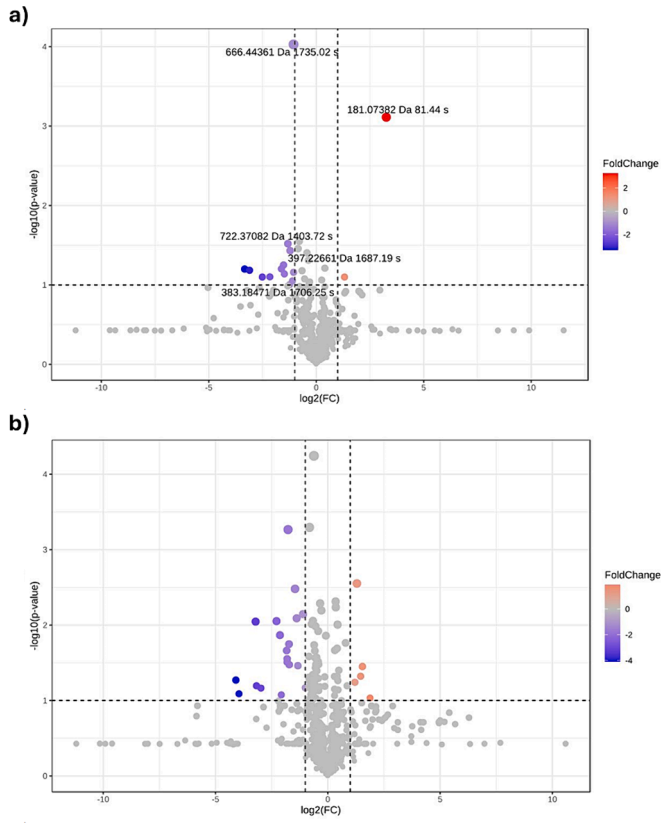

Fig. 6. Volcano plot representing the detected features in the non-targeted metabolomic analysis in water coating (a) and gum arabic (b), both *A. rabiei* infected plants. The y-axis represents the negative decade logarithm of the significance value (FDR), and the x-axis represents the log2 of fold change (coating without *T. harzianum* conidia vs. coating with *T. harzianum* conidia). Levels of features with a $-\log10(p) \leq 1.3$ and a $|\log2(FC)| \geq 1$ are considered to be differentially accumulated in different chickpea-seeds treatments. Significantly up-regulated features are represented by red circles and down-regulated features are represented by blue circles. Grey circles represent insignificant features. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

was determined that this treatment was indeed that which caused a greater presence of *T. harzianum* in the roots, fact that could be related to the greater induction of systemic plant resistance. Accordingly, it has been described how there is a direct relationship between the degree of root colonization by *T. harzianum* and the induction of systemic defenses against foliar pathogens (Poveda et al., 2019, Poveda, 2021b).

Hormonal analysis revealed how this systemic activation against *A. rabiei* in chickpea plants, induced by *T. harzianum*, could have been mediated by ET and Mel signaling. The ET defensive signaling pathway is closely related to that of JA against necrotrophic pathogens, such as *A. rabiei*, which has been described to be induced by *T. harzianum* in chickpea plants (Poveda, 2021a), but not in our work. However, the ET pathway has been described in several works as induced by *T. harzianum* in other plants, such as tomato, against nematode attack (Leonetti et al., 2017) or necrotrophic fungi, such as *Botrytis cinerea* (Martínez-Medina et al., 2013). On the other hand, Mel is a molecule initially described in animals, but also present in plants, which has been widely studied recently. This molecule, which is related to the regulation of physiological responses in plants, mainly against biotic and abiotic stresses, has been proposed by several authors as a new plant hormone (Arnao & Hernández-Ruiz, 2018, 2019, 2021). Several studies have reported how the exogenous application of Mel in plant tissues induces defensive responses against the attack of necrotrophic pathogens (Li et al., 2019, 2022). However, to date, the role of *Trichoderma* as an inducer of Mel-mediated plant responses had not been described, could be this study the first to establish a possible relationship.

Regarding the type of defensive response involved in the greater resistance of some chickpea plants versus others, treatment with *T. harzianum* conidia have produced a greater systemic accumulation of the metabolites L-tyrosine and deoxysphinganine. Although L-tyrosine may be related to an increase in plant defense against pathogens (Schenck & Maeda, 2018), it appears that this increase in its content could not be sufficient to control the advance of *A. rabiei* on the chickpea plants in our study. Deoxysphinganine is an aberrant molecule accumulated by plant in the face of stress situations (Cahoon et al., 2021), such as the attack of a necrotrophic pathogen (*A. rabiei*) of our work.

On the other hand, induction of systemic defenses in chickpea plants by coating with gum arabic and *T. harzianum* conidia was effective in reducing mortality after infection with the pathogen *A. rabiei*. This result could be linked to an increase in the systemic accumulation of two secondary metabolites, nicotinic acid and hexadienoic acid, which could be responsible for this increased resistance against the pathogen.

Table 5
Tentative identification of major metabolites with a $|\log_2 (FC)| \geq 1$ in *A. rabiei*-infected chickpea plants. With different seed treatments water and *T. harzianum* conidia (H_2O + Th) or 1 % gum arabic (GA) and *T. harzianum* conidia (GA + Th). Metabolites are sorted by ionization mode.

m/z	Chickpea seeds treatment	Neutral Mass	Ionization	RT (s)	Log2 (FC)	Fragments	Tentative identification	Molecular formula
182.0811	H_2O + Th	181.0738	$[M + H]^+$	81.44	3.26	–	L-Tyrosine	C9H11NO3
286.31111	H_2O + Th	285.3038	$[M + H]^+$	1427.07	1.32	–	Deoxysphinganine	C18H39NO
123.05279	GA + Th	122.0455	$[M + H]^+$	103.40	1.21	80.048	Nicotinic acid	C6H5NO2
145.04987	GA + Th	144.0429	$[M + H]^+$	94.33	1.30	–	Hexadienoic acid	C6H8O4
128.01937	GA + Th	254.0241	$[M + 2H]2^+$	48.47	1.46	56.964, 71,952, 84.963	–	C5H11N4O4PS
274.27454	GA + Th	273.2673	$[M + H]^+$	1277.97	1.88	–	–	C16H35NO2

Nicotinic acid has been described in numerous studies as a defense metabolite induced by the JA hormonal pathway against insect pests (Fragoso et al., 2014; Berglund et al., 2016) or pathogenic bacteria (Vogel-Adghough et al., 2013). However, hexadienoic acid had not been previously described as a plant metabolite, although its defensive role in different insects is well known (Laurent et al., 2005). Nevertheless, further studies should be performed in order to confirm the identity of those metabolites.

As conclusions, *T. harzianum* EN1 was the strain with the highest biological control capacity against *A. rabiei* on chickpea plants, among the strains used in this study. Furthermore, its application on seeds by using a GA coating is required to enhance root colonization and induce effective systemic plant defenses of chickpea plants against Ascochyta bight disease. These effective systemic defenses are ET- and Mel-mediated and might be based on the accumulation in chickpea plant tissues of nicotinic acid.

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CRediT authorship contribution statement

Javier Morcuende: Methodology, Investigation. **Jorge Martín-García:** Writing – review & editing, Supervision, Data curation. **Pablo Velasco:** Writing – review & editing, Methodology, Investigation, Data curation. **Tamara Sánchez-Gómez:** Methodology, Investigation. **Óscar Santamaría:** Writing – review & editing, Supervision. **Víctor M. Rodríguez:** Writing – review & editing, Methodology, Investigation. **Jorge Poveda:** Writing – original draft, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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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.2024.105530.

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