



Evaluation of *Trichoderma* isolates as biocontrol measure against *Claviceps purpurea*

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Abstract *Claviceps purpurea* causes ergot, a floral disease of major cereal crops, such as winter rye and winter triticale. The dark sclerotia formed by *C. purpurea* contain numerous toxic ergot alkaloids, which pose a major health risk for humans and animals when ingested. The countermeasures against ergot infection are currently limited to intensive tillage, seed cleaning, crop rotation or integration of optical sorting machines to remove ergot from cereals before processing. However, these practices confer only a minor protective effect and are highly energy-demanding. Thus, more effective and sustainable plant protection measures to combat ergot infections in cereals are needed. The application of *Trichoderma* spp. as biological control agent has already proven successful against a wide range of phytopathogenic fungi. However, there is only minor scientific evidence about its protective capacity against *C. purpurea* in cereal crops. In our study, we therefore investigated the antagonistic potential of

several *Trichoderma* isolates against *C. purpurea* and their ability to confer protection against ergot infection. In initial *in vitro* tests and confrontation assays, we quantified and compared the production of iron-chelating compounds and hydrolytic enzymes as well as the antagonistic activity against *C. purpurea* sclerotia and mycelial cultures. Several strains showed high antifungal capacity and growth inhibitory effects towards *C. purpurea*. Selected *Trichoderma* strains were then tested for their potential to protect rye (*Secale cereale*) plants from ergot infection within a greenhouse trial. Rhizosphere-inoculated plants displayed a decreased disease severity compared to plants that had been sprayed with *Trichoderma* spores above-ground. Gene expression analysis by reverse transcription quantitative PCR also indicated the induction of systemic defense reactions. Overall, our data suggest that individual *Trichoderma* isolates possess a high antagonistic potential towards *C. purpurea*, which could be effective by direct mycoparasitism and by inducing systemic plant resistance, and therefore provide important guidance towards the development of *Trichoderma* treatments of cereals as biocontrol measure against ergot.

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Introduction

Ergot, caused by the phytopathogenic ascomycete *Claviceps purpurea*, is a re-emerging plant disease affecting over 400 species of the *Poaceae* family, including economically important crops with rye most often reported (Lauber et al., 2005; Miedaner & Geiger, 2015). Ascospores and conidia of *C. purpurea* germinate on the stigma of flowering host plants. After infecting the ovaries, fungal mycelium differentiates into purplish-brown sclerotia replacing the host's ovarian tissue. While ergot infection seldom causes a significant decrease in crop yield, the most serious consequence is the contamination of harvested crops with ergot sclerotia containing various alkaloid toxins (EFSA Panel on Contaminants in the Food Chain, 2012). Ergot alkaloids are secondary fungal metabolites and are defined as derivatives of 4-(γ,γ dimethylallyl)tryptophan that are classified into three major groups as clavines, amides of lysergic acid and ergopeptines, based on their substituents (Agriopoulou, 2021; Florea et al., 2017).

By further processing the contaminated cereals, the mycotoxins enter the food and feed chain. Consumption can lead to typical clinical symptoms of ergot poisoning, such as strong vasoconstrictive and vasodilative effects, as well as severe neurological symptoms in humans and animals (Maruo et al., 2018; Merhoff & Porter, 1974). The disease caused by these alkaloids is called ergotism, also known as St. Anthony's fire, and first reports date back to the Middle ages (Grzybowski et al., 2021). Therefore, the European Commission amended maximum levels for the content of ergot sclerotia in unprocessed rye to 500 mg/kg and for other unprocessed cereals to 200 mg/kg (Commission Regulation (EU) 2021/1399). The distribution of individual ergot alkaloids in crop samples is highly variable and does not necessarily correlate with ergot sclerotia content (Grusie et al., 2017; Kodisch et al., 2020b). Therefore, the ergot alkaloid concentration, defined by the sum of the most frequent members of ergot alkaloids, namely ergotamine, ergometrine, ergosine, ergocristine, ergocryptine, ergocornine and their epimers, in milled grain products has been newly limited to 500 $\mu\text{g}/\text{kg}$ in rye and 100 $\mu\text{g}/\text{kg}$ in other cereals being further reduced by 2024 to 250 $\mu\text{g}/\text{kg}$ and 50 $\mu\text{g}/\text{kg}$, respectively (Commission Regulation (EU) 2021/1399).

A combination of physical and mechanical methods, based on gravity or optical separation, can be used to remove ergot sclerotia from cereals prior to processing, but these methods do not completely eliminate contamination with ergot alkaloids (Agriopoulou, 2021). Therefore, it is essential to apply countermeasures such as farming practices to reduce the incidence and severity of ergot formation.

The germination of overwintering structures of *C. purpurea* requires a period of cold temperatures during winter followed by a warmer period with high humidity in spring to facilitate spore production (Menzies & Turkington, 2015; Uppala et al., 2016). One of the current agricultural management strategies is therefore intensive ploughing to a depth of 5 cm to reduce the level of primary inoculum and hamper the ergot germination for following crops (Agriopoulou, 2021).

However, the fungus can survive in this dormant stage for up to three years in the soil. Crop rotation is therefore a recommended strategy to further reduce the amount of sclerotia (Agriopoulou, 2021; Menzies & Turkington, 2015). The risk of secondary infection and sclerotia formation by alternative hosts of the *Poaceae* family in the field margin can be reduced by application of herbicides or mowing. Preventive measures to counteract ergot infections include sowing of ergot-free seeds and avoidance of open-pollinated varieties with a long flowering period, which are more susceptible to ergot infections. In general, winter varieties are usually less susceptible than summer varieties, as the risk of secondary infections by neighboring alternative host plants is rather low (Menzies & Turkington, 2015). Another favorable approach is the cultivation of hybrid breeds with increased pollen production, which enhances the probability of fertilization and shortens the period of susceptibility (Kodisch et al., 2020a; Miedaner et al., 2022). Applying fungicides against ergot is less common and the main limitation of contact fungicides is the proper timing of the treatment, since to be functional, the compounds have to reach the plant's ovary (McLaren, 2003).

A commercial fungicide belonging to the triazole group was shown to be effective against *Claviceps africana* in sorghum, but multiple applications from first anthesis to the end of flowering period are required (Ryley et al., 2003), risking the development of resistance in phytopathogenic fungi. The

application of systemic antifungal compounds, which are taken up by the plant, is also restricted since the transport predominantly takes place through the xylem, limiting the amount of compounds reaching the ovary (Evans et al., 2000). However, soil-applied fungicides reduced the germination of ergot sclerotia but bears the risk to affect native beneficial microorganisms (Dung et al., 2018).

In contrast to the named countermeasures, biological control is taking advantage of beneficial organisms to control plant pests, which offers an additional strategy for a more sustainable and environmentally-friendly approach of disease management (Heydari & Pessaraki, 2010; Maruo et al., 2018; Stenberg et al., 2021; Waage & Greathead, 1988).

Trichoderma spp. (Ascomycota, Hypocreales, Hypocreaceae) are among the most commonly used fungal biocontrol agents against plant pathogens (Freeman et al., 2004; Sharma et al., 2011; Sood et al., 2020; Tyśkiewicz et al., 2022). During the versatile inter-kingdom interaction of *Trichoderma* spp. with host plants, both organisms are benefitting from this symbiotic relationship. While *Trichoderma* spp. receive photosynthates and habitat in the rhizosphere, they promote plant growth by increased nutrient availability and plant health by priming of plant defenses and alteration of root architecture (Guzmán-Guzmán et al., 2019; Naseby et al., 2000; Vinale & Sivasithamparam, 2020). The cellulolytic activity of *Trichoderma* spp. has been associated with the induction of local and systemic defense reactions during the root colonization process (Shoresh et al., 2010). Even though those plant growth-promoting properties are host plant specific and not present in every *Trichoderma* strain, it is widely common among the genus (Lorito & Woo, 2015). Further mechanisms for biological control by *Trichoderma* are the recognition and chemotrophic growth towards plant-pathogenic fungi, antibiosis through nutrient competition and release of cell wall degrading enzymes, and direct attack of fungal antagonists (Benítez et al., 2005; Degani & Dor, 2021; Sood et al., 2020). Mycoparasitism is a complex process which depends on a combination of various mechanisms, acting sequentially or combined (Viterbo & Horwitz, 2010).

The production of hydrolytic enzymes, such as proteases and chitinases, have been shown to have a crucial impact on their potential as biocontrol agents (Seidl et al., 2009). Several *Trichoderma*

species have already proven to be effective against fungal phytopathogens via various synergistic mechanisms, but scientific evidence of the biocontrol ability against ergot in rye is limited.

However, Bhosale et al. (2022) identified *T. viride*, followed by *T. hamatum* and *T. harzianum* as effective antagonists of *Claviceps fusiformis* *in vitro* by dual plate assays. Furthermore, a commercial biocontrol preparation of *T. viride* showed a 50% biological efficacy in germination inhibition of ergot sclerotia from *C. purpurea* *in vitro* (Sheshgova & Shchekleina, 2020). Another approach to identify potential antagonists of ergot is the isolation from the native mycoflora of the overwintering sclerotia structures. Ondřej et al. (2010) isolated fungal species from the surface of *C. purpurea* sclerotia and further tested them for their mycoparasitic effect on overwintering sclerotia structures in terms of germinating ability and microbial destruction. The highest effect of degradation *in vitro* was found by *T. harzianum*, but could not be verified under field conditions (Ondřej et al., 2010).

Bhuiyan et al. (2003) investigated the protective capacity of two commercial *Trichoderma*-based biocontrol products Trichopel and Trichoflow (Agrimm Technologies Limited, Christchurch, New Zealand) against *Claviceps africana* in *Sorghum bicolor*. Both culture filtrates significantly reduced conidial germination of *C. africana* macroconidia. Within a greenhouse trial, different mixtures of spores and culture filtrates of both biocontrol products were tested for their protective capacity against *C. africana* *in planta* and led to reduction of ergot severity. Summarizing, some few studies indicate biocontrol effects of *Trichoderma* spp. against *C. purpurea* vegetative growth or sclerotia germination, but were mostly performed *in vitro* (Bhosale et al., 2022; Ondřej et al., 2010; Sheshgova & Shchekleina, 2020).

The main purpose of this work was therefore to verify these observations *in vitro* and *in vivo* with several *Trichoderma* strains known for their biocontrol potential and additional isolates from agricultural land. Furthermore, the goal was to test whether any potential antagonistic effects against *C. purpurea* can be correlated to certain activities known to be associated with biocontrol and to evaluate if the antagonism against *C. purpurea* is a conserved or strain-specific property.

Materials and methods

Fungal cultures and growths conditions

For the isolation of *Trichoderma*, soil samples were taken from agricultural fields (Bavaria, Germany, 2022) and further processed as described previously (Mistry & Bariya, 2022). The obtained isolates as well as the following *Trichoderma* strains obtained from culture collection of the Chair of Phytopathology (Technical University of Munich), *T. harzianum* 20761 (A), *T. harzianum* 20770 (B), *T. atroviride* 20780 (C), *T. atroviride* 20781 (D), *T. hamatum* 20784 (E), *T. asperellum* 20866 (F) (Metz & Hausladen, 2022) and *T. harzianum* WM24a1 (G) (obtained from culture collection Austrian Institute of Technology GmbH) as well as *T. atrobrunneum* (H) (culture collection Fungal Biotechnology in Wood Science, Technical University of Munich) were routinely subcultured on ¼ potato dextrose agar (PDA) (X931.2, Carl Roth, Karlsruhe, Germany). For inoculum preparation, *Trichoderma* was grown on PDA for 7 days in darkness, followed by 3 days incubation with light to induce spore production. The spores were harvested by flooding the plates with sterile water and spores were carefully detached by using a spatula. The spore solution was then filtered through extra fine glass wool (Karl Hecht GmbH & Co KG, Sondheim, Germany) placed in a 50 ml tube to remove remaining mycelium debris.

The wild-type *C. purpurea* strain used in this experiments was isolated from sclerotium obtained of wheat cultivar (*Triticum aestivum*, Thuringia, Germany, harvest 2021) as described previously Faruk and Rahman (2022) and subcultured routinely on Mantle agar (Mantle & Nisbet, 1976). For inoculum production a pre-culture was prepared by inoculating 200 ml of 1% (w/v) wheat flour solution with five agar pieces containing mycelium and incubated at 25 °C for 10 days (Engelke, 2002). Rye grains were soaked in hot water for 30 min and autoclaved in 1200 ml mycocontainers (Mycogenetics, Everswinkel, Germany) and mixed with the fungal pre-culture. After two weeks of incubation, the overgrown grains were soaked in sterile water for 2 h and the spore suspension was then filtered through glass wool.

Molecular identification and phylogenetic analysis

Trichoderma isolates and *C. purpurea* were grown for 7 days on cellophane membranes (Natureflex 32 g/m², 80 mm diameter, HERA Papierverarbeitung, Nidda, Germany) placed on PDA and Mantle media, respectively and approximately 20 mg mycelium was scraped with a sterile spatula and transferred into a 1.5 ml tube for genomic DNA extraction using the Animal and Fungi DNA Preparation kit (PP-208S, Jena Bioscience GmbH, Jena, Germany) according to manufacturer's instructions. The quantity and quality of the obtained DNA was estimated with microplate reader (NanoQuant Plate, Tecan Infinite 200 Pro, Männedorf, Switzerland).

To confirm the identity of the fungal strains on molecular basis, a region of nuclear rDNA containing the ITS1-5.8S-ITS2 rRNA gene using the following primer pairs ITS 1: 5'-TCCGTAGGTGAA CCTGCGG-3' and ITS 4: 5'-TCCTCCGCTTAT TGATATGC-3' (White, 1990) was amplified.

For the verification of *Trichoderma* isolates the molecular identification protocol for a single *Trichoderma* isolate was applied following Cai & Druzhinina, 2021. Since ITS can only be used to identify *Trichoderma* on generic level, two additional phylogenetic markers translation elongation factor 1 α (*tef1*) and RNA polymerase B subunit II (*rpb2*) were amplified using the following primer pairs EF1: 5'-ATGGGTAAGGARGACAAGAC-3'; EF2: 5'-GGARGTACCAGTSATCATGTT-3' (O'Donnell et al., 1998) and fRPB2-5f: 5'-GAYGAYMGWG ATCAITTYGG-3' and fRPB2-7cr: 5' CCCATR GCTTGTYRCCCAT-3' (Liu et al., 1999).

PCR products were run on 1% agarose gel (1 × TAE buffer 100 ml + 5 μ l Midori Green; 140 V, 500 mA, 0.6 h) and corresponding bands were cut from the gel and purified using Hi Yield® Gel/ PCR DNA Fragment Extraction Kit (Süd-Laborbedarf GmbH, Gauting, Germany). DNA sequencing of amplified regions was performed in both directions using the Mix2Seq kit (Eurofins Genomics, Ebersberg, Germany) and consensus sequence was created by alignment of forward and reverse sequence using Benchling (Benchling, 2022, San Francisco, USA).

Phylogenetic analysis

Phylogenetic analysis of the obtained soil isolates of *Trichoderma* spp. was computed as described by Cai and Druzhinina (2021). Summarized, retrieved *tef1* and *rpb2* sequences were trimmed using *TrichoMark* 2020 and processed sequences were used for multiple sequences comparison by log-expectation using MUSCLE (Edgar, 2004). Phylogram data was computed from concatenated *tef1* and *rpb2* sequences using maximum-likelihood (ML) method in IQ-TREE web server (Nguyen et al., 2015; Trifinopoulos et al., 2016) with statistical bootstrapping of 1000 replicates and best-fit model TIM2e+G4 (Kalyaanamoorthy et al., 2017). Phylogenetic trees were visualized using the online tool Interactive Tree of Life (iTOL, version 5) (Letunic & Bork, 2021). Respective sequences of *Laccaria bicolor* KA13-0253 were included as outgroup.

In vitro screenings for biocontrol abilities

Siderophore production

The detection of fungal siderophores in liquid culture supernatants was performed according to Joshi et al. (2006). The assay is based on the formation of a blue colored complex between CAS, hexadecyltrimethylammonium (HDTMA) bromide (199,532, Sigma-Aldrich, Taufkirchen, Germany) and iron. Hydroxamate siderophores, present in the culture filtrate, scavenge the iron leading to the release of free CAS dye, visible in a color change from blue to orange, which can be determined spectrophotometrically. For the liquid CAS assay *Trichoderma* strains were inoculated with 10^6 spores/ml in 4 ml M9 Minimal Medium in 24-well-plates and incubated for one week at 30 °C, shaking at 150 rpm. The culture supernatants were obtained by filtration through glass fiber filters (Ø 55 mm, MN 85/70, MACHEREY–NAGEL, Düren, Germany) using a Büchner funnel. 100 µl of the culture filtrate were mixed with 50 µl CAS solution and after incubation in the dark for 2 h the absorbance was measured at 630 nm (Tecan Infinite 200 Pro, Männedorf, Switzerland). The uninoculated medium served as reference. The amount of produced Deferoxamine B equivalent was calculated from A/A_{ref} based on a standard curve prepared with 0 to

300 µM deferoxamine mesylate and normalized with dry biomass.

Cellulase activity

The determination of the cellulolytic activity of *Trichoderma* was conducted by a plate assay as described by Teather and Wood (1982). Summarized, the synthetic substrate Carboxymethyl cellulose (CMC) is stained with the direct dye Congo red. The hydrolysis of β-1,4-glycosidic linkages by cellulases, released into the medium, leads to the formation of a clearance halo around the fungal colony. *Trichoderma* was cultivated on solid CMC for 96 h at 25 °C followed by incubation at 45–50 °C for 18 h. Afterwards the plates were stained by adding 10 ml of a 0.2% Congo red solution (0322.1, ROTH, Karlsruhe, Germany) and incubated for 15–20 min. Staining solution was then removed by rinsing the plates with water followed by destaining with a 1 M NaCl solution for 15–20 min. Pictures were taken and the halo was measured using ImageJ V1.53e software (Wayne Rasby, National Institute of Health, USA, <http://imagej.nih.gov/ij>). The enzymatic index (EI) served as a quantitative measure of cellulase production. An EI of 1 signifies no cellulase activity and was therefore set to 0. Enzymatic indices were calculated using the following formula:

$$EI = \frac{\text{halo diameter}}{\text{colony diameter}} - 1$$

Proteolytic activity

To evaluate proteolytic activity, *Trichoderma* was inoculated on skim milk agar, previously described by Alnahdi (2012) and after incubation at 25 °C for 4 days the plates were photographed at the end. The clear zone halo, formed by hydrolyzed milk proteins, was then quantified using ImageJ V1.53e software based on pictures taken after incubation.

Chitinase activity

For the evaluation of total chitinolytic activity *Trichoderma* was inoculated with 10^6 spores/ml in 4 ml liquid chitinase detection medium as described by Agrawal and Kotasthane (2012). The cultures were

incubated at 30 °C and 150 rpm for one week and culture supernatants were obtained by filtration and used for the enzymatic assay. The reduction of 3,5-dinitrosalicylic acid to 3-amino-5-nitrosalicylic acid by the reducing sugar N-acetyl- β -D-glucosamine (NAGA) induces a shift in the absorbance at 540 nm, which serves as a quantitative measure for the NAGA concentration in the medium, a product of fungal chitinase activity (Miller, 1959). The amount of released free NAGA was determined spectrophotometrically by measuring the absorbance at 540 nm (Tecan Infinite 200 Pro, Männedorf, Switzerland) and correlating obtained values with generated standard curve of known concentrations of NAGA (A8625, Sigma-Aldrich, Taufkirchen, Germany).

Antagonistic activity

For *in vitro* antagonism assays in dual culture *C. purpurea* was inoculated on ¼ PDA in non-split and bi-compartment plates (\varnothing 9 cm, Sarstedt, Nümbrecht, Germany) and incubated for five days at 25 °C. *Trichoderma* strains were inoculated with spores on the opposite site of the plate in triplicates. Pictures were taken (Nikon, Tokyo, Japan) directly after inoculation with *Trichoderma* as well as 4 and 6 days afterwards, corresponding to pre-contact and direct contact, respectively. Plates inoculated only with *C. purpurea* served as a control. Colony area (cm²) was measured using ImageJ V1.53e software. The growth inhibition by *Trichoderma* for air contact, medium contact and direct contact stage was then calculated as follows (Raut et al., 2014):

$$\text{Growth inhibition (\%)} = \frac{(\Delta A_{\text{control}} - \Delta A_{\text{treated}})}{\Delta A_{\text{control}}} \times 100$$

Where ΔA describes the difference in the colony area of *C. purpurea* between the evaluated time point and the previous time point. A decrease in colony area of *C. purpurea* between two time points, is expressed by values above 100%.

Inhibition of germinating sclerotia

It is already known that some *Trichoderma* species are able to colonize and degrade dormant sclerotia of plant pathogenic fungi (Elad et al., 1984). To evaluate the ability of *Trichoderma* strains to hamper sclerotia

germination of *C. purpurea*, a sclerotia germination inhibition assay was conducted. Ergot sclerotia were surface sterilized by soaking them for 5 min in 2% sodium hypochlorite, washed with sterile water for 2–3 times and placed on autoclaved wet potting substrate (TS1, Klasmann-Deilmann, Geeste, Germany). After a vernalization period of 7 weeks at 4 °C with high humidity, the plates were inoculated with 2 ml of a spore solution of *Trichoderma* containing 10⁶ spores/ml and the negative control was mock inoculated with sterile water. Afterwards, the plates were incubated for at least 3 weeks at 21 °C, 70% humidity and 6 h/ 18 h dark/ light cycle until the negative control started to germinate. Pictures were taken with a dissecting microscope (MDG41, Leica Microsystems, Wetzlar, Germany) and the number of germinated sclerotia per plate was assessed (Kaur et al., 2016).

In planta application

Greenhouse experiment

The Harzianum section revealed the highest number of predicted antimicrobial peptides, identified by comparative genomic screening (Rush et al., 2021). The strain *T. harzianum* WM24a1, belonging to the Harzianum clade, has already shown to be a strong volatile organic compound (VOC) emitter (Guo et al., 2019) and was therefore chosen for the greenhouse experiment. Furthermore, *T. atrobrunneum* was chosen as a second *Trichoderma* spp. to be tested *in planta* based on the registration as an active ingredient of commercial biocontrol agents under the European Union regulation of the *T. atrobrunneum* isolate ITEM 908 (Fanelli et al., 2018). For the *in planta* application, four seeds of *Secale cereale* (ARANTES, KWS Lochow GmbH, Bergen, Germany) were planted in 3 l pots containing C700 growth substrate and fertilized through drip irrigation. One subset of 7-week-old plants was inoculated with *Trichoderma* by adding 10 ml containing 10⁶ spores/ml to each plant rhizosphere one week before infection with *C. purpurea*. The other subset was phyllosphere-treated with *Trichoderma* spore solution (10⁶ spores/ml) using a chromatography sprayer (H451.1, Carl Roth, Karlsruhe, Germany) until run off. In order to increase air humidity, the plants were covered with plastic bags after spraying. Phyllosphere treatment

was applied twice, about 3 to 5 days and one day before infection with *C. purpurea*.

For the *in planta* evaluation of *Trichoderma* as a potential biocontrol agent a phenotypic evaluation of ergot infection as well as an expression analysis was conducted. For the phenotypic evaluation, the plants were infected through spraying until run off, imitating the natural infection process, and intensity was monitored in terms of honeydew production and formation of ergot sclerotia (Menzies, 2004).

For the expression analysis one half of florets of blooming ears with ripe stigma, but not fully developed anthers, were directly inoculated between the palea and lemma with 5 µl conidial suspension of *C. purpurea* containing 10⁶ spores/ml (in total 15–20 florets per ear) (Tenberge et al., 1996). Only the plants treated with *T. harzianum* WM24a1 (G) were further investigated for RT-qPCR. In order to avoid cross contamination and to achieve an increased humidity, all ears were covered with plastic bags for at least five hours or overnight.

Expression analysis

RNA extraction and RT-qPCR

One to two ears per pot were sampled 24 h, three days and five days after infection with *C. purpurea*. Plant material stored at -80 °C was finely ground in liquid nitrogen and 100 mg was used for RNA extraction using Direct-zol™ RNA Miniprep Plus Kit (R2073, Zymo Research Europe GmbH, Freiburg, Germany) combined with TRIzol™ reagent (Invitrogen, Carlsbad, USA) following manufacturer's instruction, but expanding the DNase I

treatment to 30 min at 37 °C. The extracted RNA was tested for genomic DNA contamination by gel electrophoresis on a 2% agarose gel running at 135 V for 0.6 h and quantified with microplate reader (NanoQuant Plate, Tecan Infinite 200 Pro, Männedorf, Switzerland) and stored at -80 °C. For cDNA synthesis, 1 µg RNA per reaction was reverse transcribed using the FastGene® Scriptase Basic cDNA Kit (LS-62, NIPPON Genetics Europe, Düren, Germany) and cDNA was stored at -20 °C until further use. RT-qPCR was conducted by using the 2×qPCRBIO SyGreen Mix Separate-ROX kit (PB20.11, PCR Biosystems Ltd, London, United Kingdom) using a total reaction volume of 10 µl, including 5 µl SyGreen, 10 µM forward and reverse primer each, nuclease free water and 0.5 µl cDNA as template. The qPCR amplification program included 2 min initial denaturation at 95 °C, 45 cycles of amplification (5 s at 95 °C, 25 s at the primer-specific annealing temperature (T_a)) and a melting curve ramping from 64.5 °C to 95 °C with an increasing temperature of 0.5 °C/s. The amplification of RNase L inhibitor-like protein from rye (*Scrl1* NCBI: BE495958) (Giménez et al., 2011) and glyceraldehyde-3-phosphate dehydrogenase from *C. purpurea* (*Cpgpd1*; NCBI: X73282.1) (Oeser et al., 2017) served as an internal plant and fungal reference gene to normalize the amount of total RNA present in each reaction (Table 1). The gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ -method according to Livak and Schmittgen (2001). All samples were analyzed with three technical replicates and the amplicon lengths of all test genes as well as the respective annealing temperatures are summarized in Table 1.

Table 1 List of genes selected for differential expression analysis and the oligonucleotides used

Gene	Putative function	Primer sequences [5' to 3']	Amplicon length	T_a [°C]
<i>Scrl1</i>	RNase L inhibitor-like protein	qFw TTGAGCAACTCATGGACCAG qRev GCTTTCCAAGGCACAAACAT	84 bp	55
<i>ScxylInh</i>	Xylanase inhibitor (<i>XIP-1</i> homologue)	qFw GGACGACAAGTCCAACCAGT qRev CGAAGTATCGGTCCCAGAGC	113 bp	55
<i>Scchit</i>	Glucan endo-1,3-beta-glucosidase 8	qFw GTGCAGAACCAGAAGGACGA qRev GCAGCGATCTGTATGGGGAA	104 bp	60
<i>Cpgpd1</i>	Glyceraldehyde-3-phosphate dehydrogenase	qFw CCCCGAATATGCTGCCTACA qRev CGCTCAGTGTGGAACCTTGAC	123 bp	60
<i>Cpcell</i>	1,4-β-cellobiosidase	qFw AGGTTCCCGATGAACAAGGC qRev TTGGAGTTGGCCATTCCGTT	101 bp	60

Statistical analysis

Statistical data analysis was performed with Origin Pro (version 2021, OriginLab Corporation, Northampton, USA). The data was tested for normal distribution using Shapiro–Wilk test, followed by Levene’s test for variance homogeneity. One-way ANOVA was performed combined with Tukey’s test for pairwise comparison of means or Fisher’s least significant difference test.

Results

Isolation and phylogenetic analysis

In the present study altogether 12 *Trichoderma* strains, belonging to sections with reported biocontrol activities (Fanelli et al., 2018), were included in the *in vitro* screenings. *T. harzianum* 20761 (A), *T. harzianum* 20770 (B), *T. atroviride* 20780 (C), *T. atroviride* 20781 (D), *T. hamatum* 20784 (E), *T. asperellum* 20866 (F) were already tested for their biocontrol abilities *in vitro*, *in vivo* and in the field against *Alternaria solani* in potato and have shown to be effective (Metz & Hausladen, 2022). The available strains *T. harzianum* WM24a1 (G) and *T. atrobrunneum* (H) were also included. Furthermore, to test *Trichoderma* spp. that are naturally present on arable land, soil samples were collected for isolation of additional *Trichoderma* strains. By selective cultivation on Rose Bengal agar, a total of five *Trichoderma*-like fungal strains (designated I–L) were isolated from the soil samples and identified at the molecular level as *T. velutinum* S1 (I), *T. velutinum* S2 (J), *T. harzianum* (K) and *T. velutinum* S3 (L) (Fig. 1).

Phylogenetic analysis of concatenated *tefl* and *rpb2* sequences verified a close relationship of the soil isolate L with *T. harzianum* WM24a1 and the reference strain *T. harzianum* CBS 226.95 (Fig. 2). Furthermore, the soil isolates *T. velutinum* S1–S3 clustered together with the reference strains *T. velutinum* C.P.K. 2978 and *T. velutinum* DAOM 230013 (Fig. 2).

In vitro screenings

Within the scope of laboratory experiments, 12 *Trichoderma* strains, including the four *Trichoderma* spp. rhizosphere isolates, were evaluated *in vitro* for their biocontrol and plant-growth promoting potential. To this end, the strains were screened for the release of fungal siderophores as well as the secretion of lytic enzymes, such as chitinases, proteases and cellulases. The antagonistic potential against *C. purpurea* was evaluated in dual plate confrontation assays with different degrees of mycelium contact and during germination of *C. purpurea* sclerotia.

Siderophore production

The production and release of fungal hydroxamate siderophores has been suggested as an important mechanism of *Trichoderma* BCAs for outcompeting other fungi in the rhizosphere (Benítez et al., 2005) and the mobilization of iron has shown to contribute to plant growth and nutrition (Altomare et al., 1999). Even though those plant growth promoting properties are host plant specific and not present in every *Trichoderma* spp. strain, it is widely common among the genus (Lorito & Woo, 2015). The release of siderophores was evaluated by liquid CAS assay, to test for



Fig. 1 Morphological diversity of *Trichoderma* isolates from rhizosphere samples. *Trichoderma* spp. were grown on ¼ PDA for one week. **I:** *Trichoderma velutinum* S1, **J:** *Trichoderma velutinum* S2, **K:** *Trichoderma harzianum*, **L:** *Trichoderma velutinum* S3

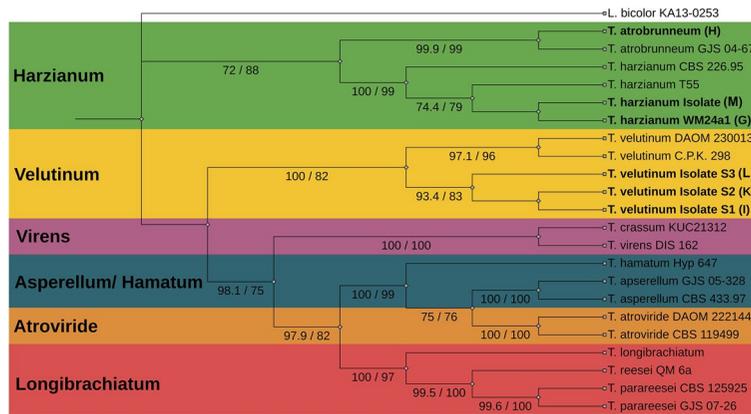


Fig. 2 Phylogenetic tree of the soil isolates *Trichoderma* spp. based on concatenated sequences of translation elongation factor 1 α (*tef1*) and RNA polymerase B subunit II (*rpb2*) of the genus *Trichoderma*. Processed sequences were aligned using Muscle 3.8.1 and maximum-likelihood phylogeny was performed with IQ-TREE web server with bootstrap val-

ues of 1000 and visualization using iTOL. Metadata values are Shimodaira-Hasegawa approximate likelihood ratio test (SH-aLRT) (≥ 70) and ultrafast bootstrap support. The strains included in this study are in bold and *Laccaria bicolor* KA 13–0253 was included as outgroup

the capacity to mobilize iron. The highest siderophore secretion was detected for *T. harzianum* 20770 (B), followed by *T. atroviride* 20781 (D), *T. harzianum* (K) and *T. harzianum* WM24a1 (G) (Fig. 3). *T. atrobrunneum* (H) showed the lowest chelating capacity.

Cellulase production

Cellulase activity is a prerequisite of *Trichoderma* spp. for the colonization of plant roots and has been shown to be associated with the induction of local and systemic defense reactions in plants (Saravankumar et al., 2018; Shoresh et al., 2010). We utilized a Congo red-based plate assay to study the secretion of cellulases in the 12 *Trichoderma* strains. The formation of clearance halos around the colonies served thereby as quantitative measure. While the two *T. atroviride* strains (20781 D and 20780 C) showed the highest EI (Fig. 4), the *T. velutinum* strains (particularly J and L) showed the lowest cellulolytic activity of all strains during the test conditions.

Proteolytic activity

Proteolytic activity of *Trichoderma* is another indicator for its antagonistic and antifungal potential. For example, it is already known that aspartyl and serine proteases are involved in mycoparasitism mechanisms of important biocontrol species such

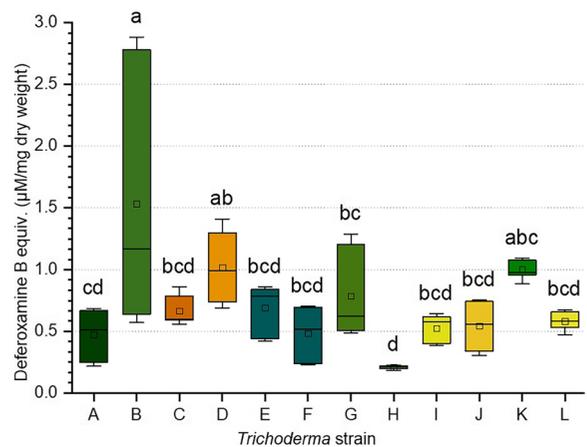


Fig. 3 Liquid chrome azurol S (CAS) assay for quantification of siderophore production. *Trichoderma* spp. were cultivated in liquid M9 medium for 7 days at 30 °C. Culture supernatants were incubated with CAS solution for 2 h and the absorbance measured at 630 nm. Uninoculated medium was used as reference. A/A_{ref} -values were correlated to a standard curve prepared with deferoxamine mesylate and normalized with dry weight. *Trichoderma harzianum* 20761 (A), *Trichoderma harzianum* 20770 (B), *Trichoderma atroviride* 20780 (C), *Trichoderma atroviride* 20781 (D), *Trichoderma hamatum* 20784 (E), *Trichoderma asperellum* 20866 (F), *Trichoderma harzianum* WM24a1 (G), *Trichoderma atrobrunneum* (H), *Trichoderma velutinum* S1 (I), *Trichoderma velutinum* S2 (J), *Trichoderma harzianum* (K), *Trichoderma velutinum* S3 (L). Different letters indicate significant differences with $p < 0.05$, $n = 9$

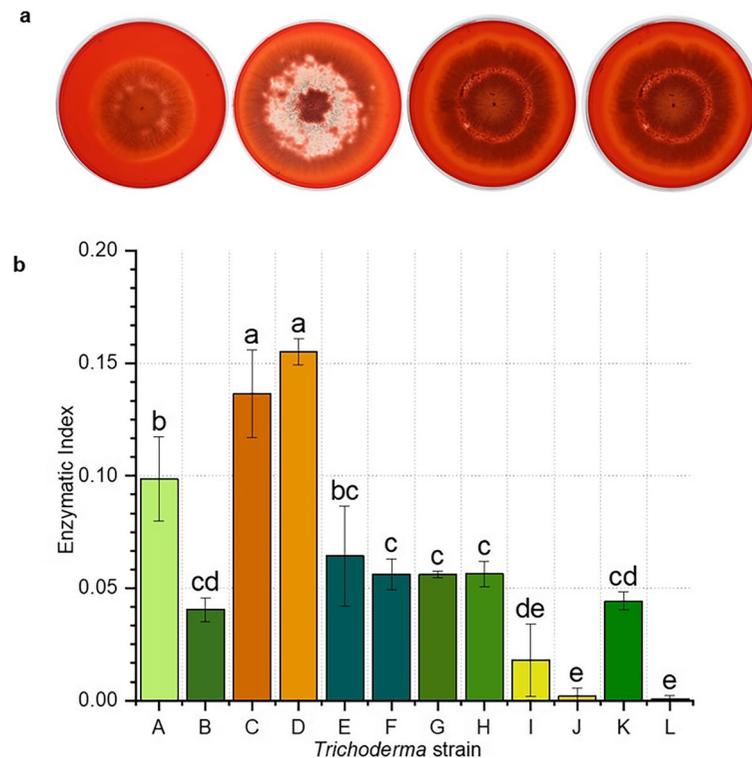


Fig. 4 Carboxymethylcellulose (CMC) and Congo red assay for the detection of cellulase activity. **a** *Trichoderma* spp. cultivated on CMC agar for 96 h at room temperature, followed by incubation at 45–50 °C for 18 h. Plates were stained with 0.2% Congo red solution. Representative photos of *Trichoderma harzianum* 20761, *Trichoderma harzianum* 20770, *Trichoderma atroviride* 20780 and *Trichoderma atroviride* 20781 after staining process. **b** Quantitative evaluation of cellulolytic activity. Enzymatic indices were estimated from the colony and

halo diameters. *Trichoderma harzianum* 20761 (A), *Trichoderma harzianum* 20770 (B), *Trichoderma atroviride* 20780 (C), *Trichoderma atroviride* 20781 (D), *Trichoderma hamatum* 20784 (E), *Trichoderma asperellum* 20866 (F), *Trichoderma harzianum* WM24a1 (G), *Trichoderma atrobrunneum* (H), *Trichoderma velutinum* S1 (I), *Trichoderma velutinum* S2 (J), *Trichoderma harzianum* (K), *Trichoderma velutinum* S3 (L). Different letters indicate significant differences with $p < 0.05$, SD, $n = 3$

as *T. harzianum* and *T. atroviride* (Atanasova et al., 2013; Benítez et al., 2005; Kubicek et al., 2011). We therefore tested the secretion of proteases in all strains by way of a skim milk plate assay. The tested *Trichoderma* spp. strains showed significant differences in their activity during the experimental conditions. *T. harzianum* WM24a1 (G) as well as the soil isolate *T. velutinum* S3 (L) showed the strongest proteolytic activity during the protease plate test (Fig. 5). In contrast, *T. asperellum* 20866 (F) was the only strain not showing any formation of a clear zone indicating no proteolytic activity under these conditions.

Chitinase activity

Chitinases are an indispensable tool of *Trichoderma* spp. to mycoparasitize phytopathogenic fungi and they act as elicitors of the plant's immune response (Kubicek et al., 2001; Nawrocka & Małolepsza, 2013). Chitinases catalyze the hydrolytic breakdown of β -1,4-glycosidic linkages between the NAGA residues of chitin. The *in vitro* analysis of total chitinolytic activity of *Trichoderma* spp. was based on the quantification of free NAGA, released from a defined concentration of colloidal chitin by the activity of fungal chitinases present in the culture supernatants.

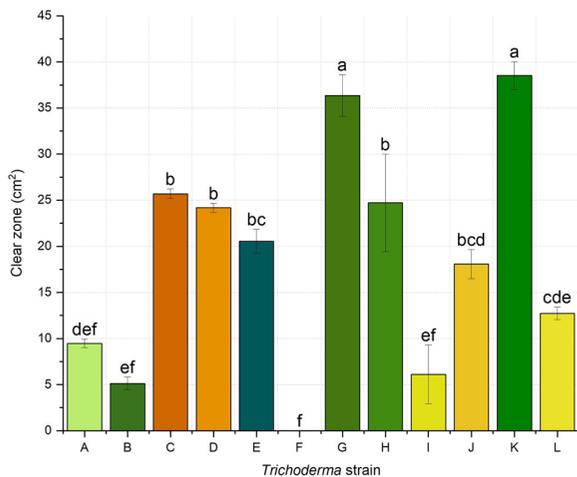


Fig. 5 Skim milk plate assay for the detection of proteases. *Trichoderma* spp. were cultivated on skim milk agar for 4 days at room temperature. The formation of a clear zone halo was quantified. *Trichoderma harzianum* 20761 (A), *Trichoderma harzianum* 20770 (B), *Trichoderma atroviride* 20780 (C), *Trichoderma atroviride* 20781 (D), *Trichoderma hamatum* 20784 (E), *Trichoderma asperellum* 20866 (F), *Trichoderma harzianum* WM24a1 (G), *Trichoderma atrobrunneum* (H), *Trichoderma velutinum* S1 (I), *Trichoderma velutinum* S2 (J), *Trichoderma harzianum* (K), *Trichoderma velutinum* S3 (L). Different letters show significant differences with $p < 0.05$, SE, $n = 3$

The soil isolate *T. harzianum* (K), *T. harzianum* 20761 (A), *T. atroviride* 20780 (C), and *T. atroviride* 20781 (D) were found to show the highest chitinolytic activity (Fig. 6). Under the given experimental conditions, the lowest chitinolytic activity was detected for *T. atrobrunneum* (H) and the soil isolate *T. velutinum* S3 (L).

In vitro antagonistic activity against *C. purpurea*

The ability of *Trichoderma* spp. to restrict *C. purpurea* growth by combined mechanisms of secreted antifungal compounds, antibiosis and direct hyphal contact is a strong indicator for the antagonistic capacity and biocontrol abilities and can be evaluated with dual confrontation assays. Furthermore, by using two-compartment petri-dishes, preventing diffusion of metabolites and direct hyphal contact, the impact of volatile organic compounds can be explored. In our study, dual-plate confrontation assays showed considerable antagonistic activity of *Trichoderma* spp. against *C. purpurea* (Fig. 7). Interestingly, at 4 days

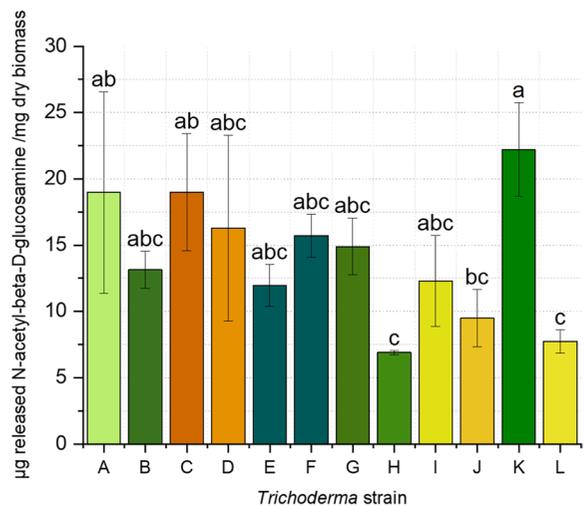


Fig. 6 Enzymatic assays for the quantification of chitinolytic activity by *Trichoderma* spp.. Strains were grown in liquid chitinase detection medium supplemented with 4.5 g/l colloidal chitin for 7 days at 30 °C. Supernatant was harvested by filtration and 1 ml mixed with 0.3 ml of 1 M sodium acetate buffer (pH 4.6) and 0.2 ml of colloidal chitin (0.5% w/v). After incubation at 40 °C for 20 h the mixture was centrifuged at 8.000 rpm for 5 min and 0.75 ml of the obtained supernatant were then mixed with 0.25 ml of a 1%-solution of 3,5-dinitrosalicylic acid. Reduction was quantified by absorbance measurements at 540 nm. The concentration of released NAGA is given per mg dry biomass. *Trichoderma harzianum* 20761 (A), *Trichoderma harzianum* 20770 (B), *Trichoderma atroviride* 20780 (C), *Trichoderma atroviride* 20781 (D), *Trichoderma hamatum* 20784 (E), *Trichoderma asperellum* 20866 (F), *Trichoderma harzianum* WM24a1 (G), *Trichoderma atrobrunneum* (H), *Trichoderma velutinum* S1 (I), *Trichoderma velutinum* S2 (J), *Trichoderma harzianum* (K), *Trichoderma velutinum* S3 (L). The graph shows the mean values \pm SE of three biological replicates. Different letters show significant differences with $p < 0.05$

after *Trichoderma* inoculation, the inhibitory effect on the *Claviceps* colonies was generally substantially higher with only air contact compared to media contact conditions, indicating a strong influence of volatile compounds. Here, *T. harzianum* 20761 (A) and *T. harzianum* 20770 (B) showed the weakest inhibitory effect. The soil isolate *T. harzianum* (K) could not be assessed for air contact conditions, since the mycelium grew over the separation after 3 days. After six days of incubation, allowing direct contact in this experimental setup, the inhibition strongly increased in all cases. Some of the tested strains even provoked *C. purpurea* to withdraw, expressed by growth inhibition values of more than 100%, as

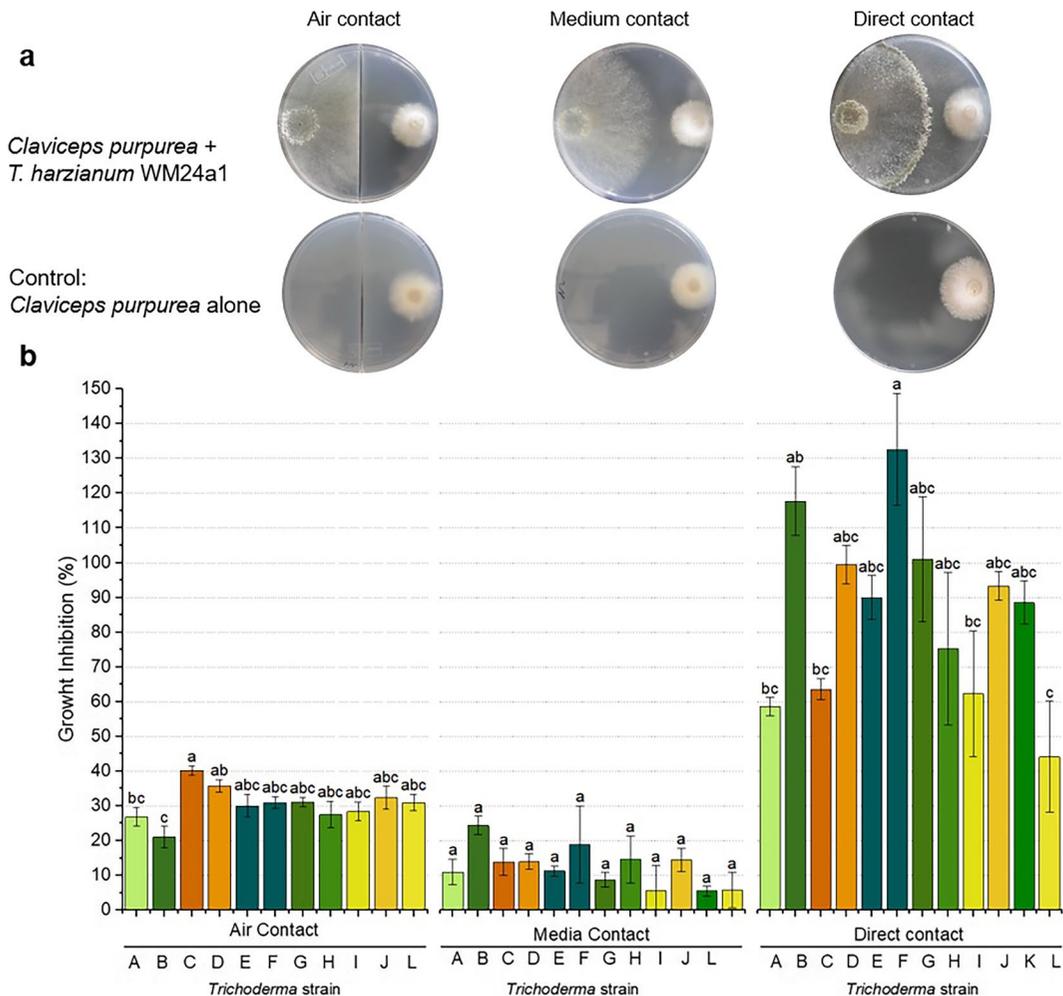


Fig. 7 Dual plate confrontation assay between *Trichoderma* spp. and *Claviceps purpurea*. Both fungi were inoculated on the opposite sides of one- or two-compartment ¼-PDA plates and incubated at room temperature. For air contact and media contact conditions, the increase in colony area (cm.²) of *Claviceps purpurea* from the time point of inoculation with *Trichoderma* spp. until 4 days post inoculation was assessed and compared to the negative control, where *Claviceps purpurea* was grown alone. For direct contact between *Trichoderma* spp. and the plant-pathogen, the change in colony area was assessed between day 4 and 6 after inoculation with the fungal antagonist, when both fungal partners were already in hyphal contact. **a** Representative pictures for the four contact stages of *Trichoderma harzianum* WM24a1 corresponding to

4 dpi, for air contact and medium contact, 6 dpi for the direct contact phase. **b** Quantitative evaluation of the growth inhibition of *Claviceps purpurea* by the respective *Trichoderma* spp. at the air, medium and direct contact stage. The size of the ergot colonies was measured with ImageJ. *Trichoderma harzianum* 20761 (A), *Trichoderma harzianum* 20770 (B), *Trichoderma atroviride* 20780 (C), *Trichoderma atroviride* 20781 (D), *Trichoderma hamatum* 20784 (E), *Trichoderma asperellum* 20866 (F), *Trichoderma harzianum* WM24a1 (G), *Trichoderma atroviride* 20780 (H), *Trichoderma velutinum* S1 (I), *Trichoderma velutinum* S2 (J), *Trichoderma harzianum* (K), *Trichoderma velutinum* S3 (L). Significances were determined for each contact stage separately, $p < 0.05$, SE, $n = 3$

e.g. for *T. asperellum* 20866 (F) and *T. harzianum* 20770 (B) with 132,58% and 117,72%, respectively. The weakest inhibitory effect during direct contact was assessed for the soil isolate *T. velutinum* S3 (L) with <45%.

Inhibitory effect on sclerotia germination

The application of *Trichoderma* spp. to agricultural fields bears the potential to not only directly antagonize *C. purpurea* mycelium, but also to reduce

sclerotia germination in the soil, which would help to minimize the risk of a following infection cycle. Some *Trichoderma* spp. have already shown potential to colonize and degrade resistant structures such as sclerotia of plant pathogenic fungi, leading to decreased germination (Elad et al., 1984; Ondřej et al., 2010).

The ergot sclerotia used in this experimental setup were collected from a winter wheat cultivar from the harvest season 2020/2021 (Thuringia, Germany). The soil isolates *T. velutinum* S1 (I), *T. velutinum* S2 (J), *T. harzianum* (K) and *T. velutinum* S3 (L) could not be included in this assay, since ergot sclerotia require a cold phase of 7 weeks for germination induction, which was performed prior to their isolation. The antagonistic capacity of the tested strains was assessed by a qualitative rating score from 0 to 6, adapted from the evaluation of antagonistic activity of *Trichoderma* against several plant-pathogens

previously described by Yu et al. (2022). Particularly *T. atroviride* 20781 (D), *T. asperellum* 20866 (F) and *T. harzianum* WM24a1 (G) revealed a high inhibitory effect on the formation of stroma of the germinating sclerotia (Fig. 8). *T. asperellum* 20866 showed an intensive mycelium growth on the surface of ergot sclerotia as well as a strong sporulation, while *T. hamatum* 20784 (E) did not show any visible impact.

Summarized *in vitro* *Trichoderma* spp. screenings

For each conducted *in vitro* screening assay, the isolates were grouped according to their performance from 0 to 3, referring to 0=no activity, 1=low activity, 2=medium activity and 3=high activity (Fig. 9). The thresholds for each group were set individually for each assay. *T. atroviride* 20781 (D) showed the overall highest total score of 18, followed by *T.*

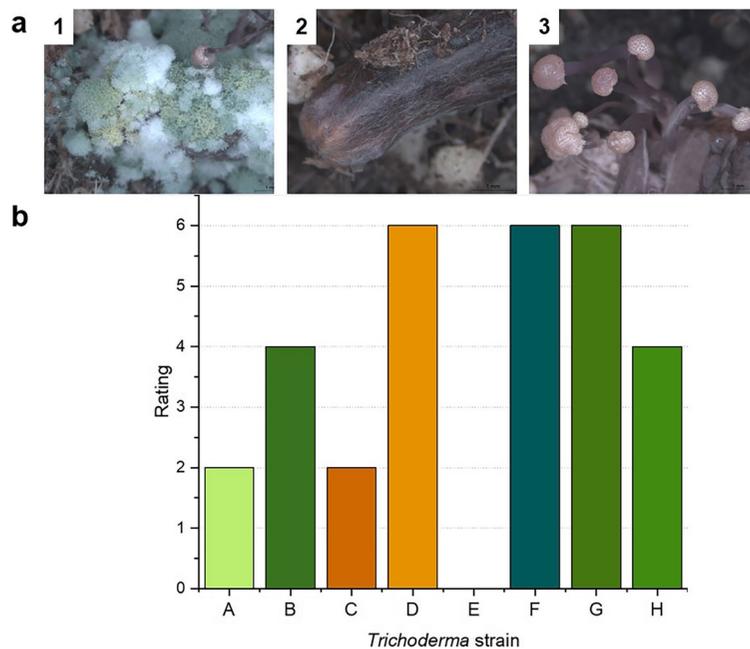


Fig. 8 Germination assay of ergot sclerotia inoculated with *Trichoderma* spp. **a** Close-up images of ergot sclerotia on TS 1 fully overgrown by sporulating **1**: *T. asperellum* 20866 (F) sporulating on ergot sclerotia, **2**: sclerotia covered with fine mycelium and **3**: stroma formed by a germinating sclerotium of control group without *Trichoderma*. **b** Qualitative rating of the antagonistic activity of *Trichoderma* spp. against ergot sclerotia according to Yu et al. (2022). A score of 0 means no impact of the respective *Trichoderma* strain; 2 indicates growth

and mycelium formation on the ergot sclerotia; 4 means sporulation on the ergot surface and 6 implies an inhibitory effect on germination of the ergot sclerotia (given by <30% germinating sclerotia). *Trichoderma harzianum* 20761 (A), *Trichoderma harzianum* 20770 (B), *Trichoderma atroviride* 20780 (C), *Trichoderma atroviride* 20781 (D), *Trichoderma hamatum* 20784 (E), *Trichoderma asperellum* 20866 (F), *Trichoderma harzianum* WM24a1 (G), *Trichoderma atroviride* 20781 (D)

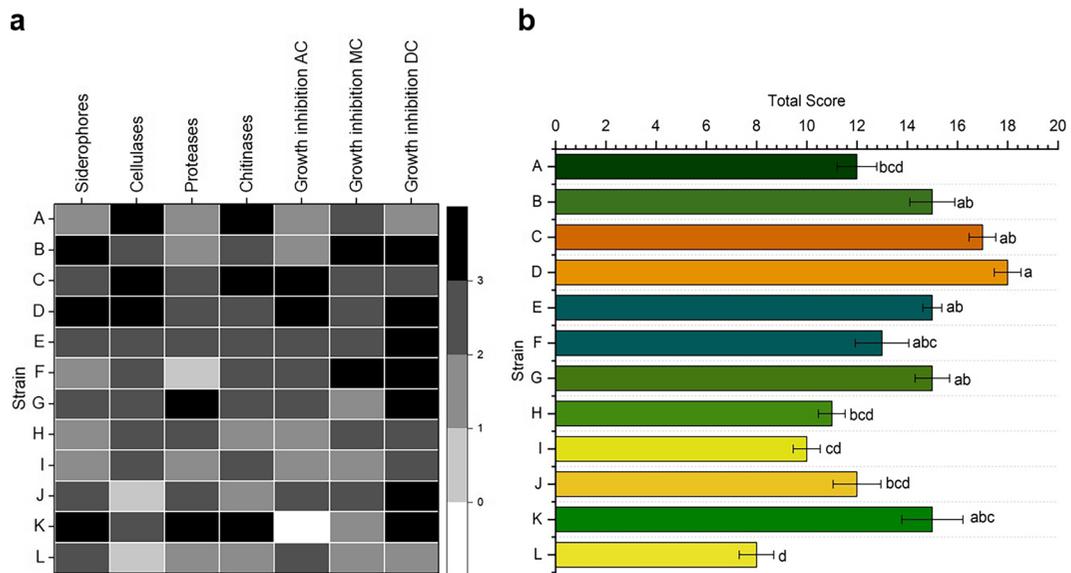


Fig. 9 Summarized results of the *in vitro* screening of *Trichoderma* spp. **a** The thresholds for each group were set for each assay individually as follows: siderophore production (μM deferoxamine B equiv.): low: <0.5 , medium: $0.5\text{--}1.0$, high: >1.0 ; cellulase activity (EI): low: <0.025 , medium: $0.025\text{--}0.075$, high: >0.075 ; protease activity (clear zone, cm^2): low: <15 , medium: $15\text{--}30$, high: >30 ; chitinase activity (NAGA released, $\mu\text{g/ml}$): low: <10 , medium $10\text{--}17$, high: >17 ; growth inhibition (%): air contact (AC): low: <30 , medium: $30\text{--}35$, high: >35 , medium contact (MC): low: <10 ,

medium: $10\text{--}15$, high: >15 , direct contact (DC): low: <55 , medium: $55\text{--}85$, high: >85 . red = not determined **b** Total score for each *Trichoderma* strain. *Trichoderma harzianum* 20761 (A), *Trichoderma harzianum* 20770 (B), *Trichoderma atroviride* 20780 (C), *Trichoderma atroviride* 20781 (D), *Trichoderma hamatum* 20784 (E), *Trichoderma asperellum* 20866 (F), *Trichoderma harzianum* WM24a1 (G), *Trichoderma atrobrunneum* (H), *Trichoderma velutinum* S1 (I), *Trichoderma velutinum* S2 (J), *Trichoderma harzianum* (K), *Trichoderma velutinum* S3 (L)

atroviride 20780 (C) with a score of 17, and *T. harzianum* 20770 (B), *T. hamatum* 20784 (E) and *T. harzianum* WM24a1 (G), all with a total score of 15 (Fig. 9). The soil isolates *T. velutinum* S1(I) and S3 (L) showed the lowest total score with a value of 9 and 8, respectively.

In planta application

Phenotypic infection assay

For the *in planta* application, two *Trichoderma* strains from the Harzianum section were chosen based on the total score of the *in vitro* screenings: *T. harzianum* WM24a1 (G) as a top-scoring strain and *T. atrobrunneum* (H) as a representative of the medium-performing strains (Fig. 9). Two different treatments were included to test for their protective capacity in terms of reduced signs of ergot infection. Spores of both strains were applied either foliar by spraying the ears

until run-off, or by direct inoculation of the rhizosphere prior to infection with *C. purpurea*.

Honeydew production was tested, as it is mainly responsible for secondary infection cycles of ergot after successful infection of *C. purpurea* by containing fungal conidia which are further spread by rainsplashes or insects (Tudzynski & Scheffer, 2004). Furthermore, exclusively the sphaelial stage in the ergot development is characterized by production of honeydew, which stops with the formation of sclerotia. The initiation, duration as well as the amount of honeydew produced can therefore be used as indicators for a proper development of the ergot fungus and a balanced host–pathogen interaction (Mower & Hancock, 1975). Timing and amount of honeydew formation by the infected ears was assessed in a daily manner starting with the appearance of honeydew on the first ear (Fig. 10). Furthermore, the number of sclerotia produced per infected ear was counted.

No striking differences could be detected between both applied strains. The high variability in disease

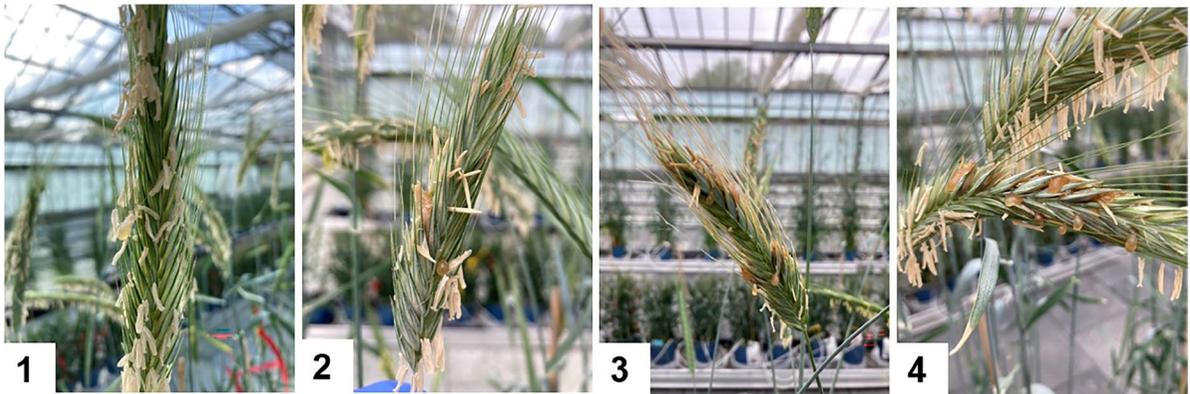


Fig. 10 Scaling scheme of honeydew production. Representative pictures for the rating of honeydew production with 1 = very low, 2 = low, 3 = mediate and 4 = high

development did not allow a statistic evaluation, but overall, a reduced infection severity, in terms of appearance of honeydew and intensity of honeydew

production, could be observed for plants that had been rhizosphere-treated compared to phyllosphere-treatment (Table 2). Furthermore, the number of

Table 2 Quantification of honeydew production and sclerotia formation after treatment with *Trichoderma*. The monitoring of honeydew production was started 7 days after infection. One pot containing four rye plants was denoted as one biological replicate. The rating of honeydew production was conducted 3–4 days after the first appearance of honeydew, based on the assessment scheme given in Fig. 10, with + very low, ++ low, +++ mediate and ++++ high. The number of

sclerotia represents the mean of all infected ears per pot and was determined at 39 days after infection. Some ears of the control plants, as well as both phyllosphere-treated groups, did show formation of sclerotia without any sign of honeydew production. Since only one biological replicate of the control group showed visible signs of ergot infection, no statistical analysis could be assessed

Treatment	Biological replicate (4 plants/pot)	Ears infected (per pot)	Appearance of honeydew (dpi)	Honeydew production	Number of sclerotia (per infected ear)	
Positive control	Only <i>Claviceps</i>	1	0	0	0	
		2	0	0	0	
		3	2*	9	++++	3.5 (±2.5)
Phyllosphere	<i>T. harzianum</i> WM24a1 + <i>Claviceps</i>	1	3	9.3 (±0.47)	++++	5.7 (±3.3)
		2	4**	9	+++	3 (±1.9)
		3	0	0	0	0
Phyllosphere	<i>T. atrobrunneum</i> + <i>Claviceps</i>	1	0	0	0	0
		2	4	10.8 (±2.0)	++	1.8 (±0.8)
		3	2*	10	++++	8.5 (±7.5)
Rhizosphere	<i>T. harzianum</i> WM24a1 + <i>Claviceps</i>	1	1	9	++	2
		2	2	11.5 (±0.5)	+	0.5 (±0.5)
		3	0	0	0	0
Rhizosphere	<i>T. atrobrunneum</i> + <i>Claviceps</i>	1	1	9	++	1
		2	0	0	0	0
		3	2	10.5 (±1.5)	++	1

*One ear did not show honeydew production

**Two ears did not show honeydew production

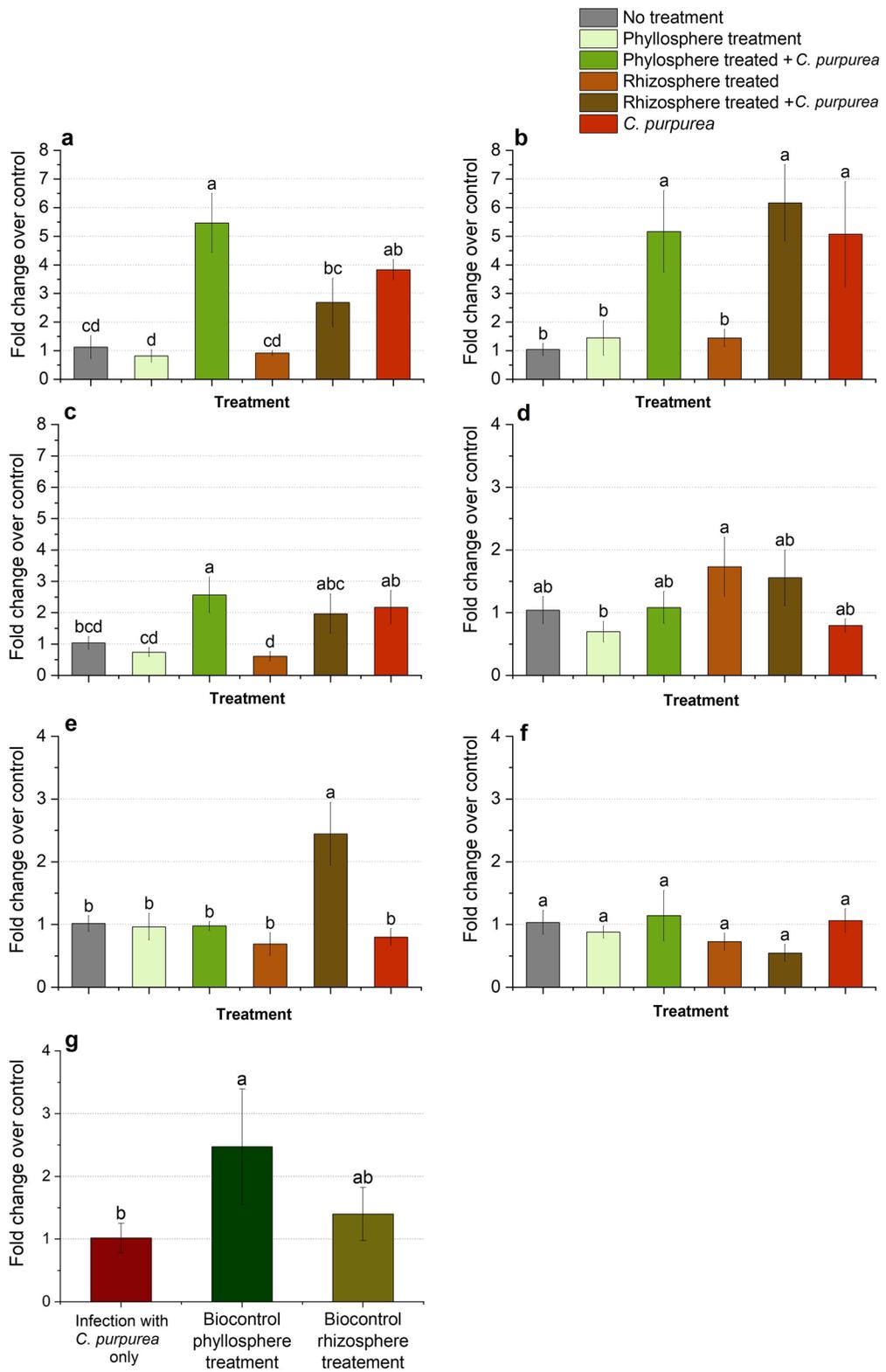


Fig. 11 Relative expression level of xylanase inhibitor (*Scxylinh*) 24 h (a), 3 d (b) and 5 d (c) and relative expression of glucan endo-1,3-beta-glucosidase 8 (*Scchit*) 24 h (d), 3 d (e) and 5 d (f) post infection with *Claviceps purpurea*. Plants were phyllosphere treated with *Trichoderma harzianum* WM24a1 by spraying and rhizosphere treated by direct inoculation of the growth substrate with a conidial suspension containing 10^6 spores/ml. For infection flowers were directly inoculated with a conidial suspension of *Claviceps purpurea* (10^6 conidia/ml) using a micropipette. The expression of the test genes was determined by RT-qPCR analysis and the $2^{-\Delta\Delta C_t}$ method. The rye gene RNase L inhibitor-like protein (*Scrli*) served as internal reference plant gene, and the untreated and uninfected plants were used as control (grey). Relative expression of the ergot gene 1,4- β -cellobiosidase (*Cpcell*) in rye ears at 5 dpi (g) and the ergot gene glyceraldehyde-3-phosphate dehydrogenase (*Cpgpd1*) served as reference gene and the plants only infected with *Claviceps purpurea* were used as control. Different letters show significant differences with $p < 0.05$, SE, $n = 3$

produced sclerotia per infected ear showed a tendency to be decreased in rhizosphere-treated plants compared to phyllosphere-treated ones.

Expression analysis

To detect relative changes within the transcription of pathogenesis-related (PR) genes in the ears of rye, a RT-qPCR analysis was conducted for the plant defense genes *xylinh* and *chit* (in rye) as well as for the pathogenicity factor *cell* in *C. purpurea*. Samples for RNA extraction were only collected from plants treated with *T. harzianum* WM24a1 (G), since we expected an increased biocontrol ability based on the *in vitro* screenings. The expression of *Scxylinh* was found to be significantly increased in all ergot-infected plants and across all time points (Fig. 11 a-d). Those that were additionally phyllosphere-treated with *T. harzianum* WM24a1 spores showed the strongest induction compared to the non-treated controls after 24 h and after 5 d.

The temporal changes in the expression of the rye gene *chit* (Fig. 11 d-f) revealed less dynamics than in the analysis of *xylinh*. After 24 h, significant differences could only be detected between phyllosphere-treated and rhizosphere-treated groups but without ergot infection. Three days after the infection with *C. purpurea* the *chit* transcript level of the rhizosphere-treated and ergot-infected plants was significantly increased compared to all other test groups. However, this effect could not be detected anymore

after five days of infection, where no significant differences between all groups were observed.

Due to the limited amount of *C. purpurea* mRNA within the harvested plant biomass of the early time points, the expression analysis for the ergot gene *cell* was only performed for the 5 day samples after infection. At this time point, the expression of *cell* was increased in both *Trichoderma*-treated groups vs. the control plants without any biocontrol treatment (Fig. 11 g). The transcript level of *Cpcell* was most strongly increased in the previously phyllosphere-treated plants with an average fold-change of 2.5 compared to the rhizosphere-treated plants which showed an average fold-change of 1.4.

Discussion

Biological control of plant diseases caused by fungal pathogens is a promising alternative to chemical fungicides (Thambugala et al., 2020). As an opportunistic and avirulent plant symbiont, *Trichoderma* is by far the most extensively studied biocontrol agent against a broad spectrum of aerial and soil-born fungal pathogens but antagonism is highly species- and host-specific (Alfiky & Weisskopf, 2021; Nur & Noor, 2020). Therefore, to identify *Trichoderma* strains which are effective as biocontrol agents against specific pathogens, a broad variety of species should be evaluated individually (Rush et al., 2021). The isolation of *Trichoderma* spp. from rhizosphere soil, followed by their characterization and screening for biocontrol activities, such as production of hydrolytic enzymes and siderophores, is a quite common practice and has already proven successful in the identification of some promising biocontrol strains (Chen et al., 2021; Rai et al., 2016; Yu et al., 2022). Although *in vitro* screenings always bear the risk to accidentally exclude strains by not properly inducing the tested mycoparasitism-related gene clusters under laboratory conditions (Fanelli et al., 2018; Hermosa et al., 2004; Li et al., 2021), they are nevertheless the first choice when several different *Trichoderma* isolates have to be rated for their characteristic biocontrol abilities to identify the strains with the highest potential for follow-up experiments (Bedine Boat et al., 2020; Imran et al., 2022; Yassin et al., 2022).

During our *in vitro* screenings of cellulolytic, proteolytic, and total chitinolytic activity, significant differences could be detected between the tested *Trichoderma* strains under the given test conditions. However, strains belonging to the Harzianum section, such as *T. harzianum* 20761 (A), *T. harzianum* WM24a1 (G), and the soil isolate *T. harzianum* (K) displayed highest protease activities and showed a strong inhibitory effect on sclerotia germination. *T. atroviride* 20780 (C) and *T. atroviride* 20781 (D), belonging to the Atroviride section, performed also well in protease and cellulase activity plate assays, and *T. atroviride* 20781 (D) showed a strong inhibitory effect on sclerotia germination. Besides those enzymatic *in vitro* screenings testing for biocontrol properties, we aimed to evaluate the inhibitory effect of the 12 strains towards the specific plant-pathogen *C. purpurea* using direct confrontation assays with different degrees of contact.

In dual plate confrontation assays, all tested *Trichoderma* strains displayed the lowest inhibitory effect during media contact and the highest at direct contact. The increased inhibitory effect on *C. purpurea* during air contact compared to media contact is indicating that VOCs are involved in the interaction and have an impact in the process of antagonism of *Trichoderma* (Bae et al., 2016; Rajani et al., 2021). *Trichoderma* spp. produce VOCs that inhibit growth of other fungi, promote plant growth, and induce plant defense responses (Bruce et al., 2000; Phoka et al., 2020; Ruangwong et al., 2021; Salwan et al., 2019; Wonglom et al., 2020). Furthermore, Guo et al. (2019) showed by multivariate analysis that VOC profiles were highly species-specific among the three tested *Trichoderma* strains *T. velutinum* GL1561, *T. hamatum* QL15d1 and *T. harzianum* WM24a1 and that the latter strongly emitted the most diverse compound patterns. During fungus–fungus interaction VOC emissions and perceptions are comprising a complex host–pathogen cross-talk and can lead to a host-specific mycoparasitism initiation in *Trichoderma* spp. (Sharma et al., 2017; Zeilinger & Omann, 2007).

In summary, our findings showed that all *Trichoderma* spp. significantly suppressed mycelium growth of *C. purpurea* during *in vitro* conditions. During direct contact, the strains *T. harzianum* 20770 (B), *T. atroviride* 20781(D), *T. harzianum* WM24a1 (G) and *T. asperellum* 20866 (F) were not only able to inhibit

C. purpurea growth, but even led to an active reduction of the *C. purpurea* colony size. The same strains, except *T. harzianum* 20770 (B), had a similarly strong inhibitory effect on the germination of ergot sclerotia *in vitro*. Smith et al. (2013) tested 21 *Trichoderma* isolates and *T. atroviride* Th002 and *T. harzianum* Th203 showed strong mycoparasitic activity against the plant-pathogen *Sclerotinia sclerotiorum* and prevented sclerotia germination of more than 70%. Haddad et al. (2017) also evaluated 120 *Trichoderma* spp. regarding their potential to inhibit sclerotia germination of *S. sclerotiorum in vitro* and identified 22 strains, including two *T. atroviride* strains, that caused 100% inhibition of sclerotia germination, which implies that large-scale screenings are worthwhile to identify efficient *Trichoderma* isolates against specific fungal pathogens.

The expression of genes connected with carbohydrate metabolism is associated with pathogenicity in plant pathogenic fungi such as *Aspergillus flavus*, *Botrytis cinerea*, and *Alternaria* sp. (Annis & Goodwin, 1997; Have et al., 1998; Shieh et al., 1997). As a plant-pathogenic fungus, *C. purpurea* is dependent on various carbohydrate-active enzymes (CAZymes), such as the investigated putative cellulase, to penetrate the plant cell walls in the rye ovary (Oeser et al., 2017; Schardl et al., 2013). Expression analysis revealed that this putative cellulase gene is induced during the first days of infection in rye (Müller et al., 1997). Furthermore, Oeser et al. (2002) showed that mutants lacking two polygalacturonase genes nearly lost their complete pathogenicity towards rye.

Trichoderma-induced resistance is not only achieved when applied directly to rhizosphere, but also after foliar application to the plants surface (Korolev et al., 2008; Perazzolli et al., 2008). Furthermore, foliar application of *T. harzianum* WM24a1 onto the ears surface is taking advantage of the *in vitro* observed antagonistic activity towards *C. purpurea in planta*. Therefore, the increased expression of *Cpcell* in the phyllosphere-treated ears could be influenced by the antagonist being present, leading to nutrient competition. However, since we obtained data for only one late time point and little is known about the direct interaction of *Trichoderma* and *C. purpurea in planta*, further studies need to be conducted to explore the observed phenomena. Xylanases from *C. purpurea* were found to be upregulated *in planta* during all infection stages (Giesbert

et al., 1998). Xylanase inhibitors, expressed by the plant, inhibit the hemicellulose degradation of those secreted fungal xylanases (Tundo et al., 2022). Expression analysis revealed significantly increased mRNA levels of the xylanase inhibitor *xylinh* in rye plants that were infected with *C. purpurea*, irrespective of the *Trichoderma* treatment. In contrast, plants that were only treated with *Trichoderma* did not show an induced expression of *xylinh*. The expression of *chit* in rye was enhanced in rhizosphere-inoculated plants, but the effect was not detectable later than 3 days after infection with *C. purpurea*. Assigned to three families of PR proteins, PR3, PR8 and PR11, chitinolytic enzymes represent a major part of the plant defense system effective against a wide range of phytopathogenic fungi (Wang et al., 2018). In the control plants only infected with *C. purpurea*, no significant differences could be detected compared to the not-infected and not-treated plants, which is contradictory to the observations of an increased expression of chitinases during the first days of infection by Oeser et al. (2017).

However, this could be indicative for a strategy of host immune suppression by *C. purpurea* through sequestering chitin oligosaccharides (Jonge et al., 2010), which remains to be elucidated. Nonetheless, the increased expression of *chit* in rhizosphere-inoculated plants is supporting the results of the phenotypic evaluation of the infection severity. In contrast to the control plants, the rhizosphere-treated plants showed a delayed and reduced honeydew production as well as a decrease in sclerotia formation. This effect could be explained by an induced systemic resistance in rye stimulated by the interaction with *Trichoderma* in the rhizosphere as it was already seen in wheat (Esmail et al., 2022; Jaroszuk-Ścisiel et al., 2019). Furthermore, Pocięcha and Dziurka (2015) were able to show that rhizosphere inoculation of winter rye with *T. viride* and *T. harzianum* increased resistance to the snow mold *Microdochium nivale* when applied after cold acclimation treatment, by a decreased carbohydrate content in the shoot and increased carbohydrate content in the roots.

A combination of different *Trichoderma* species, as it is the case in the commercial product Trichopel, has been previously shown to promote growth of plants in agriculture and forestry more effectively compared to single strain application (Chen et al., 2021; Chirino-Valle et al., 2016). Similarly, two mixed *Trichoderma*

isolates showed synergistic effects and enhanced disease control of *Fusarium solani* in tomato plants compared to separate application (Awad-Allah et al., 2022). This could therefore also be considered for biocontrol applications against ergot. PR genes play an important role in the process of plant disease resistance and there is ample evidence that *Trichoderma* can induce plant defense responses, but it remains to be elucidated which biocontrol treatment and which time point of application will lead to an effective defense response in rye and subsequently to an increased resistance against ergot in the field.

Conclusion

The objective of this work was to evaluate the biocontrol ability of *Trichoderma* spp. against *C. purpurea* *in vitro* and the potential to protect against ergot infection of rye *in planta*. Taken together, a clear antagonistic activity against *C. purpurea* could be verified in several of the tested *Trichoderma* strains *in vitro*, and a trend for protection against ergot could be seen for rhizosphere-treated rye in the greenhouse trial. Based on the *in vitro* screenings and their robust ability to inhibit the growth of *C. purpurea*, we have identified *T. harzianum* 20770 (B), *T. atroviride* 20780 (C), *T. atroviride* 20781 (D), *T. hamatum* 20784 (E), and *T. harzianum* WM24a1 (G) as strong candidates for biocontrol agents against ergot. Our results therefore indicate that the application of suitable *Trichoderma* isolates could provide an alternative and sustainable disease management strategy for ergot infection. In the future, a mixture of several candidate strains should be evaluated regarding their effect on disease severity. Additionally, the efficacy of *Trichoderma* isolates in managing ergot in rye, as well as inhibiting sclerotia germination within the soil, should be further investigated and explored under field conditions.

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Data Availability All data generated or analysed during this study are included in this published article and its supplementary information files or can be obtained from the corr. author upon reasonable request.

Declarations

Competing interests The authors have no competing interests to declare that are relevant to the content of this article.

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