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First study on the root endophytic fungus *Trichoderma hamatum* as an entomopathogen: Development of a fungal bioinsecticide against cotton leafworm (*Spodoptera littoralis*)

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ABSTRACT

Cotton leaf worm (*Spodoptera littoralis*) is a pest that produces important losses in horticultural and ornamental crops in greenhouse, being classified as quarantine pest A2 by EPPO. One of the strategies proposed to control agricultural pests in a health and environmentally friendly way is biological control with entomopathogenic fungi. The genus of filamentous fungi *Trichoderma* includes different species with direct (infection, antibiosis, anti-feeding, etc.) and indirect (systemic activation of plant defenses) insecticidal capacity, however, the species *T. hamatum* has never been described previously as entomopathogenic. In this work, the entomopathogenic capacity of *T. hamatum* on *S. littoralis* L_3 larvae was analyzed by applying spores and fungal filtrates (topically and orally). Infection by spores was compared with the commercial entomopathogenic fungus *Beauveria bassiana*, obtaining similar results with respect to the production of larval mortality. Oral application of spores reported high mortality and fungal colonization of larvae, however, *T. hamatum* did not show chitinase activity when grown in the presence of *S. littoralis* tissues. Therefore, infection of *S. littoralis* larvae by *T. hamatum* is through natural openings such as mouth, anus or spiracles. With respect to the application of filtrates, only those obtained from the liquid culture of *T. hamatum* in contact with *S. littoralis* tissues reported a significant reduction in larval growth. Metabolomic analysis of the filtrates determined that the filtrate with insecticidal capacity presented the siderophore rhizoferrin in large quantities, which could be responsible for this activity. However, the production of this siderophore had never been previously described in *Trichoderma* and its insecticidal capacity was unknown. In conclusion, *T. hamatum* presents entomopathogenic capacity against *S. littoralis* larvae through the application of spores and filtrates, and both ways could be the basis for the development of efficient bioinsecticides against the pest.

1. Introduction

Spodoptera littoralis or cotton leaf worm (Boisduval) (Lepidoptera: Noctuidae) is a highly polyphagous pest, whose larvae can feed on 87 plant species of great economic importance, especially Solanaceae, Brassicaceae vegetables, artichoke, strawberry, corn and cotton (CABI, 2022). Currently, *S. littoralis* is present throughout Africa, southern Europe and Asia Minor, climatic zones where winter frosts are rare (CABI, 2022). Since the pest can spread even into the temperate zone due to the international transport of ornamentals and vegetables, EPPO has listed *S. littoralis* as a quarantine pest A2 (OEPP/EPPO, 2015). In Europe, the pest is a serious phytosanitary problem when it appears in greenhouses, causing important losses in vegetable and ornamental crops (CABI, 2022). Nowadays, the most widespread method for *S. littoralis* control is the use of chemical insecticides (benzoylureas, oxadiazines, pyrethroids, pyrazoles, spinosins, carbamates or

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organophosphates), although the insect develops resistance quickly and effectively (EFSA-PLH, 2015; CABI, 2022).

The need to develop new insect pest control strategies in agriculture highlights biological control as the most effective, safe and environmentally friendly alternative. In this sense, the use of entomopathogenic fungi is spreading within the development of sustainable agriculture and of new integrated pest management strategies (Mantzoukas and Eliopoulos, 2020). The main fungal genera used as bioinsecticides are *Beauveria*, *Metarhizium*, *Paecilomyces*, *Hirsutella*, *Verticillium*, or *Nomuraea*, being *Beauveria bassiana* the most widely used fungus in the world (Sinha et al., 2016). Entomopathogenic fungi can act against pest insects by direct infection, by entering the interior of the animal, colonizing it internally until death, or by various indirect mechanisms. Direct infection requires fungal enzymes that degrade the cuticle of the insect to actively penetrate, or entry through natural orifices (mouth, anus, or spiracles). These fungi can also produce a wide diversity of directly toxic or repellent/anti-feedant secondary metabolites, can attract predators/parasitoids by producing volatiles, or activate plant defenses (Sinha et al., 2016; Poveda, 2021a). In addition, many of these fungi have an endophytic lifestyle, providing their host plants with other important benefits, such as plant growth promotion, pathogen antagonism, mycoremediation, or the fight against attack by other herbivores (e.g., synthesis of toxic metabolites) (Dara, 2019; Litwin et al., 2020). Therefore, entomopathogenic fungi are being studied in recent years for the development of effective bioinsecticides against pests within the genus *Spodoptera* (Guo et al., 2020; Paredes-Sánchez et al., 2021). Specifically, against *S. littoralis*, the following species have been described as effective entomopathogenic fungi: *B. bassiana* (Fergani and Refaei, 2021), *Metarhizium anisopliae* (El Husseini, 2019), *M. brunneum* (Garrido-Jurado et al., 2020), *Isaria fumosorosea* (syn. *Paecilomyces fumosoroseus*) (Hussein et al., 2013), and different specific endophytic fungi (Saad et al., 2019; El-Sayed et al., 2020).

Trichoderma is a genus of filamentous fungi widely distributed worldwide as a plant symbiont that colonizes the rhizosphere and/or plant tissues (endophyte) (Abdullah et al., 2021). This fungal genus includes species widely studied and used as biological control agents (BCAs) in agriculture, mainly against soilborne fungal pathogens (Ferreira and Musumeci, 2021). Besides as BCA, *Trichoderma* is used in agriculture as a growth and yield promoter of crops (Poveda et al., 2019; Tyśkiewicz et al., 2022), as an inducer of tolerance under conditions of abiotic stresses (such as salinity or drought) (Poveda, 2020), and/or as an inducer of systemic plant defenses (Poveda et al., 2020a). In 2021, we published the first review so far on the use of *Trichoderma* as a bioinsecticide for agricultural pests (Poveda, 2021b). There are several *Trichoderma* species described as effective entomopathogenic agents, both through direct (infection, antibiosis, anti-feeding, etc.) and indirect (systemic activation of plant defenses) mechanisms. Among these species, *T. harzianum* and *T. atroviride* stand out as the most studied, and there is no work to date on the species *T. hamatum* as an entomopathogen (Poveda, 2021b).

T. hamatum strain H690 was isolated as an endophyte from kale (*Brassica oleracea* var. *acephala*) roots (Poveda et al., 2020b). After reinoculation on kale, its ability to activate plant systemic defenses against pathogen attack on leaves, specifically against the bacterium *Xanthomonas campestris* pv. campestris, was reported (Poveda et al., 2020b). Subsequently, the ability of *T. hamatum* to promote the growth of different *Brassica* leaf crops, increasing the glucosinolate content and the antioxidant potential of these functional foods, by colonizing their roots, has been described (Velasco et al., 2021).

In view of the great potential of *Trichoderma* species to be used as effective bioinsecticides against agricultural insect-pests and of the benefits for their host plant reported for *T. hamatum*, the main objective of this work is to analyze the possible entomopathogenic capacity of *T. hamatum* on *S. littoralis* larvae. For this purpose, *T. hamatum* was applied on larvae using different forms (spores and filtrates) and following different methodologies (topical or oral application), trying to identify its effectiveness as bioinsecticide and the biochemical, metabolomic and genomic mechanisms of action involved.

2. Materials and methods

2.1. Biological material

The microorganisms used were the filamentous fungi *T. hamatum* (MT641233 and OL389793 sequences) and *B. bassiana*. The endophytic fungus *T. hamatum* was isolated from kale roots in Galicia (Northwestern Spain) (Poveda et al., 2020b). *B. bassiana* isolated from the commercial product NATURALIS® (BIOGARD, Barcelona, Spain) was used as a control entomopathogenic fungus for infection studies. Both fungi were routinely grown on potato dextrose agar (PDA, Sigma-Aldrich, Madrid, Spain) in the dark at 25 °C.

S. littoralis L_3 larvae, from the Insectarium of the Public University of Navarra, were used for all tests. The *S. littoralis* population is maintained in the Insectarium under controlled conditions of photoperiod (16 h of light and 8 h of darkness), temperature (25 ºC) and relative humidity (RH 50%), fed with a semisynthetic diet based on wheat germ (Greene et al., 1976), with modifications (Table S1).

2.2. Spore infection assays

Fungal spores were obtained from one-week-old (*T. hamatum*) and two-week-old (*B. bassiana*) Petri-PDA plates, at which time the entire surface is completely covered with mycelium and spores. Five mL of sterile milli-Q water was added per plate, scraping the surface to release all spores. The mycelium and spore solutions were passed through a Miracloth filter (22–25 μm pore size) (Calbiochem®, Darmstadt, Germany), obtaining a suspension with all collected spores, free of mycelium and PDA debris. The number of spores per mL was determined using a hemocytometer.

For the study of fungal spore infection, two types of approaches were used: topical application and dietary application. Topical application used *T. hamatum* and *B. bassiana* spore concentrations of $1 * 10^4$-10^{10} spores mL^{-1} (seven different concentrations), while dietary application used concentrations of $1 * 10^4$-10^8 spores mL^{-1} (five different concentrations). All treatments were performed on 28 larvae of *S. littoralis* L_3, in triplicate (84 larvae in total per treatment).

Topical application of the spores was performed in 90 mm Petri dishes. Twenty-eight *S. littoralis* L_3 larvae per plate were deposited and 5 mL of a spore suspension of each fungus in sterile milli-Q water were applied to each of the concentrations (10^4-10^{10} spores mL^{-1}) and incubated for 5 min. Oral application of the spores was performed directly on the diet. From each of the spore suspensions/concentrations of both fungi (10^4-10^8 spores mL^{-1}) 35 μL were applied on the individual diet of each larva. The containers were then tilted from one side to the other in order to homogenize the solution over the entire surface of the diet and allowed to dry for 5–10 min. Larvae from both types of treatments were deposited individually in 5 mL plastic containers with ~500 μL of semi-synthetic diet (Greene et al., 1976; Table S1). Larvae were kept in darkness, at 25 ± 2 °C, for 10 days, when the mortality was quantified. Both topical and oral spore application trials used two controls without spores: applying the same amount of water without spores (C+W) and without applying water (C).

Fungal-induced mortality results were subjected to Probit analysis using the Polo-Plus program (LeOra Software, 1987). The percentages of mortality obtained for each spore concentration and fungi were statistically analyzed by Tukey's test, while the comparison between topical and oral application was carried out using Student's t-test, using the statistical program SPSS vr. 27 (IBM Informatics, Armonk, USA).

2.3. Quantification of fungal colonization in S. littoralis larvae

To quantify the internal colonization of *S. littoralis* larvae by

T. hamatum, the qPCR technique was used, following the methodology described by Poveda (2021c), with modifications. Larvae exposed to fungal spores (previous section) were superficially washed with sterile milli-Q water, immediately frozen in liquid nitrogen, lyophilized and pulverized.

From 100 mg of larvae tissues, DNA was extracted using the cetyltrimethyl-ammonium bromide (CTAB) extraction method. For qPCR, a mix was prepared in a 10 μL volume using 5 μL of Brilliant SYBR Green QPCR Master Mix (Roche), 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* genes from *Trichoderma* and *S. littoralis* were used as reference genes, whose primers are listed in Table 1. Amplifications were performed in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) programmed for 40 cycles under the following conditions: denaturation, 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 three pools of 28 insects per treatment. Cycle threshold values served to calculate the amount of fungal and insect DNA using standard curves from insects without fungal infection and *T. hamatum* mycelium from a PDA plate. Values of *Trichoderma* DNA were referred to the amount of *S. littoralis* DNA in every corresponding sample. The *Trichoderma*-insect colonization obtained for each spore concentration were statistically analyzed by Tukey's test, using the statistical program SPSS vr. 27 (IBM Informatics).

2.4. *T. hamatum* liquid culture

To obtain the secondary metabolites that *T. hamatum* could produce when recognizing *S. littoralis* larvae, liquid culture of the fungus was performed under different conditions. All cultures were grown in 50 mL of potato dextrose broth medium (PDB, Sigma-Aldrich, Madrid, Spain) on an orbital shaker at 150 rpm for 7 days. Each culture condition was performed with 5 replicates. The culture conditions were:

- PDB: culture medium only.
- PDB+Th: culture medium inoculated with 2.5×10^5 spores mL^{-1} of *T. hamatum*.
- PDB+Chi: culture medium with 0.75 g of chitin (from shrimp shells) (Sigma-Aldrich, Madrid, Spain).
- PDB+Th+Chi: culture medium with 0.75 g chitin, inoculated with *T. hamatum* spores.
- PDB+Sl: culture medium with 0.75 g of *S. littoralis* larvae frozen in liquid nitrogen, freeze-dried and powdered.
- PDB+Th+Sl: culture medium with 0.75 g of *S. littoralis* larvae inoculated with *T. hamatum* spores.

The addition of chitin or *S. littoralis* powder was intended to determine whether the activity of *T. hamatum* was modified by sensing the insect or by sensing chitin. After 7 days, all cultures were passaged through a sterile 0.2 μm filter (Sigma-Aldrich, Madrid, Spain). The filtrates were stored at − 20 °C until used for topical and oral application on *S. littoralis* larvae and for enzyme activity.

2.5. Chitinase activity analysis

Chitinase enzyme activity was assayed in *T. hamatum* liquid culture filtrates (50 μL) using the commercial Chitinase Assay Kit (Sigma-Aldrich, Madrid, Spain), which allows quantification of endochitinase and exochitinase (chitobiosidase and β-N-acetylglucosaminidase) activity. The analysis was based on the enzymatic hydrolysis of different substrates at 4.8 pH, releasing p-nitrophenol, which can be quantified colorimetrically at 405 nm (Multiskan SkyHigh Microplate Spectrophotometer; Thermo Fisher, Waltham, USA). The substrates used were 4-Nitrophenyl N,N′-diacetyl-β-D-chitobioside (chitobiosidase activity), 4-Nitrophenyl N-acetyl-β-D-glucosaminide (β-N-acetylglucosaminidase activity) and 4-Nitrophenyl β-D-N,N′,N″-triacetylchitotriose (endochitinase activity). The enzyme activity is represented as units mL^{-1}, data statistically analyzed by Tukey's test, using the statistical program SPSS vr. 27 (IBM Informatics). The analysis was performed in triplicate from filtrates of five different cultures per condition.

2.6. Filtrates application on larvae

In order to determine whether *T. hamatum* could produce insecticidal secondary metabolites (repellent or toxic), the different filtrates obtained previously were applied topically and orally on *S. littoralis* L_3 larvae. For this purpose, a methodology similar to that carried out with fungal spores was used. The filtrates were applied at different concentrations: intact filtrate and dilutions with sterile milli-Q water 1:10, 1:100 and 1:1000.

For topical application, 250 μL of each filtrate and dilution were applied on 14 *S. littoralis* L_3 larvae in cell culture plates (Corning™ Costar™ 6-well, Thermo Fisher, Waltham, USA), holding them for 5 min. Oral application of the filtrates was performed by applying 35 μL of each of the filtrates and dilutions to the semi-synthetic diet of *S. littoralis* L_3 larvae. The containers were then tilted from one side to the other in order to homogenize the solution over the entire surface of the diet and allowed to dry for 5–10 min. All treatments were performed on 28 larvae of *S. littoralis* L_3, in triplicate (84 larvae in total per treatment).

Larvae from both types of treatments were deposited individually in 5 mL plastic containers with ~500 μL of semi-synthetic diet (Greene et al., 1976; Table S1). Larvae were kept in darkness, at 25 ± 2 °C, for 10 days, when the weight was quantified. Both topical and oral filtrates application trials used two controls without filtrates: applying the same amount of water without filtrates (C+W) and without applying water (C). In both types of application, the weight of the larvae was measured at the beginning and at the end of the trial, comparing the weight gain with that obtained in the C larvae. The relative decrease in larval weight gain for each filtrate dilution were statistically analyzed by Tukey's test, while the comparison between all treatments (filtrates and dilutions) and C+W was carried out using Student's t-test, using the statistical program SPSS vr. 27 (IBM Informatics, Armonk, USA).

2.7. Metabolomic analysis

Metabolite analysis was performed using the methodology previously described by Poveda et al. (2021). Filtrate (50 mg) was dissolved in 500 mL of 80% aqueous methanol and then centrifugated 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 a ultra–performance liquid chromatography (UHPLC) (Thermo Dionex Ultimate 3000 LC; Thermo Fisher Scientific, Waltham, MA, USA) coupled

Table 1
Oligonucleotides used in this work.

Code	Sequence (5′−3′)	Use	Reference
Act-T-F	TGAGAGCGGTGGTATCCACG	*Trichoderma* endogenous gene	Wang et al. (2021)
Act-T-R	GGTACCACCAGACATGACAATGTTG		
Act-S-F	ATCATGTTCGAGACCTTCAAC	*S. littoralis* endogenous gene	Hamama et al. (2016)
Act-S-R	GCACGATTTCTCTCTCGG		

with electrospray ionization quadrupole time–of–flight mass spectrometry (ESI–Q–TOF–MS/MS) (Bruker Compact™). Chromatographic separation was performed in 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), from 3% to 25% B (4–16 min), from 25% to 80% B (16–25 min), from 80% to 100% B (25–30 min), hold 100% B until 32 min, from 100% to 3% B (32–33 min), hold 3% B until 36 min. The injection volume was 5 μL, the flow rate was established at 0.4 mL/min and column temperature was controlled at 35 °C. MS analysis was operated in spectra acquisition range from 50 to 1200 *m/z*. Both polarities (±) of ESI mode were used under the following specific conditions: gas flow 9 L/min, nebulizer pressure 38 psi, dry gas 9 L/min, and dry temperature 220 °C. Capillary and end plate offset were set to 4500 and 500 V, respectively. The instrument was calibrated externally with a calibration solution of 1 mM sodium formate/acetate in iPrOH/H_2O 50/50 with 0.2% formic acid directly infused to the source. Before sample injections, LC-qTOF system stability was tested by three consecutive injections of chloramphenicol (ESI – mode; ΔRT= 0.02 min; $\Delta m/z$ = 0.002) and triphenyl phosphate (ESI + mode; ΔRT= 0.02 min; $\Delta m/z$ = 0.001). The calibration solution was injected at the beginning of each run and all the spectra were calibrated prior to statistical analysis. MS/MS analysis was performed based on the previously determined accurate mass and RT and fragmented by using different collision energy ramps to cover a range from 15 to 50 eV. The algorithm T–Rex 3D from the MetaboScape 4.0 software (Bruker Daltonics, Billerica, MA, USA) was used for peak alignment and detection.

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.

To compare the performance of the four treatments, a partial least squares discriminant analysis (PLS-DA) was done to find the metabolic differences between the different groups. PLS-DA models were cross-validated using R2 and Q2 parameters. The quality assessment (Q2) and R-squared (R2) statistics provide a quantitative measure of consistency between the predicted and original data, or in other words, estimates the predictive ability of the model. A PLS-DA model is believed to be reliable when Q2 > 0.5 and R2 > Q2. The PLS-DA model using the three first principal component of VIP (variable importance in the projection) values was used to find differentially expressed metabolites. Based on VIP > 2, metabolites related to the treatment (Th + insect) were distinguished. Besides, Univariate analysis (one-way ANOVA) with a p value ≤ 0.05 was carried out to find differentially expressed metabolites followed by a mean comparison. From the VIP list, only metabolites which concentration was significant higher for Th + insect treatment than the rest of the treatments were selected.

For tentative identification, a consensus molecular formula was assigned to each molecular feature based on exact mass data and isotopic pattern distributions for the precursor using MetaboScape 4.0 and Sirius v4 (Dührkop et al., 2019) software. Molecular formula was used to perform identification analysis on publicly available databases: PubChem (Kim et al., 2019), MassBank (Horai et al., 2010), Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa et al., 2016), KNApSAcK (Afendi et al., 2012), Metlin (Guijas et al., 2018), and Chemspider (Ayers, 2012). When available, the ms/ms fragmentation spectrum of reference compounds identified on databases was compared to that obtained experimentally (Table 2).

Table 2
Tentative identification of metabolites selected after a PLS-DA analysis, responsible of the insecticidal capacity of *T. hamatum* filtrates.

Mass	RT (min)	mz	Adduct	VIP Score	Tentative name	Fragments
220.0562	0.93	203.0524	M - H_2O + H	5.21	–	50.049, 56.049, 70.064, 84.081
436.1337	4.28	437.1405	M + H	4.21	Rhizoferrin	182.081, 199.108, 245.114, 319.093, 355.113, 373.1211

3. Results

3.1. Direct infection of S. littoralis larvae with fungal spores

In order to test the direct infection capacity of *T. hamatum* on *S. littoralis* larvae, fungal spores were applied topically and orally, using the fungus *B. bassiana* as a positive infection control.

In topical application, the concentration of 10^4 spores mL^{-1} had no effect on larval mortality with any of the fungi. The concentration of 10^5 spores mL^{-1} reported mortalities of 12–19% for both fungi, increasing with increasing spore concentration to 43–50% mortality with a concentration of 10^{10} spores mL^{-1}. In the case of both fungi, there are no significant differences between the concentrations 10^8 and 10^{10} spores mL^{-1}. In topical application, there were no significant differences in mortality caused by both fungi at the same spore concentration (Fig. 1a). Since it was not possible to obtain data on mortality percentages higher than 50% in topical spore application, it was not possible to determine the LC_{50} for any of the fungi used. Therefore, it is predicted that the LC_{50} would be greater than 10^{10} spores mL^{-1}. However, we were unable to obtain fungal concentrations greater than 10^{10} spores mL^{-1}.

In the oral application (in the diet), with the concentration 10^4 spores mL^{-1} there was larval mortality (36–52%), increasing according to the concentration up to mortality percentages of 77–85% with 10^7 spores mL^{-1}. At 10^4 and 10^5 spores mL^{-1} the fungus *B. bassiana* caused higher mortality (52% and 55%, respectively) than *T. hamatum* (36% and 50%, respectively). However, at 10^8 spores mL^{-1} *T. hamatum* caused significantly higher larval mortality (85%) than *B. bassiana* (77%) (Fig. 1). Regarding the way of applying the spores to the larvae, oral application always reports significantly higher mortality percentages than those obtained with topical application (Fig. 1a). Fig. 1b shows larvae infected by *T. hamatum,* where their bodies have been colonized by the fungus, which sporulates on the cadaver.

In the oral application of spores, LC_{50} and relative potencies were calculated for both fungi. The test for equality (χ^2 =5.11; l.g.=2; $P = 0.077$) and parallelism (χ^2 =2.11; l.g.=1; $P = 0.146$) were statistically equal, therefore, *B. bassiana* and *T. hamatum* exhibited the same activity (Table 3).

To know the levels of colonization of *S. littoralis* tissues by *T. hamatum*, DNA quantification by qPCR was performed. Regarding the topical application of spores, the concentration of 10^4 spores mL^{-1} did not report fungal colonization of the insect. The concentration of 10^5 spores mL^{-1} reported colonization levels (0.12), which increased along with the spore concentrations, until 10^8-10^{10} spores mL^{-1}, which maintained fungal colonization without increasing significantly (0.44–0.48) (Fig. 2).

In *T. hamatum* spores' oral application, all concentrations (10^4-10^8 spores mL^{-1}) reported colonization of *S. littoralis* larvae. Increasing spore concentration application reported significantly higher levels of fungal colonization, with the greatest statistical differences between 10^4, 10^6 and 10^8 spores mL^{-1} (0.38, 0.57 and 0.89, respectively) (Fig. 2). When

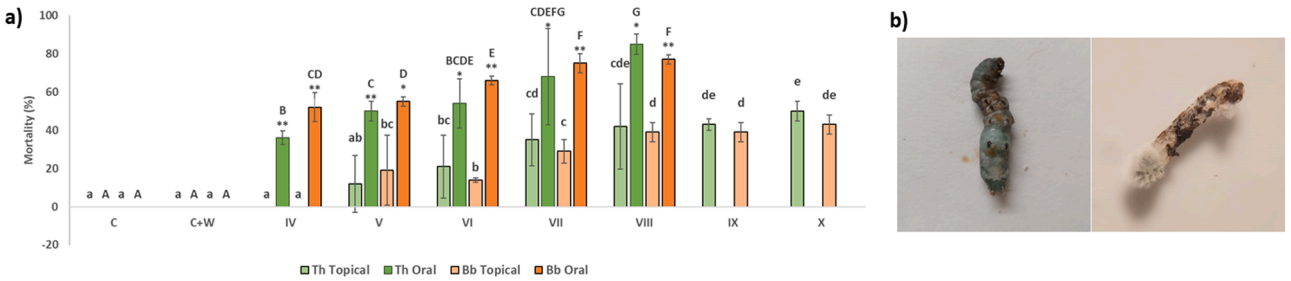

Fig. 1. : Percentages of mortality of *S. littoralis* L_3 larvae (a) by topical or oral application of *T. hamatum* (Th) or *B. bassiana* spores at different concentrations: 10^4 (IV), 10^5 (V), 10^6 (VI), 10^7 (VII), 10^8 (VIII), 10^9 (IX) and 10^{10} (X) spores mL^{-1}. Data are the mean of 84 larvae with the corresponding standard deviation. Tukey's test was performed between spore concentrations and fungi; different letters represent significant differences ($P < 0.05$) (lower case letters for topical application and upper case letters for oral application). Student's t-test was performed between topical and oral treatments; asterisks denote significant differences at $P \leq 0.05$ (*) or $P \leq 0.01$ (**). Representative photographs of symptoms observed in *S. littoralis* larvae infected by *T. hamatum* (b).

Table 3
Probit analysis of concentration–mortality responses used to estimate LC_{50} and relative potency values for *T. hamatum* and *B. bassiana* spores in oral application on *S. littoralis* larvae.

Fungus	LC_{50} (spores mL^{-1})	95% Conf. Interval		Relative potency	95% Conf. Interval	
		Lower	Upper		Lower	Upper
B. bassiana	8.31×10^4	$1{,}14 \times 10^2$	$7{,}88 \times 10^5$	-	-	-
T. hamatum	$1{,}6 \times 010^6$	$3{,}50 \times 10^4$	$1{,}11 \times 10^7$	0,05	0002	1,58

Relative potency values were calculated as the ratio of LC50 values relative to that of B. bassiana.

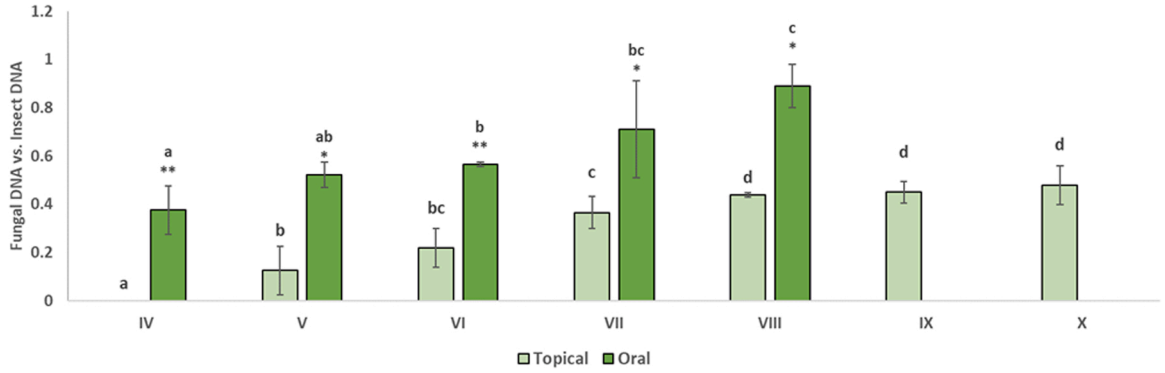

Fig. 2. Quantification of *T. hamatum*-insect tissue-colonization in *S. littoralis* infected larvae by topical or oral application of spores at different concentrations: 10^4 (IV), 10^5 (V), 10^6 (VI), 10^7 (VII), 10^8 (VIII), 10^9 (IX) and 10^{10} (X) spores mL^{-1}. Columns represent the values of fungal DNA vs. insect DNA ratio being the means of 3 pools (from three independent experiments) with 28 larvae each, with corresponding standard deviations. Tukey's test was performed between spore and fungal concentrations; different letters represent significant differences ($P < 0.05$). Student's t-test was performed between topical and oral treatments; asterisks denote significant differences at $P \leq 0.05$ (*) or $P \leq 0.01$ (**).

comparing the levels of fungal colonization between the different ways of applying the spores, significantly higher levels of colonization were always obtained with the oral application (Fig. 2).

3.2. Chitinase activity of T. hamatum

In order to test whether exposure of *T. hamatum* to *S. littoralis* larvae increased fungal chitinase activity (related to its ability to penetrate the insect body), this enzyme activity was measured in liquid culture filtrates using a commercial kit. The culture medium alone (PDB) and with chitin (PDB+Chi) did not report chitinase activity. The culture medium with larval *S. littoralis* tissues (PDB+Sl) showed reduced chitinase activity, without significant differences with the two previous ones. In the three enzyme activities analyzed, the culture medium with growing *T. hamatum* (PDB+Th) reported a significant increase (2.14–5.35) in comparison with PDB, PDB+Chi or PDB+Sl. With the addition of *S. littoralis* tissues to culture medium with growing *T. hamatum* (PDB+Th+Sl), no significant differences were quantified (2.56–5.17) in comparison with the same culture without *S. littoralis*. The culture medium with chitin and growing *T. hamatum* (PDB+Th+Chi) had the highest activity of the three enzymes (6.89–9.56), being significantly higher than the rest of the filtrates (Fig. 3).

3.3. Filtrates effect on S. littoralis larvae

In order to determine whether *T. hamatum* produces secondary metabolites with insecticidal capacity, topical and oral application of fungal liquid culture filtrates on *S. littoralis* L_3 larvae was performed. A dose-effect was not determined for either route of application (Fig. 4). However, in the topical application of the filtrate of *T. hamatum* growing in PDB medium with *S. littoralis* tissues (PDB+Th+Sl), the application of the insect filtrate reported a significantly greater reduction in larval weight increase than the 1:10 (1:X) dilution, which in turn was significantly greater than the 1:1000 (1:M) dilution (Fig. 4a).

On topical application of filtrates, the 1:10 (1:X) and 1:1000 (1:M) doses of the filtrate of *T. hamatum* growing in PDB medium with chitin

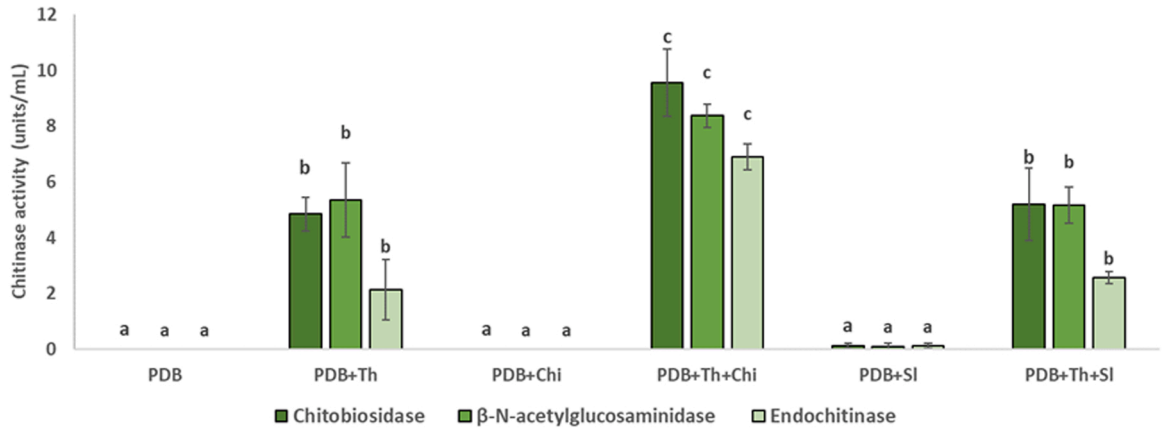

Fig. 3. : Chitinase activity of different filtrates of liquid cultures of *T. hamatum* (Th) in PDB medium with/without chitin (Chi) or *S. littoralis* tissues (Sl). Endochitinase and exochitinase (chitobiosidase and β-N-acetylglucosaminidase) activities were analyzed. Data are the mean of 15 measurements (5 filtered in triplicate) with the corresponding standard deviation. Tukey's test was performed between filtrates; different letters represent significant differences ($P < 0.05$).

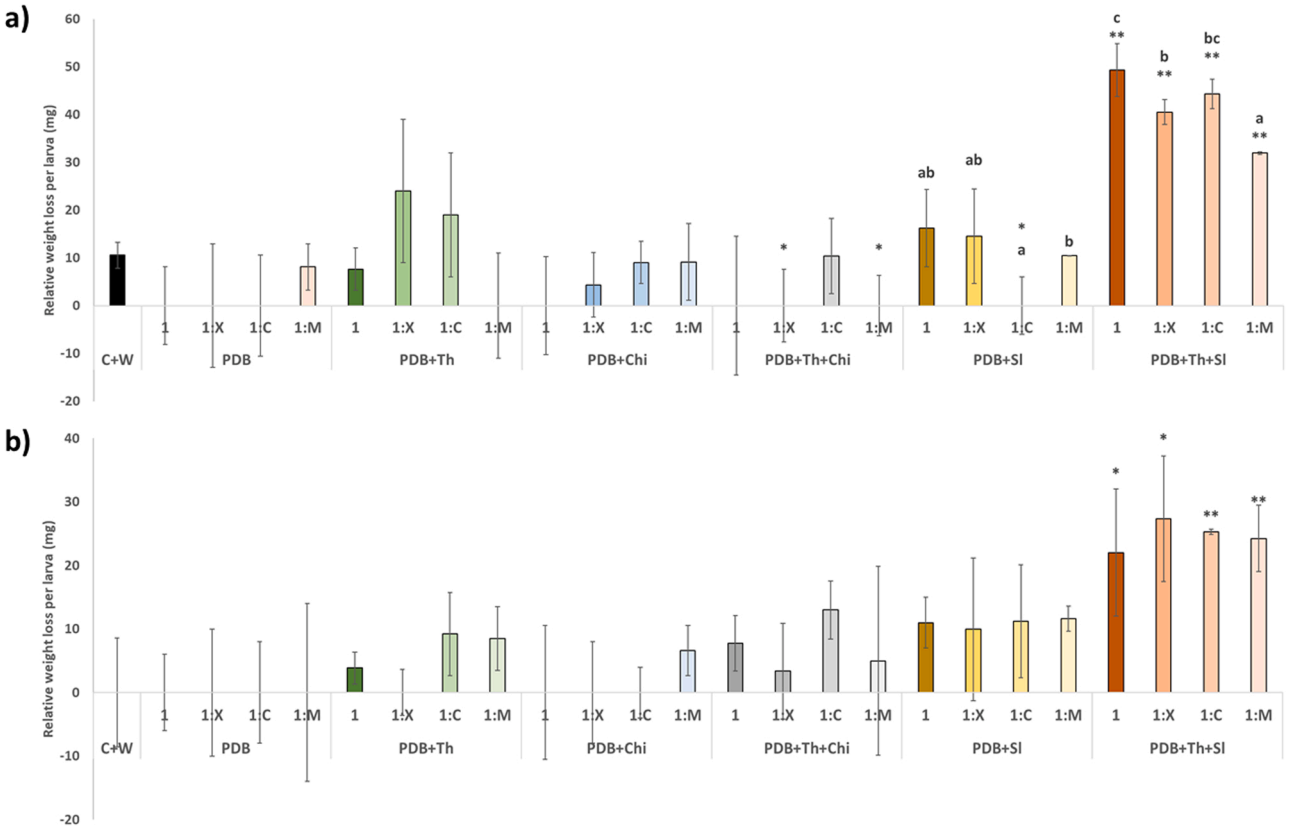

Fig. 4. : Relative weight loss of *S. littoralis* larvae exposed to different filtrates topically (a) and orally (b). The filtrates consisted of PDB medium (PDB), with chitin (+Chi) or *S. littoralis* larvae tissues (+Sl) and with growing *T. hamatum* (+Th), being applied intact (1) or with different dilutions 1:10 (1:X), 1:100 (1:C) and 1:1000 (1:M). Weight gains were relative to those obtained with control larvae. Data are the mean of 84 larvae with the corresponding standard deviation. Tukey's test was performed between filtrate dilutions; different letters represent significant differences ($P < 0.05$). Student's t-test was performed between filtrates and water control (C+W); asterisks denote significant differences at $P \leq 0.05$ (*) or $P \leq 0.01$ (**).

(PDB+Th+Chi) and the 1:100 (1:C) dose of PDB medium with *S. littoralis* tissues (PDB+Sl) reported a significant increase in larval growth (Fig. 4a). In both topical and oral application, all doses of filtrate of T. *hamatum* growing in PDB medium with *S. littoralis* tissues (PDB+Th+Sl) caused a significant decrease in weight gain of *S. littoralis* larvae (Fig. 4).

3.4. Metabolomics of T. hamatum filtrates

An untargeted metabolomics analysis was made to search the metabolites implicated in the higher resistance of PDB+Th+Sl filtrates, compared to the other treatments. PLS-DA analysis gave the best model with 3 principal components, explaining a 81.5% of the variance. A PLS-DA model is believed to be reliable when Q2 > 0.5 and R2 > Q2. In this case, value of Q2 = 0.88 and R2 = 0.96. Besides, permutation test using separation distance model had a p = 0.07. So, these results indicate a good prediction power of the model. In the Fig. 5, a plot with the 3 first components is shown.

From this analysis, we selected 63 features with a VIP score > 2.

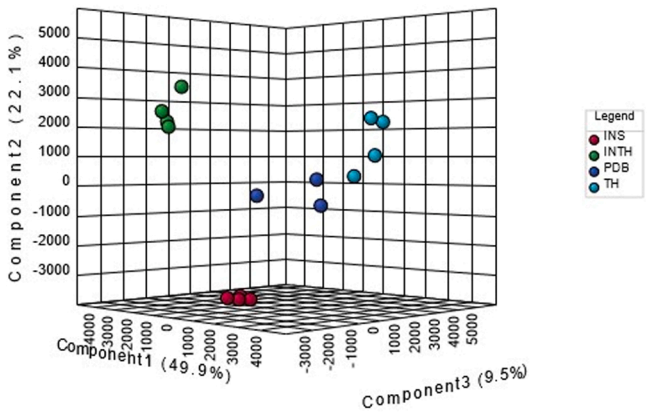

Fig. 5. : Score-plot of the three principal components from a PLSD analysis of the untargeted metabolomics. The four treatments used in the analysis were PDB medium (PDB), with *S. littoralis* larvae tissues (INS) and with growing *T. hamatum* (TH). Four biological replicates were used per treatment.

These features were subjected of an ANOVA and Posthoc Fisher's LSD test to select only those metabolites significantly higher in the treatment PDB+Th+Sl than any other treatment. Only two features were selected simultaneously as shown in Table 2 and Fig. 6.

4. Discussion

The environmental and health problems associated with chemical pesticides (Budzinski and Couderchet, 2018) make it necessary to look for new alternatives in the control of agricultural pests, being entomopathogenic fungi a potential alternative (Rajula et al., 2021). In this sense, the fungal genus *Trichoderma* includes several species described as effective biological control agents of agricultural pests (Poveda et al., 2021b). However, the species *T. hamatum* has never been previously described as entomopathogenic, being our work the first approach, using the insect pest *S. littoralis*.

In the infection of *S. littoralis* larvae with fungal spores of *T. hamatum* and *B. bassiana* we were able to determine that both fungi cause very similar insect mortality data by topical or oral application. Being aware that *B. bassiana* is one of the most effective entomopathogenic fungi currently on the market and in the scientific landscape (Mahankuda and Bhatt, 2019; Amobonye et al., 2020), the results obtained with *T. hamatum* pose a great potential as a bioinsecticide.

Within the topical application of the spores, we did not report significant differences between the concentrations 10^8-10^{10} spores mL^{-1}, being 10^8 the maximum concentration effective against *S. littoralis* larvae, although without exceeding 50% mortality. In the case of *B. bassiana*, its ability to infect *S. littoralis* L_3 larvae with mortality of up to 90% at 10^9 spores mL^{-1} has been previously described, however, our commercial isolate did not report such efficacy (Fergani and Refaei, 2021). In the case of *T. hamatum*, our study is the first performed with this fungal species and with *S. littoralis* and any *Trichoderma* species, but a previous study with the species *T. longibrachiatum* and the lepidopteran *Leocinodes ordinalis* obtained mortality percentages of 85% with 10^8 spores mL^{-1} (Ghosh and Pal, 2016).

The application of spores orally (through the diet) reports significantly higher mortality data than topically, reaching percentages higher than 80%, which allowed the calculation of the LC_{50} for both fungi. These dietary efficacy results have been reported previously for *B. bassiana* colonizing wheat tissues and being attacked by *S. littoralis* larvae (Sánchez-Rodríguez et al., 2018). And for other *Trichoderma* species against other non-lepidopteran insects, such as *T. harzianum* spores on wheat grains consumed by *Sitophilus oryzae* beetle (Gad et al., 2020).

These results of mortality by topical and oral application of *T. hamatum* spores on *S. littoralis* larvae agree with those reported for the analysis of fungal colonization of larvae by qPCR. *T. hamatum* topically colonized the larval body in a reduced way compared to its oral application, obtaining its maximum colonization levels at a concentration of 10^8 spores mL^{-1}. These results suggest that the mechanism of *T. hamatum* entry into the insect body is favored by natural openings, as opposed to direct penetration through the action of chitinase enzymes. Therefore, the analysis of chitinase activity of *T. hamatum* in contact with *S. littoralis* was carried out. The chitinase activity of *T. hamatum* in the presence of *S. littoralis* was not increased, which was not the case if *T. hamatum* recognized chitin in the culture medium. These results, together with those obtained so far, lead us to believe that *T. hamatum* does not penetrate the body of *S. littoralis* by direct penetration of the cuticle, but must do so through natural openings, such as spiracles, mouth or anus. This hypothesis would also explain the higher degree of mortality reported for the oral application of spores, as opposed to the topical way.

In addition to fungal spore formulations, another formulation and application of entomopathogenic fungi as bioinsecticides can be by their

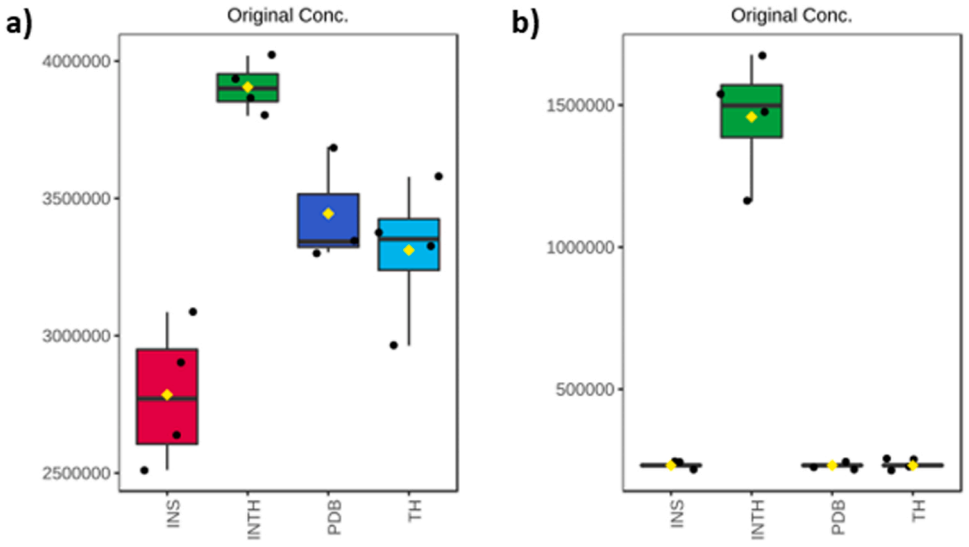

Fig. 6. : Concentration of the significant features after PLSDA and ANOVA analyses. a) concentration of the mass 220.05, and b) concentration of the mass 436.13 (rhizoferrin).

insecticidal (toxic or repellent) metabolites (Zhang et al., 2020). In our work we obtained different liquid culture filtrates from *T. hamatum* elicited with chitin or *S. littoralis* tissues. Of all the filtrates used, only those obtained from the culture medium in which *T. hamatum* had grown in the presence of *S. littoralis* tissues reported a decrease in larval size, both in oral and topical application. Therefore, *T. hamatum* would produce insecticidal secondary metabolites when recognizing the presence of *S. littoralis*. The production of insecticidal metabolites has been previously reported in other *Trichoderma* species against lepidopteran larvae, such as *T. harzianum* and *Pectinophora gossypiella* topically (El, Massry et al., 2016) and *T. viride* and *Helicoverpa armigera* orally (Chinnaperumal et al., 2018).

In the metabolomic analysis carried out, it was determined that the filtrate obtained from the culture medium with *T. hamatum* in interaction with *S. littoralis* tissues presents a metabolite in large quantities that is absent in the rest of the filtrates analyzed, rhizoferrin. Therefore, the insecticidal activity reported for these filtrates could be a direct consequence of this secondary metabolite. Rhizoferrin is a siderophore that was first isolated and described in the fungus *Rhizopus microsporus* var. *rhizopodiformis* (Drechsel et al., 1991). This siderophore is a carboxylate consisting of two molecules of citric acid linked to 1,4-diaminobutane through two amide bonds (Ghorai et al., 2021). Rhizoferrin is produced by fungi of the order Mucorales (division Zygomycota), being indispensable for their growth but not to produce disease (mucormycosis) in humans (Škríba et al., 2020).

Although rhizoferrin production has been reported in entomopathogenic zygomycetes, such as the order Entomophthorales (Thieken and Winkelmann, 1992), neither its role in the infection process nor its insecticidal capacity has been described so far. In our work we have been able to relate the insecticidal capacity of *T. hamatum* filtrates to the presence of rhizoferrin, however, until now, the production of this siderophore by *Trichoderma* species had never been described and its insecticidal capacity had never been reported. Therefore, our work represents the first description of rhizoferrin production by *Trichoderma* and its possible first relationship with insecticidal activity, requiring a more specific study with this molecule in the future for its absolute confirmation.

In conclusion, *T. hamatum* presents entomopathogenic capacity against *S. littoralis* larvae by direct infection by spores at similar levels to the commercial fungus *B. bassiana*, possibly being its route of infection through natural openings. In addition, *T. hamatum* produces insecticidal secondary metabolites upon recognition of *S. littoralis* tissues, and the siderophore rhizoferrin has been described as the main one involved in this activity. Although more research is needed on *T. hamatum* and its insecticidal capacity, both using spores and filtrates, effective bioinsecticides could be developed against *S. littoralis* and possibly other lepidopteran pests.

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CRediT authorship contribution statement

J.P. conceived the research idea and designed most of the experiments. M.L. and J.P. performed all the experiments and collected the data. M.L., O.S. and J.P. analyzed the data obtained. O.S. assisted in the experiments design. P.V. and V.M.R. performed the metabolomic analysis. P.C. obtained most of the funding used and contributed with the knowledge on the bioinsecticides field. J.P. wrote the manuscript. M.L., O.S., P.V., V.M.R. and P.C. contributed to the manuscript correction and critical reading. All authors have read and agreed to the published version of the manuscript.

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.

Data Availability

No data was used for the research described in the article.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.micres.2023.127334.

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