A glimpse into a green future

Examples of secreted proteins of *Trichoderma harzianum*

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1 Layman summary

In the future a growing world population will put more pressure on food security. Increasing crop losses due to droughts, storms and plant diseases on the other hand threaten this food security. Fungal diseases, which is likely to increase in the future, are one of the main causes of crop losses. The excessive use of chemical pesticides and fertilizers is one of the driving factors for the increase in fungal diseases and has a negative impact on our environment. That's why interest in biological pesticides has increased.

Trichoderma harzianum is a soil fungus which is known to be useful against many fungal plant diseases. *T. harzianum* produces a wide variety of secreted proteins. These are useful in agriculture, but also in the biofuel, paper industry and other fields. *T. harzianum* can grow in the soil together with the plants or their proteins can be extracted and used separately.

T. harzianum has many methods to fight against fungal plant diseases. The first way is to directly attack the hostile fungus. This is called mycoparasitism. *T. harzianum* coils around or sticks to the hostile fungus. Then it releases enzymes that target the cell wall of the hostile fungus, these are called fungal cell wall lysing enzymes. In one study it was found that the different enzymes adapt to the hostile fungi. A second way to fight against other microorganisms, is the production of antimicrobial substances. One of them is LAAO, L-amino acid oxidase, which is as effective as the antibiotic kanamycin against the two bacteria *E. coli* and *Staphylococcus aureus* and stops their growth. Moreover, the hyphae of other fungi are disrupted which reduces their growth. Also, LAAO showed a positive effect on the growth of *T. harzianum* itself. Another substance is harzianic acid, which also stops the growth of bacteria.

Besides helping in defending against plant diseases, *T. harzianum* is in contact with the plant and supports them. It helps the plant to deal with abiotic stresses like droughts, high salt concentration or pollution of the soil. In an experiment with mung beans growing in the heavy metal chromium polluted soil, the substance harzianopyridone reduced the uptake of chromium into the plant and increased the growth of it. 6PP (6-Pentyl-α-pyrone), is also a metabolite that stimulates growth. In an experiment with common grape vine the harvest almost doubled in the plants which got 6PP. In addition, it stimulates the immune system of the plant. This process is also called the "priming effect". It means that the immune system is already in a higher state of alert and can react faster when pathogens attack the plant. For example, the protein Epl-1 has an impact on the regulation of defence genes in tomato plants.

Lastly, *T. harzianum* produces many enzymes to break down plant biomass, such as cellulases and hemicellulases. This is useful for the production of biofuel and in the paper industry. It is shown that it has advantages over already used enzyme producers like *Trichoderma reesei.* For example, the amount of hemicellulases is higher, which makes the process more efficient. Moreover *T. harzianum* has an earlier cellulase activity, which reduces process time.
Furthermore*, harzianum* not only produces enzyme that breaks down plant material but also some protein that assist this process, called accessory proteins. LPMO (lytic polysaccharide monooxygenases) is a group of enzymes, that helps cellulase to break down more resistant structures. How exactly LMPO do this is unknown, but when plant biomass got treated with these LPMO first the cellulase mixture releases double the amount of soluble sugar than without this pretreatment. Another one is swollenin, which can loosen the structure of the plant material so that the breakdown enzymes have better access and thus make the process more effective.

Not all *T. harzianum* strains have all these positive characteristics. To create a fungus that is well adapted to a certain environment, while at the same time having the optimal fighting capabilities against pathogens and enhancing crop properties, combining traits from different strains is needed. For this genetic modification is necessary. This is possible by using a method called CRISPR-Cas9. This opens many opportunities for the research and use of *T. harzianum* and its secreted proteins in the future.

2 Abstract

Trichoderma harzianum is a well-known soil fungus, which produces a wide variety of proteins. It has been long used as a biocontrol agent against a wide range of plant pathogenic genera and has a positive effect on plants in general. Additionally, it is a good producer of cellulolytic enzymes which could be used in the biofuel and paper industries. To gain a better understanding of the possibilities of *T. harzianum*, this review provides examples of secreted proteins and their functions.

This review covers actions against phytopathogenic organisms, which includes fungal cell wall lysing enzymes like chitinase and glucanases, the antimicrobial substances harzianic acid and L-amino acid oxidase (LAAO), and Epl-1, that is involved in coiling and self-recognition and is also involved in the "priming effect" in plants. Further, the plant growth promoting effect of 6-Pentyl-α-pyrone (6PP) and the reduced effect of chromium pollution through harzianopyridone are described. As the final topic plant biomass degradation with cellulolytic enzymes and the accessory proteins lytic polysaccharide monooxygenases (LPMO) and swollenin will be discussed.

To know which proteins are involved in the different processes gives a better understanding of the biocontrol and cell factory activity of *T. harzianum.* It offers the possibility to create strains with a variety of favoured characteristics to enhance their efficiency. Likewise, the proteins can be used individually, for example as plant stimulants or in enzyme preparations. The creation of optimal strains is limited by the asexual reproduction of *T. harzianum*. Genetic editing is possible, but at the same time limits the field of use. Only a few proteins that *T. harzianum* produced are mentioned and the possibilities for future research are plenty.

3 Introduction

As the human population grows, food security becomes a bigger problem. In 2015, with a population of 7.3 billion, there were approximately 800 million people worldwide that experienced food shortage and more than 2 billion people who had micronutrient deficiency (Pérez-Escamilla, 2017; *The World Population Prospects*, 2015). The UN expects that in 2030 8.5 billion people will be living on earth, which means even more food must be produced or even more people have to suffer. At the same time more and more previously used farmland cannot be used for food production anymore due to land degradation (Webb et al., 2017). Also, due to climate change, extreme weather events like drought, flooding and storms occur at a higher frequency and intensity, which often has high crop losses as a result (Lawrence & Vandecar, 2015; Taylor et al., 2017; Walsh et al., 2016; Webb et al., 2017). On top of this, about 20% of crop yield is lost through pre-harvest fungal infection, making fungi the leading cause of losses due to pathogenic infections (Fisher et al., 2018; Silva et al., 2019). Fungicides are widely used to save the crops, but with the practice of monocultures and genetically uniform crops, fungi have easy play to develop resilience to the chemicals (Gomes et al., 2015). The similarities that fungi share with animals make it more complicated to find target mechanisms that can be tackled by fungicides without posing a health risk to animals and humans (Fisher et al., 2012). Consequently, finding an alternative for the use of fungicides is of great importance.

The ascomycete *Trichoderma* genus includes many common fungal species in various ecosystems around the world and are well known for their production of substances with biological activity (Li et al., 2019). *Trichoderma harzianum* is used to improve crop yield and on top of that is used as a biocontrol agent against a wide variety of plant pathogenic genera such as *Botrytis, Fusarium, Pythium, Rhizoctonia, Sclerotina, and Verticillium* (Gomes et al., 2015; Inglis et al., 2020). By mycoparasitism, *T. harzianum* parasitizes pathogenic fungi (Silva et al., 2019). For this the target hyphae are attacked by coiling hyphae which coincides with the release of cell wall-degrading enzymes (CWDEs) like chitinases, glucanases and proteases. The proteases are also involved in the inactivation of enzymes of the pathogen (Chakraborty et al., n.d.; Elad, 2000). Also, *T. harzianum* produces different compounds with for instance antibacterial, antifungal and nematicidal activity (Li et al., 2019). In addition to the suppression of pathogens *T. harzianum* promotes the growth and quality of plants through influencing the plant metabolism (Mona et al., 2017; Pascale et al., 2017). This change in metabolism also leads to resistance against abiotic factors like drought and salinity (Mona et al., 2017; Yasmeen & Siddiqui, 2017). Also, the secreted molecules of *T. harzianum* activate plant defence responses (Gomes et al., 2017). Furthermore, *T. harzianum* produces an array of cellulolytic enzymes, such as cellulases and hemicellulases, to break down plant biomass (Zhang et al., 2020).

In this review examples are presented of the way in which *T. harzianum* acts against phytopathogenic fungi, interacts with plants, and produces cellulolytic enzymes, with a focus on extracellular molecules.

4 Action against phytopathogenic organisms

Since the 1920s it has been known that soil fungi from the genera *Trichoderma* are suited for use as a biocontrol agent (BCA) against plant pathogens (Rajesh et al., 2016). Mechanisms such as mycoparasitism, the competition for nutrients and the production of antifungal/antibiotics are the likely reasons for successfully controlling phytopathogenic organisms (Gomes et al., 2017). In a study by Braun *et al.* (2018) different fungicides were compared to *T. harzianum* MRI349 in their ability to inhibit the growth of important phytopathogenic fungi. After seven days of growth, Mancozeb showed the best results of the tested fungicides. It was able to inhibit the fungal pathogens *Verticillium dahliae, Penicillium verrucosum and Alternaria alternata*, whereas *Fusarium oxysporum and Aspergillus carbonarius* were only weakly inhibited (App. 1). However, towards a longer growth period of 29 days this inhibitory effect was seen to be reduced. The experimental co-cultivation with *T. harzianum* MRI349 showed that after seven days of incubation inhibition of growth was observed in the case of *Aspergillus flavus*, *A. carbonarius, A. alternata* and *F. oxysporum* (no data was shown of the fungicide efficiency against *A. flavus* and the efficiency of *T. harzianum* MRI349 against *V. dahliae* and *P. verrucosum*). Over time *T. harzianum* MRI349 overgrows the target fungi, adheres to it, and induces cell lysis, penetration and disintegration of conidiophores and mycelia. Another study showed similar results; within 96 h *T. harzianum* ALL42 had “completely destroyed the target fungi and sporulated” (Monteiro et al., 2010). They also analysed the secreted proteins after growth on the purified cell wall of different phytopathogens and found that the response to the different phytopathogens was different. In both studies the hyphae of *T. harzianum* stick to or coiled around the target hyphae. Figure 4.1,C shows the hyphae from *T. harzianum* in green and the target hyphae in light brown (Braun et al., 2018). This is part of mycoparasitism where first the cell wall is degraded and then with the help of an appressorium the target gets penetrated. Both the difference in the secreted proteins and the way the hyphae of *T. harzianum* adhere to or coil around the target hyphae is adjusted to the target fungus. This shows that *T. harzianum* is a potential alternative to fungicide and we will now look deeper into the different actions of *T. harzianum* starting with the degradation of the cell wall.

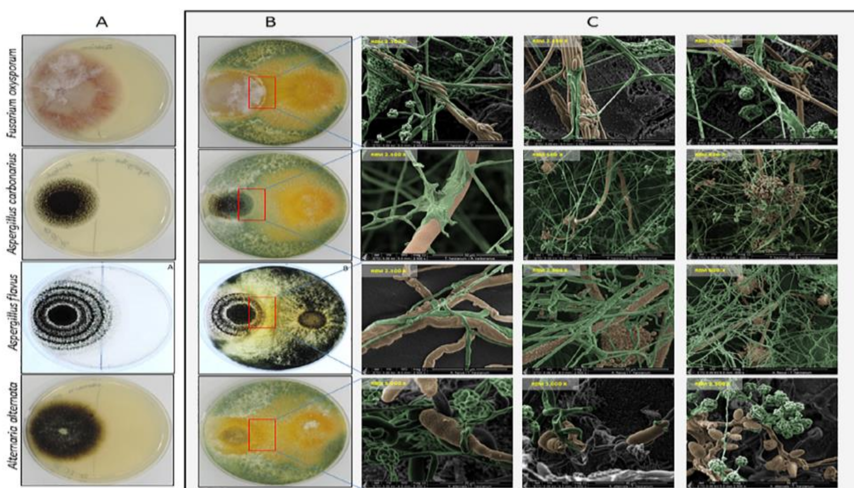

Figure 4.1 Interaction of *T. harzianum* with *Fusarium oxysporum, Aspergillus flavus, Aspergillus carbonarius, Alternaria alternata*. Phenotypical growth on YES medium after 7 days at 25 °C of either (A) the fungal species alone or (B) under competition with *T. harzianum*. (C) Post-colored SEM pictures made from the contact zone shows *T. harzianum* in green and the target fungi in brown. *T. harzianum* stick and coiled around target fungi. Adapted from: (Braun et al., 2018)

4.1 Fungal cell wall lysing enzymes

The fungal cell wall plays an important role in the functioning and ecology of the fungus. The composition can differ between different species and is highly regulated in response to environmental conditions and imposed stresses (Gow et al., 2017). However, all fungal cell walls have a similar basic composition. They contain polysaccharides like chitin and glucan, and various (glyco-) proteins (App. 2). The cell walls, or parts of them, are recognized by *T. harzianum* as non-self. Monteiro *et al.* did an experiment growing *T. harzianum* ALL42 in liquid culture containing purified cell walls from *Rhizoctonia solani* (CWRS), *Macrophomina phaseolina* (CWMP) and *Fusarium sp.* (CWFU) and measured the enzyme activity of *T. harzianum* (Monteiro et al., 2010). Striking is that the enzyme activity depends on the co-cultured fungus (Table 4.1). The increase in activity for acid phosphatase was higher in CWRS and CWFU (80, 125 times respectively) than in CWMP (20 times increased) compared to a control without purified cell walls. On the other hand, β-1,3-glucanase increased more in CWMP (118 times) and CWRS (114 times) compared to CWFU (25 times), while chitinase increased evenly 30-32 fold when compared with the control. Even without cell walls in the liquid culture, small enzyme activities were observed. It would be interesting to see if *T. harzianum* would also produce higher enzyme activities when grown on its own cell walls. This could show if it also can differentiate between living and dead cells. In the next section I will elaborate on the self-recognition.

Table 4.1 Enzyme activities of secreted enzymes by *T. harzianum* during growth in liquid medium containing purified cell wall from the phytopathogens *R. solani* (CWRS), *M. phaseolina* (CWMP), and *Fusarium sp*. (CWFU). (a) *T. harzianum* grown in TLE medium without cell wall of phytopathogens. Adapted from: (Monteiro et al., 2010)

Enzyme activity (U ml^{-1})	Control[a]	CWRS	CWMP	CWFU
β-1,3-Glucanase	0.06 ± 0.02	6.81 ± 0.04	7.05 ± 0.01	1.52 ± 0.02
Chitinase	0.05 ± 0.02	1.59 ± 0.03	1.48 ± 0.05	1.47 ± 0.01
Alkaline carboxypeptidase	0.05 ± 0.10	0.74 ± 0.02	1.72 ± 0.03	0.97 ± 0.05
β-Glucosidase	0.14 ± 0.04	7.33 ± 0.03	3.96 ± 0.04	4.25 ± 0.08
Acid phosphatase	0.66 ± 0.02	52.55 ± 0.21	13.49 ± 0.36	82.46 ± 0.68
α-Mannosidase	0.92 ± 0.03	21.76 ± 0.38	10.79 ± 0.54	3.14 ± 0.48

4.2 Coiling and hyphal recognition

T. harzianum interacts with the target fungus by coiling or sticking to their hyphae. There is no correlation between the number of coils and the production of chitinases, *N*-acetyl-β-D-glucosaminidase and β-1,3-glucanases (Almeida et al., 2007). Yet, proteins are important to identify whether hyphae are self or non-self. One protein involved in hyphal coiling is Epl-1 (Gomes et al., 2015). The *T. harzianum* ΔEpl-1 strain does not show the typical coiling around the target *Sclerotinia sclerotiorum* hyphae as the wildtype (figure 2.3). The coiling process could be restored after complementing the strain with the gene encoding Epl-1. The involvement of Epl-1 in hyphal recognition can be seen clearly when growing ΔEpl-1 together with a wild type strain of *T. harzianum*. When growing two wildtype strains of *T. harzianum* in the same dish the mycelium is homogeneous, and no hyphal degradation occurs. However, when the wildtype strain is grown together with the deletion mutant, no recognition takes place and both hyphae show signs of degradation (App. 3). Epl-1 is a cerato-platanin protein (CPPs), that is exclusively found

as fungal secreted proteins (Bonazza et al., 2015). It is known that CPPs are chitin-binding proteins and form highly ordered monolayers at hydrophobic liquid/surface interface and also form a protein layer at an air/water interface.

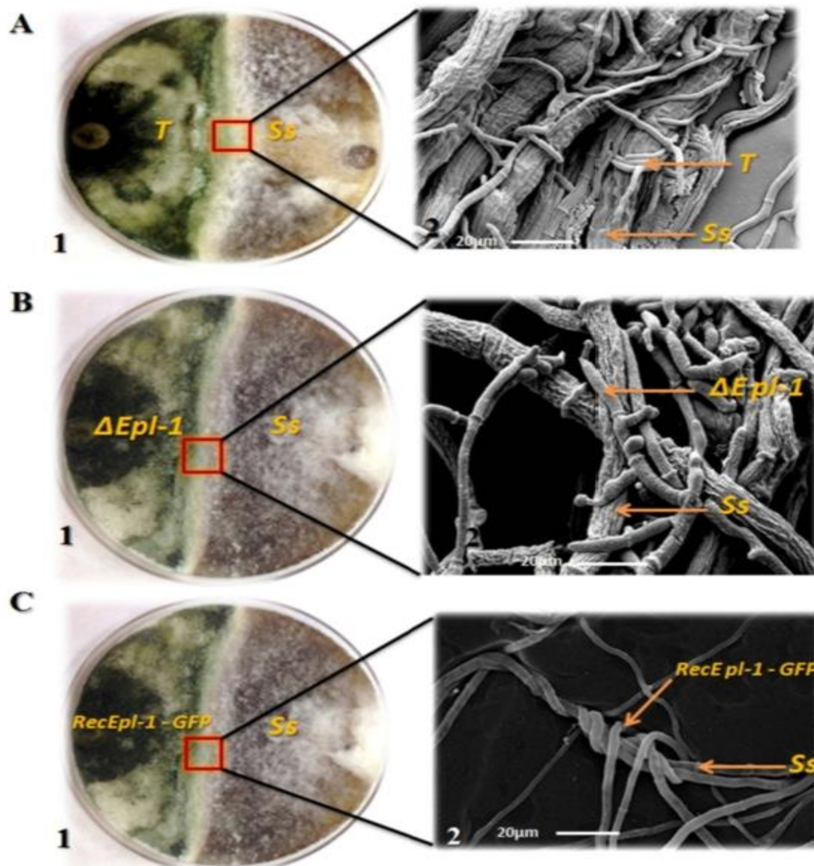

Figure 4.2 Scanning electron microscopy of the influence of EPL-1 in interaction between *T. harzianum* and *S. sclerotiorum.* (A) *S. sclerotiorum* and *T. harzianum* wild type 1: dual culture plate; 2: SEM 900x; (B) *S. sclerotiorum* and *T. harzianum* wild type ΔEpl-1 ; (C) *S. sclerotiorum* and *T. harzianum* wild type RECΔEpl-1 1: dual culture plate; 2: SEM 900x; Adapted from: (Gomes et al., 2015)

4.3 Antimicrobial substances

A different mechanism with which *T. harzianum* defends itself against other microorganisms is by producing antimicrobial substances. A total of 11 secondary metabolites of two *T. harzianum* strains were tested for their antifungal effects (Ahluwalia et al., 2015) against the phytopathogenic fungi *Macrophomina phaseolina, Rhizoctonia solani, Sclerotium rolfsii and Fusarium oxysporum.* The identified harzianopyridone showed the strongest response with an EC_{50} of 35.9– 50.2 µg/mL and inhibited growth more than 90% in *R. solani, F. oxysporum* and *S. rolfsii* (App. 4). They also tested the antifungal activity of Bavistin, a standard fungicide, which had the strongest antifungal effect of the tested substances.

For the first time isolated from *T. harzianum* SY-307 in 1994, harzianic acid (HA) demonstrated to have antimicrobial activities against the bacterium *Pasteurella piscicida sp. 6395* (Sawa et al., 1994). Further, HA shows antimicrobial activity in different pathogenic organisms

like the oomycete plant pathogen *Pythium irregulare* and the plant pathogenic fungi *S. sclerotiorum* and *R. solani* (Vinale et al., 2009). The antifungal working was tested by putting 5 mm pathogen plugs in the center of a petri dish containing potato dextrose agar. A droplet with harzianic acid in a concentration ranging from 0.01 to 200 µg was applied to each sample. A concentration of 10 µg was enough to completely inhibit the growth of *P. irregulare* and *S. sclerotiorum,* whereby *R. solani* was only mildly affected. An amount of 100 µg was enough to completely stop the growth of *R. solani.* Harzianopyridone is a good examples of a substance that has more than one function. Additional to the antimicrobial properties they reduce influence of abiotic stress, that will be further described in section 4 plant interaction.

L-amino acid oxidase (LAAO) is known to have antibacterial properties, but the mechanisms behind it were unknown. A LAAO isolated from *T. harzianum* ETS 323 (Th-LAAO), was used to study the mode of action in the Gram-negative bacterium *Escherichia coli* BCRC10675 and the Gram-positive bacterium *Staphylococcus aureus* BCRC10780 (C.-A. Yang, Cheng, Liu, et al., 2011). Compared to the antibiotic Kanamycin, Th-LAAO had a similar reduction in growth in both bacteria (Figure 4.3). The maximum exposure of 6 µM for 24 h resulted in the biggest reduction in growth with 75% and 60% in *E. coli* and *S. aureus,* respectively. They looked into different mechanisms and concluded that two essential mechanisms were the most likely: permeabilization of the bacterial membrane and the production of H_2O_2.

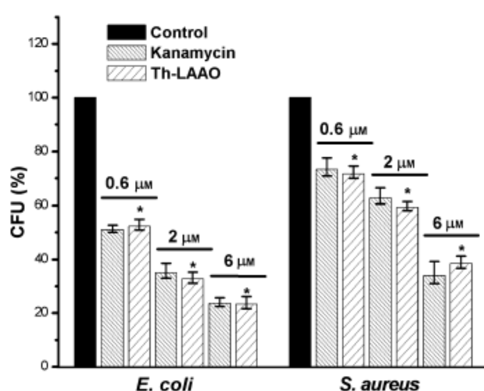

Figure 4.3 Antibacterial effects of Th-LAAO and the antibiotic Kanamycin on *E. coli* and *S. aureus.*; CFU= Colony forming units; Data are expressed as the mean ± SD; Adapted from: *(C.-A. Yang, Cheng, Liu, et al., 2011)*

First, they tested if Th-LAAO binds to the membrane with a fluorescein isothiocyanate (FITC)-labelled Th-LAAO. Fluorescens on the surface of both strains was detected. Incubation of both strains with free FITC showed no fluorescens. Propidium iodide (PI) uptake was monitored to assess whether Th-LAAO binding to the membrane coincides with loss of membrane integrity. After a 5 h treatment of *E. coli* and *S. aureus* with Th-LAAO the fluorescence compared to the negative control (no treatment) was 4.2- and 1.92-fold higher, respectively (see Table 4.2). The positive control with SDS had a 3.77- and 2.02-fold rise in fluorescence, respectively. The results indicate that the binding of Th-LAAO permeabilizes and destabilize the bacterial membrane structure.

The second mechanism was the exogenous production of H_2O_2. After an exposure of 6 µM Th-LAAO for 10h *E. coli* and *S. aureus* produced 0.275 µM and 0.175 µM H_2O_2, respectively, while the negative control did not show H_2O_2 production (App. 5a). H_2O_2 is known to induce reactive oxygen species (ROS) accumulation, which is also the case in exposure to Th-LAAO.

ROS can be measured with the fluorescent dye 2,7-dichlorofluorescin diacetate (DCF-DA).Table 3.2 shows that Th-LAAO and the positive control (H_2O_2) show an increase in fluorescence. As a consequence, ROS will result in lipid peroxidation of cell membranes and DNA damage in bacterial cells.

Table 4.2 Impact of Th-LAAO exposure to *E. coli* and *S. aureaus* on cell ROS accumulation (measured with the fluorescent dye 2,7-dichlorofluorescin diacetate (DCF-DA)), and cell permeability (Propidium iodide (PI) uptake). NT: not tested. Adapted from:(C.-A. Yang, Cheng, Liu, et al., 2011).

	Detection method	
	DCF-DA	PI
	Fluorescence intensity over control	Fluorescence intensity over control
E. coli + H_2O_2	7.75 ± 0.07	NT
S. aureus + H_2O_2	2.34 ± 0.07	NT
E. coli + SDS	NT	3.77 ± 0.12
S. aureus + SDS	NT	2.02 ± 0.26
E. coli + Th-LAAO	5.23 ± 0.25	4.11 ± 0.1
S. aureus + Th-LAAO	3.21 ± 0.05	1.97 ± 0.02

Another study researched the antimicrobial activity of Th-LAAO against the soil-borne plant pathogen *Rhizoctonia solani* (C.-A. Yang, Cheng, Lo, et al., 2011). A 6 mm sterile filter paper disc with different concentrations of Th-LAAO (3, 6, or 12 µg per disc) was put on a 2 mm disc of fungal hyphae.). At day 4 on the plates with 6µg Th-LAAO disc, hyphal lysis could be observed. After day 6 an inhibition zone of 20 mm could be seen (App. 6.A,c). Hyphae of *R. solani* showed hyphal distortion, like malformed hyphae, swelling and vacuolation (Figure 4.4,b). After vacuolation, hyphal lysis was observed (Figure 4.4, c and d). No signs of malforming or hyphal lysis was observed in the control (exposure to water) (Figure 4.4,a).

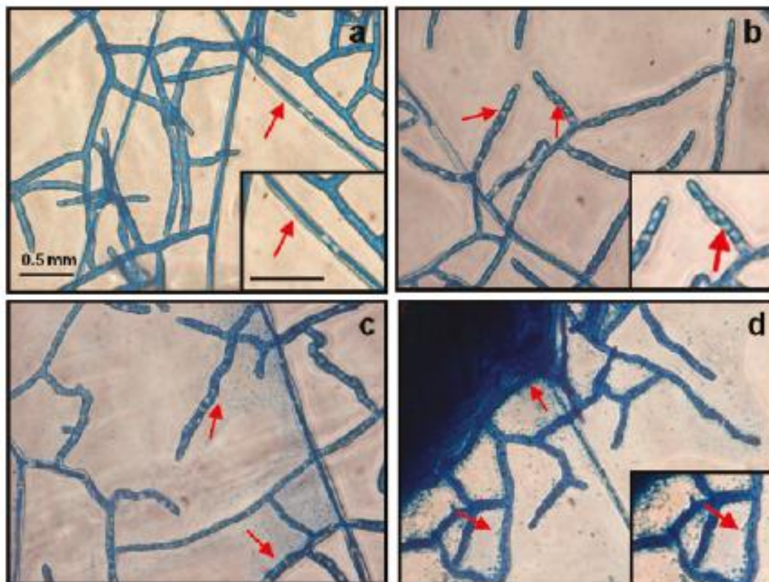

Figure 4.4 Microscopy observation on the effect of Th-LAAO on the hyphal growth of *R. solani* on day 5. a) control treatment, b-d 6 µg Th-LAAO, b) arrow point at malformed and vacuolated hyphae of *R. solani*, c) arrow points at lysis of malformed hyphae after vacuolation, d) arrow points at large amount of hyphal lysis. All panels and inset panels are in the same magnification; Adapted from: *(C.-A. Yang, Cheng, Lo, et al., 2011)*

They did the same test with *T. harzianum.* Contrary to the inhibition in growth of *R. solani* and malforming of its hyphae and hyphal lysis*, T. harzianum* showed an increase in growth. In Figure 4.5, the arrows in a and b point at the higher hyphal density and sporulation upon exposure to 6 µg Th-LAAO. Also, no morphological differences between hyphae and spores could be found between treatment and control.

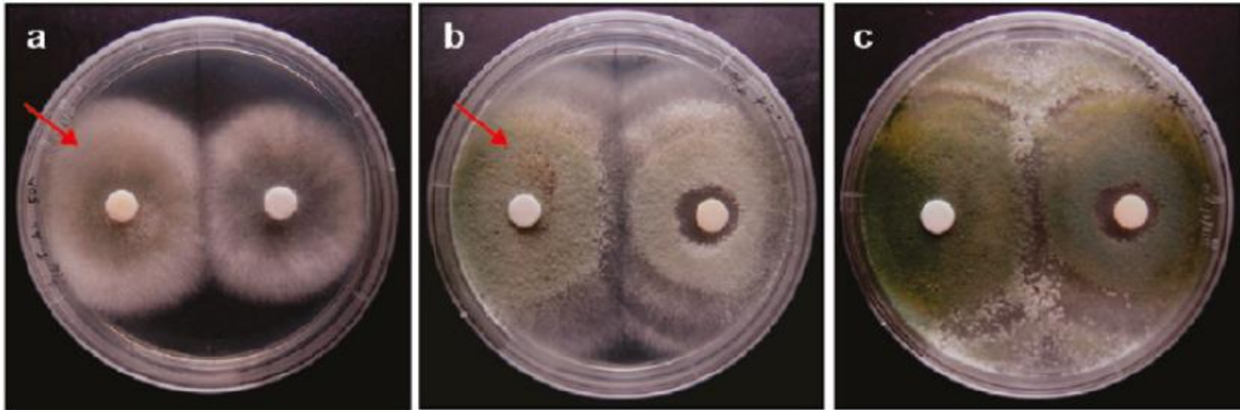

Figure 4.5 Effect of Th-LAAO on the hyphal growth and sporulation of *T. harzianum* ETS 323. *T. harzianum* on PDA medium with paperdisk, left with and right without Th-LAAO (6 µg/disk) on (a) 3rd, (b) 4th, and (c) 5th day. Arrows point to enhanced hyphal density and sporulation. Adapted from: *(C.-A. Yang, Cheng, Lo, et al., 2011)*.

4.4 Inactivation of pathogen enzymes by proteases

Many articles mention that proteases from *T. harzianum* can inactivate enzymes of pathogens, like hydrolytic enzymes produced by *Botrytis cinerea* (Cherkupally et al., 2017; Howell, 2003; Silva et al., 2019; Suárez et al., 2007; Viterbo et al.,2002). However, only one article actually studied this phenomenon(Elad & Kapat, 1999). Equal volumes of *B. cinerea* culture filtrate containing the hydrolytic enzymes (i.e. exo-polygalacturonase (exoPG), endo-polygalacturonase (endoPG) and carboxymethyl cellulase CMCase) and an aqueous solution of protease (50mU/ml) from *T. harzianum* T39 and NCIM1185 were mixed and incubated for 16 h at 22 °C. EndoPG activity was reduced by 24 and 35%, respectively, and ExoPG activity by 58 and 62%. CMCase activity was reduced only by the protease from *T. harzianum* NCIM1185 (41%) (App. 7). Furthermore, the effect of protease on disease development on bean leaves was tested. The reduction of disease with protease from *T. harzianum* T39 and NCIM1185 was 56 and 86 %, respectively. Heat deactivated protease had no effect. Moreover, protease (54 mU/ml) from *T. harzianum* T39 decreased the germination of *B. cinerea* conidia by 55% after 17h and the average germ-tube length was reduced from 100 µm to 16 µm. Notably, the secreted acidic protease from *T. harzianum* also inactivates its own chitinases, glucanases and cellulases, which are produced during mycoparasitic activities of *T. harzianum* (Viterbo et al., 2002). Together, proteolytic activity of *Trichoderma* seems not to have evolved to degrade hydrolytic enzymes of target fungi but seems to be “collateral damage”.

5 Plant interaction

Beside phytopathogenic organisms, abiotic factors like drought, flood, heavy metal pollution or salinisation of the ground are a growing problem for the agricultural industry. Furthermore, through interaction with *T. harzianum* the plant immune response and growth is stimulated.

As an opportunistic symbiont *Trichoderma* spp. interacts closely with plants by colonizing and penetrating plant roots. Intrusion happens usually to the apoplast, epidermis, and the first few cortical cell layers (Mendoza-Mendoza et al., 2018). In a hydroponic setting cucumber seedlings (*Cucumis sativus* L. cv. Delila) were inoculated with *T. harzianum* T-203. Hyphae settled at the root surface and penetrated the root epidermis (Figure 5.1,a). By mostly intercellular growth the hyphae advanced to the cortical area. *T. harzianum* can penetrate root cells by locally disrupting the cell wall. This can be seen in Figure 5.1,b, where the little black dots label cellulose. Directly to the side of the penetration no cellulose depletion is visible. The hyphal intrusion is restricted by the plant by forming wall appositions (WA) in the invaded cells and noninvaded cells below the colonized area. These WA consist mostly of callose, a plant polysaccharide (β-1,3-glucan). Hyphae show morphological chances while penetrating these WA and were stopped in their growth or even died off. Moreover, most intercellular spaces in the epidermis and outer root cortex were blocked with a dense material which varies in its compactness and hinder the hyphal growth. Furthermore, colonization of the epidermis and cortex does not lead to host cell alterations or (non-locally) cell wall digestion (Figure 5.1,a). Furthermore, secreted molecules by *T. harzianum* can influence plants as it will be described in this chapter.

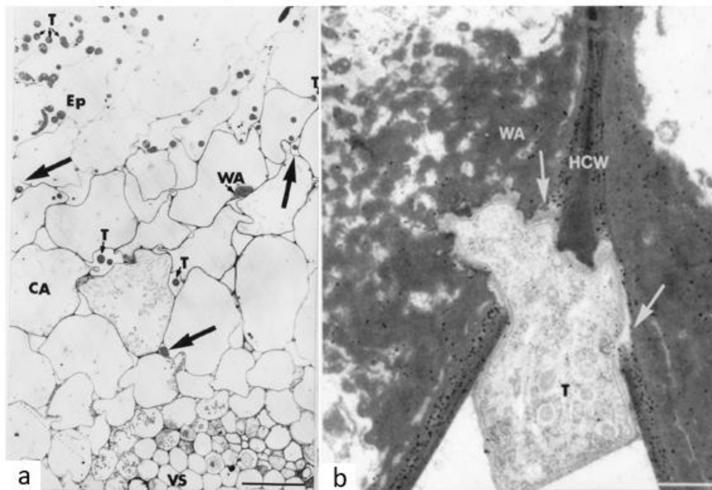

Figure 5.1 Transmission electron micrographs of *T. harzianum*-inoculated cucumber root tissues. (a) *T. harzianum* hyphae (T) on the root surface penetrate the root epidermis (Ep) and advanced primarily by growth in intercellular spaces (IS) to the cortical area (CA) pointed by arrows. (b) Labelling with b-1,4-exoglucanase–gold complex to localize cellulose (little black dots). The host cell walls (HCW) are densely labelled. Wall appositions (WA) is lightly labelled *T. harzianum* can locally disrupt the cellulose enriched HCW (arrows); Bars: a, 10 μm; b, 0.25 μm; Adapted from: (Yedidia et al., 1999)

5.1 Abiotic factors

Many studies have been published on the influents of *T. harzianum* and the change in plant metabolites in different crops under abiotic stresses (Ahmad et al., 2015; Mona et al., 2017; Singh et al., 2021).

Mona *et al.* (2017) investigated the changes in the biosynthesis of secondary plant substances in tomato plants (Solanum lycopersicum L., *var.* Rio Grande) infected with *T. harzianum* Rifai (Tr) under drought stress (Mona et al., 2017). The experiment was divided into 4 treatments: irrigated (Tr-), irrigated (Tr+), drought (Tr-), drought (Tr+). The roots of tomato seedlings got immersed in a conidial suspension (5.0×109 conidia m^{-1}) of *T. harzianum* Rifai for 1 hour and were planted in pots. For 10 weeks all treatments were grown under the same controlled condition, following 10 weeks separated into drought or irrigated treatment. Drought had a negative effect on the root and shoot length and dry weight, and the leaf area (App. 10). For example, a reduction in shoot length of approximately 52% in the drought (Tr-) treatment, while plants from the drought (Tr+) treatment only had a reduction of approximately 26% compared to the irrigated (Tr-) treatment. Moreover, plants from the irrigated (Tr+) treatment had an increase in shoot length of approximately 31% compared to the irrigated (Tr-) treatment. Similarly, an increase in photosynthetic pigments, proline content, and a positive effect on secondary plant metabolites was measured, which they assumed was the reason for the growth. However, very little is known about how *T. harzianum* induced these changes.

An example where it was examined how *T. harzianum* influences the plant, was in context of heavy metal pollution. Heavy metal pollution of soil can decrease the growth of plants and forms a danger to human health, through accumulation in the food chain (Hossini et al., 2022; Oliveira, 2012). Chromium (Cr) is one of the most potent pollutants.(Oliveira, 2012) To test the potential of harzianopyridone (HP), a volatile secondary metabolite of *T. harzianum*, to reduce Cr toxicity, mung bean (*Vigna radiata)* seeds were primed with a HP solution (Shah et al., 2022).To replicate the contaminated state, Cr (50 mg kg^{-1}) was added to the soil and irrigated with toxic effluents from the Hudiara drain in India, distilled water serving as a control (C). Priming of the seeds with 2 ppm HP reduced Cr uptake in root and shoot by 80.7% and 78.9%, respectively (Table 4.1,a). They also tested the influence of HP on plant growth under Cr stress. As expected, Cr stress decreased the growth of root and shoot length compared to the control by 57 % and 38%, respectively (Table4.1,b). Root and shoot length increased by 85% and 33%, respectively, with Cr+HP (2 ppm) treatment compared to Cr-only treatment. HP also had a positive effect on growth, without Cr stress, compared to the control. App. 9 gives an overview of negative influences from Cr stress on *V. radiata* seedlings and positive influences from HP.

Table 5.1 Effect of harzianopyridone (HZRP) on *V. radiata* under Chromium (Cr) stress. (a) Cr content in the root and shoot, (b) root and shoot length; Adapted from: (Shah et al., 2022)

a

Treatments	Cr content in root ($\mu g\ g^{-1}$ DW)	Cr content in shoot ($\mu g\ g^{-1}$ DW)
C	ND	ND
Cr	1.97 ± 0.056a	0.34 ± 0.034a
HZRP1	ND	ND
HZRP2	ND	ND
Cr + HZRP1	1.17 ± 0.029b	0.21 ± 0.073ab
Cr + HZRP2	1.09 ± 0.062bc	0.19 ± 0.081b

Different letters indicate significant differences among the treatments ($p \leq 0.05$). Cr, 50 mg kg^{-1}, HZRP1, 1 ppm; HZRP2, 2 ppm.

b

Treatments	Root length (cm)	Shoot length (cm)
C	11 ± 0.89bc	29 ± 1.76bc
Cr	7 ± 0.54d	21 ± 1.05d
HZRP1	14 ± 1.03ab	33 ± 2.18b
HZRP2	15 ± 0.72a	38 ± 2.89a
Cr + HZRP1	10 ± 0.38c	25 ± 1.27cd
Cr + HZRP2	13 ± 1.28b	28 ± 1.47c

Different letters indicate significant differences among the treatments ($p \leq 0.05$). Cr, 50 mg kg^{-1}, HZRP1, 1 ppm; HZRP2, 2 ppm.

5.2 Plant growth

In addition to protection against abiotic and biotic threats, an increase in growth is of great importance for food production. In the earlier described study of Mona *et al.* (cross ref),inoculation with *T. harzianum* lead to a significant increase in growth (Mona et al., 2017). An increase in shoot length of approximately 31%, root length of approximately 10%, shoot dry weight by approximately 31%, root dry weight by approximately 35%, and leaf area by approximately 38% compared to the control was measured. (cross ref table in app).

The influence of *T. harzianum* T22 and its metabolite 6-pentyl-a-pyrone (6PP) on *Vitis vinifera*, Common Grape Vine, was studied in terms of improvement in yield and grape quality (Pascale et al., 2017). 6PP is an important volatile organic compound (VOC) produced by *Trichoderma* spp., and can account for a large part of the VOC profile (57.94% in *Trichoderma atroviride)* (Garnica-Vergara et al., 2016). In the field experiment, the plants were drenched with a spore suspension of T22 (10^8 spores/ litre) or a solution of 1µM 6PP. There was no additional fertilization. The increase in yield was 63% with T22 and 97% with 6PP, compared to the control, which uses only water (Figure 5.2, a). The grape quality was measured by antioxidant activity and the total polyphenols amount. The increase in antioxidant activity with *T. harzianum* T22 and 6PP was 48.7 and 60.3%, respectively (Figure 5.2b). Furthermore, there was a similar increase of polyphenol content in the fruits of the two treatments, compared to the control, (no data given, Figure 5.2,c).

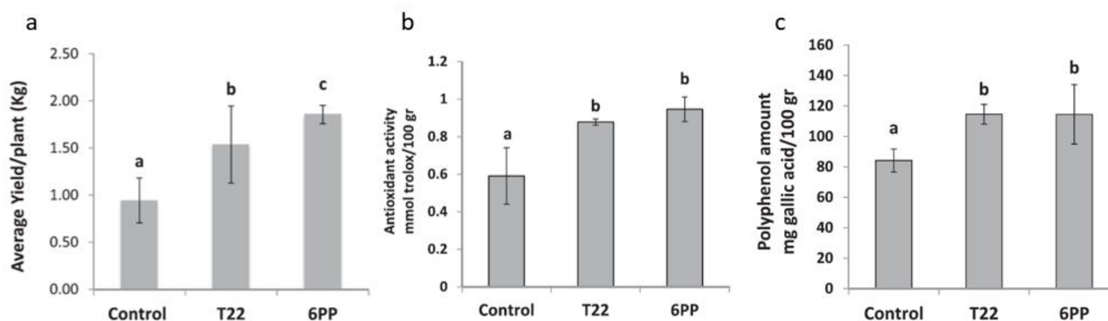

Figure 5.2 Effect of T. harzianum strain T22 (T22) and 6-pentyl-a-pyrone (6PP) treatments on Vitis vinifera. *a) Grape yield. Bars indicate the mean of yield per plant in terms of Kg of grapes produced. b) Grape fruits antioxidant activity. Bars indicate the mean on 100 gr of products, expressed as mmol equivalents of Trolox. c) Grape fruits total polyphenols amount. Bars indicate the mean of polyphenols amount on 100 gr of products expressed as mg equivalents of gallic acid. Different letters on the bars indicate significative* differences according to LSD test (p _ 0,05). Control: H2O treated ; Adapted from: *(Pascale et al., 2017)*

To look deeper into the working of 6PP in roots, *Arabidopsis thaliana* (L., Heynh.) Columbia (Col-0) ecotype, and different transgenic *A. thaliana* lines were used (Garnica-Vergara et al., 2016). For the determination of plant growth and development, 6PP (0, 50, 75, 100, 125, 150, 175 and 200 µM 6PP dissolved in ethanol) was added to the plant growth medium (agar plates with 0.2 x Murashige and Skoog (MS) medium). Plants grown in 50 - 175 µM 6PP had an increase of shoot, root, and total plant biomass (specific numbers not given). In contrast, the highest concentration of 200 µM 6PP had no increase in biomass. A concentration of 125 µM and above inhibited the primary root length. No cell-damaging effect was found from higher doses of 6PP. They concluded that the decline in primary root growth at high 6PP concentration was due to a reduction in the

cell division zone in the primary root meristem. Furthermore, the lateral root number increased at 50-150 µM 6PP, 3-fold increase by 150 µM in 6-day old seedlings and was similar to the control at higher concentrations. The lateral root density increased drastically at concentration above 125 µM.

In the study of Shah *et al.* (2022), which is described in 4.1 Abiotic factors in more detail, harzianopyridone (HP) increased growth in plants. Root length in plants primed with HP solution increased by approximately 27% (1.0 ppm HP in priming solution), and approximately 36% (2.0 ppm HP in priming solution) compared to the control. Shoot length increased by approximately 14% (1.0 ppm HP), and approximately 31% compared to the control. The increase in growth might be influenced by an increase of net photosynthetic rate through *i.e.* enhanced stomatal conduction, a decrease in chlorophyllase and reduction in ROS by antioxidant activity.

5.3 Promoting plant resistance

The ability to prime crops against pathogens is a highly studied field, which focus on what happens in the plant and not how *T. harzianum* induced it. The priming effect is the induction of a “strong and faster transcription of defence-related genes” upon pathogen attack (Ahn et al., 2007).

The involvement of Epl-1 protein (more information see 2.2) in the regulation of defence genes in tomato plants (Solanum lycopersicum var. Marmande) infected with Botrytis cinerea was examined using a Epl-1 Knockout (ΔEpl-1) *T. harzianum* strain (Gomes et al., 2017). In the pot experiment tomato seeds were coated with a *T. harzianum* spore suspension (wild type or ΔEpl-1 mutant) (2 x 10^8 ml^{-1} per 30 seeds). 4-week-old tomato plants were infected with *B. cinerea* B05.10 and after 4 days of incubation, leaves were collected for RNA extraction. Marker genes involved in the salicylic acid (SA)- (*PR1b1* and *PR-P2*) and jasmonate/ethylene (JA/ET)- mediated signalling pathway (*PINI, PINII,* and *TomLoxA*) were analysed.

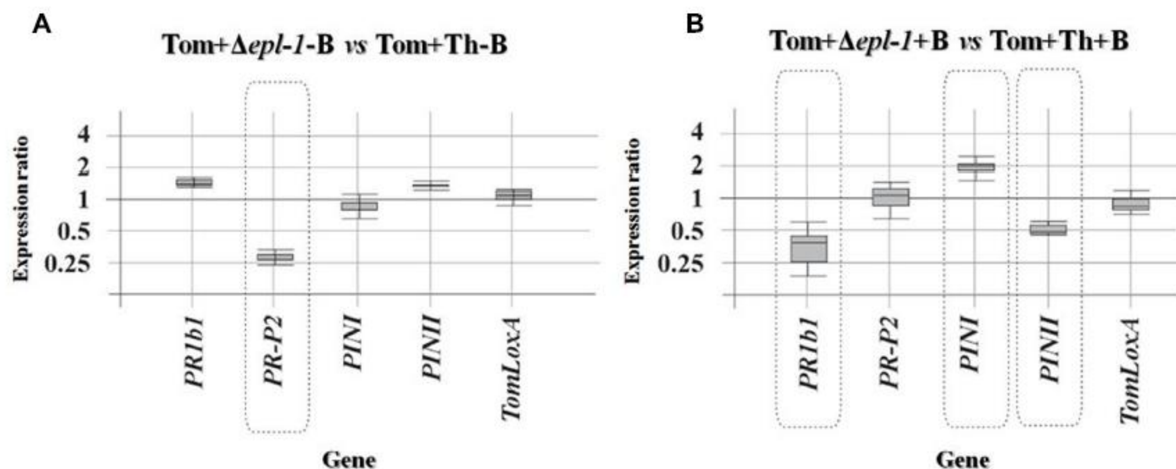

Figure 5.3 Expression ratios of marker genes of the SA- and JA/ET-pathways in tomato plants (Tom) inoculated with the *T. harzianum* ΔEpl-1 mutant (Δepl-1) versus plants inoculated with *T. harzianum* wild-type strain (Th). (A) Plants not infected with *B. cinerea* (–B). (B) Plants infected with the pathogen *B. cinerea* (+B); Adapted from: (Gomes et al., 2017)

The gene expression in non-infected plants with and without *T. harzianum* wild type, and with and without *T. harzianum* ΔEpl-1 mutant were tested. *TomLoxA* was downregulated in both settings, which indicates an involvement of *T. harzianum* unrelated to Epl-1(
App. 8). Further, the biggest difference was in the expression of *PR-P2,* which was slightly upregulated in *T. harzianum* plants and downregulated in ΔEpl-1 mutants. This is also evident when comparing non-infected plants colonized with ΔEpl-1 mutant against plants colonized with wild type *T. harzianum, PR-P2* was down-regulated (Figure 5.3,A). Whereas in infected plants, *PR-P2* remained unchanged, *PINI* was upregulated, and *PR1b1* and *PINII* were down-regulated (Figure 5.3,B). The results suggest an involvement of the Epl-1 protein in the regulation of gene expression related to the SA response, in the presence (PR1b1) as wel as absence (PR-P2) of the pathogen.

6 Plant biomass degradation

A bioinformatic approach identified 430 carbohydrate-active enzymes (CAZymes) in the *T. harzianum* genome, of which 47% were classified as secreted proteins (Ferreira Filho et al., 2017). For the enzymatic breakdown of lignocellulosic material, a variety of enzymes are necessary such as cellulases, hemicellulases and glycosidases (Zhang et al., 2020). Also, non-catalytic proteins are important.

6.1 Cellulolytic activity

Lignocellulose has a complex structure and not only the amount of enzyme produced is important in the process of degradation, but the composition of the enzyme cocktail and their synergistic effects is as well (Zhang et al., 2020). Enzyme activity of commercial enzyme preparations (C 9748 from *Trichoderma longibrachiatum* and Celluclast 1.5L from *T. reesei*) were compared with the enzyme cocktail of *T. harzianum* EM0925 when grown on corn stover (Zhang et al., 2020). The Filter Paper activity (FPA) is a common way to measure cellulase activity (Yu et al., 2015). *T. harzianum* EM0925 has an FPA which is 1.73 and 1.46-fold higher than C9748 and Celluclast 1.5 L, respectively (Table 4.1 blue box). In particular the high level of β-glucosidase, which was expressed 311 times higher than in Celluclast 1.5L and 2.9 times in C 9748, was noticeable (Table 4.1 green box). The article stated that the low protein amount of β-glucosidase in the protein cocktail of *T. reesei* limited the effective alteration of the product cellobiose into glucose (de Castro et al., 2010). The hemicellulase β-xylosidase and to a lesser extent xylanase, arabinofuranosidase and mannanase activity of *T. harzianum* EM0925 were drastically higher than in the commercial preparations (Table 4.1 red box). Hemicellulases are essential for the degradation process, because even small amounts of hemicellulose can inhibit cellulose conversion and make the process inefficient (Chylenski et al., 2019). Cellulose is derived entirely from glucose, whereas hemicellulose contains less favorable sugars like xylose and arabinose (Heinze, 2005).

Table 6.1 Enzyme activity of *T. harzianum* EM0925 and commercially used enzyme preparations of *T. longibrachiatum* (C 9748) and *T. reesei* (Celluclast 1.5L) (enzyme activity given in U/mg protein). Adapted from: (Zhang et al., 2020)

Cocktail	FPA	EG	CBH	βG	Xyl	βX	Ara	Man	Amy
C 9748	1.60 ± 0.20 a	19.70 ± 2.90 a	52.20 ± 1.90 a	29.0 ± 2.70 a	26.60 ± 3.20 a	34.80 ± 1.40 a	0.03 ± 0.00 a	0.71 ± 0.00 a	0.30 ± 1.00 a
Celluclast 1.5 L	1.90 ± 0.02 a	8.33 ± 0.18 b	32.70 ± 1.80 b	0.27 ± 0.002 b	10.80 ± 0.14 b	0.61 ± 0.02 b	0.31 ± 0.00 b	3.90 ± 0.11 b	ND
EM0925	2.77 ± 0.08 b	11.20 ± 0.13 c	33.76 ± 2.40 b	84.97 ± 0.62 c	314.70 ± 3.23 c	203.60 ± 18.60 c	11.72 ± 0.33 c	6.70 ± 0.12 c	3.92 ± 0.02 b

FPA: filter paper activity; EG: endoglucanase; CBH : cellobiohydrolase, βG: β-glucosidase; Xyl: xylanase;βX: β-xylosidase; Ara: arabinofuranosidase; Man: mannanase; Amy: amylase ND: not detected; Standard deviation of three replicates; letters indicate statistical significance by ANOVA ($p<0.05$);

Zhang *et al*. also measured the glucose and xylose yield from *T. harzianum* EM0925 and Celluclast 1.5L. They used UGCS (Ultrafine grinded pretreated corn stover) as substrate and added three different dosages, 5, 10, 30 FPU/g (Filterpaper unit/g) of enzyme preparation. After 72h, all three dosages of cellulose were completely converted to glucose by the enzyme mix of *T. harzianum* EM0925 (fig. 4.1a). Furthermore, the xylan conversion at the low dosing was already 75.35% and increased when dosage increased to 100% conversion at the highest dosage (fig. 4.1b). Meanwhile, Celluclast 1.5L had a maximal cellulose and other glucans conversion of 85.59% and xylan conversion of 66.18% at the highest dosage.

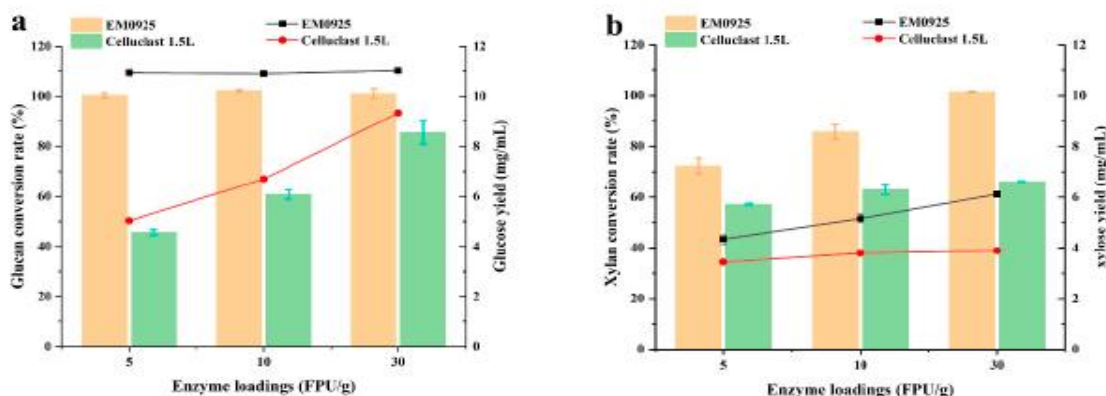

Figure 6.1 (a)Conversion of Cellulose and other glucans and xylan and sugar yield from UGCS by Celluclast 1.5L and *T. harzianum* EM0925 enzyme cocktail. (a) Cellulose and other glucans (in figure called Glucan) conversion and glucose yield, (b): xylan conversion and xylose yield. Error bars show standard deviation from three replicas; Adapted from:(Zhang et al., 2020)

In another study *T. harzianum* strain LZ117 was compared to two strains of *T. reesei* in terms of enzyme activity (Li et al., 2019). For the evaluation of major cellulolytic enzymes and extracellular proteins a wild type strain (QM6a) of *T. reesei* and a hyper-producing strain (QM9414) were used. LZ117 has an earlier induction of enzyme production with 60.67% of its peak value after only 48 hours and the highest enzyme activity after 144h, 24h prior to *T. reesei* QM9414 (fig. 4.2). Moreover, *T. harzianum* LZ117 has a higher cellobiohydrolase (pNPCase), β-glucosidase (pNPGase) and xylanase production than the two *T. reesei* strains.

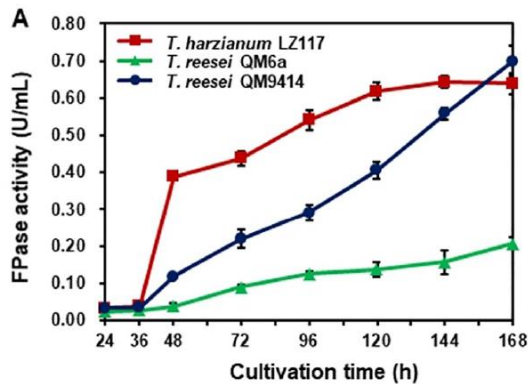

Figure 6.2 Cellulase activity by *T. harziahum* LZ117 and the *T. reesei* strains QM6a and QM9414 throughout time cultivation on 2% cellulose; Adapted from: (Li et al., 2019)

6.2 Accessory proteins

The main purpose in plant biomass degradation is often the conversion of cellulose, as it is derived entirely from glucose, whereas hemicellulose contains less favorable sugars like xylose and arabinose (Heinze, 2005). Xylanase, pectinase, and β-glucosidase can therefore be seen as accessory proteins. Another group of enzymes that is important are lytic polysaccharide monooxygenases (LPMO). This group of enzymes was first discovered in 2010 and the mode of action is not fully understood (Chylenski et al., 2019; Johansen, 2016). Nevertheless, LPMOs are already used in enzyme preparations for cellulose degradation. LPMOs make up 3.2% of the total quantified CAZymes secreted by *T. harzianum* (Zhang et al., 2020). LPMOs are copper-dependent enzymes most of which are known for their involvement in the degradation of cellulose and chitin, by catalyzing the oxidative breaking of glycosidic bonds (Johansen, 2016). In cellulose the sugar carbons are oxidized at the C1 or C4 position creating both oxidized and reduced glucose monomers (Dixit et al., 2019). LPMO facilitates the cellulases to break down crystalline structure, which is otherwise highly resistant to enzymatic conversion (Eibinger et al., 2014). In an experiment to find out the synergism between LPMO and cellulases researchers used purified LMPO from *Neurospora crassa* and a cellulase mixture from *T. reesei* on Avicel (Eibinger et al., 2014). After pretreatment with the purified LPMO, the cellulase mixture had a two times higher release of soluble sugar than the samples without pretreatment (fig.4.3).

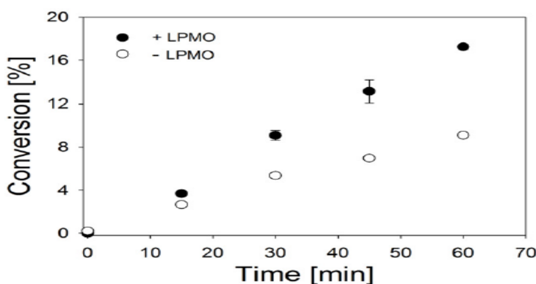

Figure 6.3 Effect of LPMO pretreatment on cellulose degradation. Cellulose with and without LPMO pretreatment was incubated with *T.reesei* cellulase mixture for 1h at 50 °C; D-glucose concentration measured in supernatant; error bars show standard deviation for three replicas; Adapted from: (Eibinger et al., 2014)

A protein with a different mode of action is swollenin. Swollenin is an expansin-like protein (Santos et al., 2017). Expansins are non-enzymatic proteins which are known for their involvement in the degradation of plant biomass. The expansin family includes a group of proteins in amoebas, bacteria, fungi, nematodes, and plants. Expansins play a role in breaking up cellulose and other cell wall polysaccharides by loosening macrofibrils. By loosening macrofibrils, also known as amorphogenesis, other enzymes involved in biomass degradation have better access and thus make the process more effective (Rocha et al., 2016; Santos et al., 2017). The swollenin gene of *T. harzianum* (ThSwo) was expressed in *E. coli* (Santos et al., 2017). The purified swollenin creates a rough and deformed surface on Avicel (microcrystalline cellulose), which is not seen in the control sample (Fig 4.4).

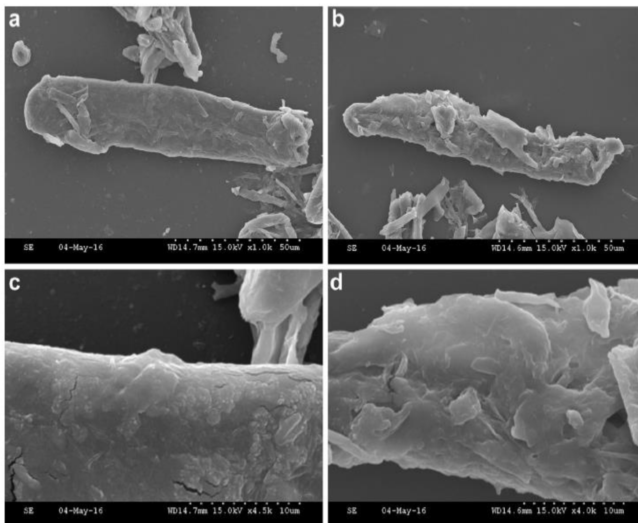

Figure 6.4 **Disruptive action of swollenin on Avicel.** Scanning electron micrographs of Avicel without swollenin (a) 1000x,(c) 4500x , and with swollenin (b) 1000x, (d) 4000x after incubated for 72h at 45 °C; Adapted from: (Santos et al., 2017)

The activity of commercial xylanase from *Trichoderma viride* on a xylan sample was followed with and without ThSwo. The results show that the xylanase hydrolysis was enhanced reaching up to 147 ± 7% (fig 4.5). There was no difference between the negative control and the samples with only xylanase. Furthermore, in xylan incubated with just ThSwo a sugar reduction was not observable. Therefore, there is no evidence of a degrading effect of ThSwo, however it has a supporting function by promoting amorphogenesis. ThSwo represents 1.18 mol% of the total secretome when growing *T. harzianum* on delignified sugarcane bagasse (DSB), which is a cellulose and hemicellulose rich agricultural waste product (Loh et al., 2013; Santos et al., 2017). For comparison the most abundant protein detected in the secretome was cellobiohydrolase II with 8.32 mol%.

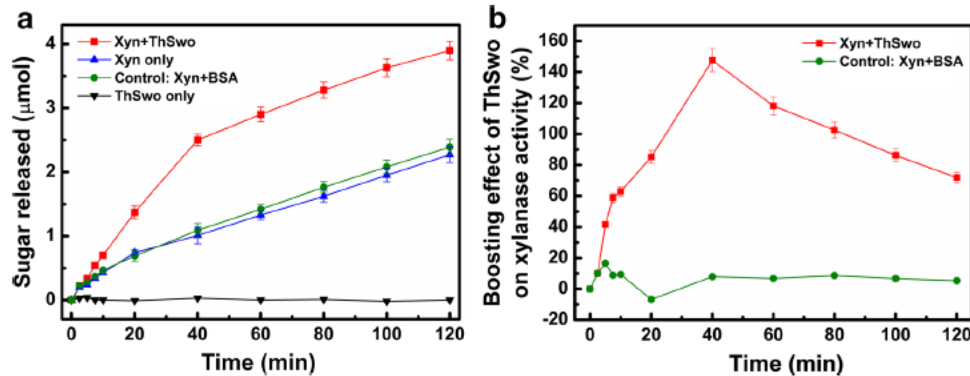

Figure 6.5 Synergistic effect of swollenin on xylanase activity. (a) Reduction of sugar over time released by xylanase in the presence or absence of swollenin (b) Boosting effect of swollenin on xylanase activity; error bars show standard deviation for three replicas; Adapted from: (Santos et al., 2017)

The swollenin gene of *T. harzianum* is located in a CAZymes enriched genomic region that contains three consecutive CAZy genes, including a xylanase gene (xyn) and cbh1 (cellobiohydrolase I). This structural organization, in terms of gene position within the genome, is also observed in *T. reesei*. Furthermore, in both *T. harzianum* and *T. reesei*, the cbh1-swo-xyn genomic region was co-induced. Likewise, Gourlay *et al.* (2013) showed that swollenin and xylanases act synergistically with an increase of approximately 300% xylose release. All these findings suggest that the synergistic effect between swollenin and xylanases is well coordinated, and the removal of xylan raises the overall efficiency of enzymatic deconstruction of biomass.

7 Discussion and Conclusion

T harzianum is used as a BCA for many years and is known to produce a wide variety of secreted proteins. It shows comparable or better results than commonly used pesticides against different phytopathogenic organisms (Braun et al., 2018). Furthermore, in contrast to a living organism the working of chemical pesticides often is reduced over time, as seen in the experiment, and would need to be reapplied (Braun et al., 2018). This excessive use can lead to environmental pollution, developing of resistance in pathogens and can form a health risk for humans and animals (Das et al., 2020; Fisher et al., 2018; Qin et al., 2014)

Another advantage of using a living organism instead of chemical pesticides is its adaptability. *T. harzianum* showed the ability to adjust their fungal cell wall lysing enzymes to the target fungi (Monteiro et al., 2010). This adaptability could possibly also apply to the production of the multitude of antimicrobial compounds released while in contact with other microorganisms, however, this still needs to be investigated. Additionally, *T. harzianum* is known to not produce mycotoxins, making it suitable for food production (Braun et al., 2018). Furthermore, some studies show plants grown together with *T.* harzianum had an increase in growth and crop yield (Pascale et al., 2017). This can be induced by growth stimulation through secretion of substances like 6PP. Also, the reduction in stress through pathogens and environmental factors contributes to the increase in yield. However, in other studies, such as a field experiment with wheat in Spain, no impact on crop yield were found (Illescas et al., 2020). Unfortunately, little is known about the way

T. *harzianum* induces these changes, because most studies focus on the changes in the plant, not what induced these changes.

Aside from using the living organism, secreted proteins can be used to create or enhance pesticides, which are more effective and safer for the environment and humans (Blauth de Lima et al., 2017). An example is *Trichoderma reesei,* which is commonly used to produce enzymes for the degradation of lignocellulose into glucose (Li et al., 2019; Rocha et al., 2016; Zhang et al., 2020). A main difficulty is the high cost of lignocellulolytic enzymes with a low saccharification efficiency (Zhang et al., 2020). *T. harzianum* has better results in this aspect. Cellulolytic enzymes are also used in other industries, like pulp and paper, textile, laundry, agriculture, and the food industry (Ejaz et al., 2021).

Nevertheless, all these positive aspects of *T. harzianum* mentioned in this review are tested using different strains of *T. harzianum.* Some strains are better adapted to certain environments than others or are better at promoting certain benefits. To have a strain of *T. harzianum* that is well adapted to a certain environment, while at the same time having the optimal fighting capabilities against pathogens and enhancing crop properties, combining traits from different strains is needed. *Trichoderma* is a genus of asexually reproducing filamentous fungi, sexual reproduction to combine traits is not possible. (Çam & Küçük, 2020). The Gene-editing techniques polyethylene glycol (PEG) and *Agrobacterium*-mediated transformation have been used for a long time to create mutants in *T. harzianum* (Bae & Knudsen, 2000; Cai et al., 2021; L. Yang, Yang, Sun, et al., 2011). Gene-editing techniques have developed significantly in recent years and the more precise gene modification technique CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) can be applied. For the first time in 2021*,* CRISPR/Cas9-based genome editing was performed in *T. harzianum* to generate a uracil-deficient strain by inactivating the *pyr4* gene (Vieira et al., 2021). In 2023, CRISPR/Cas9-based genome editing, was used to construct a marker-free strain of *T. harzianum* (Cortes et al., 2023). One result was a strain with a deletion of the *ku70* gene, which is involved in non-homologous end joining, and showed no loss of fitness and displayed increased efficiency in subsequent gene deletions. This could accelerate and ease future research. In addition, there is no need to introduce foreign DNA, which could represent a limitation for the use of GMOs.

In conclusion, *T. harzianum* has a lot of potential for future applications in different fields, like agriculture, biofuel production and pulp industry. Future research of the working of secreted proteins will bring a deeper understanding of how *T. harzianum* influences plants, fights against pathogens and breaks down cellulose and how to apply it.

8 References

Ahluwalia, V., Kumar, J., Rana, V. S., Sati, O. P., & Walia, S. (2015). Comparative evaluation of two *Trichoderma harzianum* strains for major secondary metabolite production and antifungal activity. *Natural Product Research*, *29*(10), 914–920. https://doi.org/10.1080/14786419.2014.958739

Ahmad, P., Hashem, A., Abd-Allah, E. F., Alqarawi, A. A., John, R., Egamberdieva, D., & Gucel, S. (2015). Role of Trichoderma harzianum in mitigating NaCl stress in Indian mustard (Brassica juncea L) through antioxidative defense system. *Frontiers in Plant Science*, *6*. https://www.frontiersin.org/articles/10.3389/fpls.2015.00868

Ahn, I.-P., Lee, S.-W., & Suh, S.-C. (2007). Rhizobacteria-Induced Priming in Arabidopsis Is Dependent on Ethylene, Jasmonic Acid, and NPR1. *Molecular Plant-Microbe Interactions®*, *20*(7), 759–768. https://doi.org/10.1094/MPMI-20-7-0759

Bae, Y.-S., & Knudsen, G. R. (2000). Cotransformation of Trichoderma harzianum with β-Glucuronidase and Green Fluorescent Protein Genes Provides a Useful Tool for Monitoring Fungal Growth and Activity in Natural Soils. *Applied and Environmental Microbiology*, *66*(2), 810–815. https://doi.org/10.1128/AEM.66.2.810-815.2000

Blauth de Lima, F., Félix, C., Osório, N., Alves, A., Vitorino, R., Domingues, P., da Silva Ribeiro, Rute. T., & Esteves, A. C. (2017). Trichoderma harzianum T1A constitutively secretes proteins involved in the biological control of Guignardia citricarpa. *Biological Control*, *106*, 99–109. https://doi.org/10.1016/j.biocontrol.2017.01.003

Braun, H., Woitsch, L., Hetzer, B., Geisen, R., Zange, B., & Schmidt-Heydt, M. (2018). Trichoderma harzianum: Inhibition of mycotoxin producing fungi and toxin biosynthesis. *International Journal of Food Microbiology*, *280*, 10–16. https://doi.org/10.1016/j.ijfoodmicro.2018.04.021

Cai, F., Kubicek, C. P., & Druzhinina, I. S. (2021). Genetic Transformation of Trichoderma spp. In C. Basu (Ed.), *Biofuels and Biodiesel* (pp. 171–185). Springer US. https://doi.org/10.1007/978-1-0716-1323-8_12

Çam, S., & Küçük, K. (2020). The Effect of Salinity on Growth, Antagonistic Potential, Protease Activity, and Proline Content of Trichoderma harzianum. *Commagene Journal of Biology*, *4*, 62–66. https://doi.org/10.31594/commagene.738313

Cortes, M. V. de C. B., Barbosa, E. T., Oliveira, M. I. de S., Maciel, L. H. R., Lobo Junior, M., Contesini, F. J., Filippi, M. C. C. de, & Silva-Lobo, V. L. da. (2023). Trichoderma harzianum marker-free strain construction based on efficient CRISPR/Cas9 recyclable system: A helpful tool for the study of biological control agents. *Biological Control*, *184*, 105281. https://doi.org/10.1016/j.biocontrol.2023.105281

Das, S., Hageman, K. J., Taylor, M., Michelsen-Heath, S., & Stewart, I. (2020). Fate of the organophosphate insecticide, chlorpyrifos, in leaves, soil, and air following application. *Chemosphere*, *243*, 125194. https://doi.org/10.1016/j.chemosphere.2019.125194

Eibinger, M., Ganner, T., Bubner, P., Rošker, S., Kracher, D., Haltrich, D., Ludwig, R., Plank, H., & Nidetzky, B. (2014). Cellulose Surface Degradation by a Lytic Polysaccharide Monooxygenase and Its Effect on Cellulase Hydrolytic Efficiency. *Journal of Biological Chemistry*, *289*(52), 35929–35938. https://doi.org/10.1074/jbc.M114.602227

Elad, Y., & Kapat, A. (1999). The Role of Trichoderma harzianum Protease in the Biocontrol of Botrytis cinerea. *European Journal of Plant Pathology*, *105*(2), 177–189. https://doi.org/10.1023/A:1008753629207

Fisher, M. C., Hawkins, N. J., Sanglard, D., & Gurr, S. J. (2018). Worldwide emergence of resistance to antifungal drugs challenges human health and food security. *Science*, *360*(6390), 739–742. https://doi.org/10.1126/science.aap7999

Fisher, M. C., Henk, Daniel. A., Briggs, C. J., Brownstein, J. S., Madoff, L. C., McCraw, S. L., & Gurr, S. J. (2012). Emerging fungal threats to animal, plant and ecosystem health. *Nature*, *484*(7393), 186–194. https://doi.org/10.1038/nature10947

Garnica-Vergara, A., Barrera-Ortiz, S., Muñoz-Parra, E., Raya-González, J., Méndez-Bravo, A., Macías-Rodríguez, L., Ruiz-Herrera, L. F., & López-Bucio, J. (2016). The volatile 6-pentyl-2H-pyran-2-one from Trichoderma atroviride regulates Arabidopsis thaliana root morphogenesis via auxin signaling and ETHYLENE INSENSITIVE 2 functioning. *New Phytologist*, *209*(4), 1496–1512. https://doi.org/10.1111/nph.13725

Gohel, N. M., Raghunandan, B. L., Patel, N. B., Parmar, H. V., & Raval, D. B. (2022). Role of Fungal Biocontrol Agents for Sustainable Agriculture. In V. R. Rajpal, I. Singh, & S. S. Navi (Eds.), *Fungal diversity, ecology and control management* (pp. 577–606). Springer Nature. https://doi.org/10.1007/978-981-16-8877-5_28

Gomes, E. V., Costa, M. do N., de Paula, R. G., Ricci de Azevedo, R., da Silva, F. L., Noronha, E. F., José Ulhoa, C., Neves Monteiro, V., Elena Cardoza, R., Gutiérrez, S., & Nascimento Silva, R. (2015). The Cerato-Platanin protein Epl-1 from Trichoderma harzianum is involved in mycoparasitism, plant resistance induction and self cell wall protection. *Scientific Reports*, *5*(1), Article 1. https://doi.org/10.1038/srep17998

Gomes, E. V., Ulhoa, C. J., Cardoza, R. E., Silva, R. N., & Gutiérrez, S. (2017). Involvement of Trichoderma harzianum Epl-1 Protein in the Regulation of Botrytis Virulence- and Tomato Defense-Related Genes. *Frontiers in Plant Science*, *8*. https://doi.org/10.3389/fpls.2017.00880

Hossini, H., Shafie, B., Niri, A. D., Nazari, M., Esfahlan, A. J., Ahmadpour, M., Nazmara, Z., Ahmadimanesh, M., Makhdoumi, P., Mirzaei, N., & Hoseinzadeh, E. (2022). A comprehensive review on human health effects of chromium: Insights on induced toxicity.

Environmental Science and Pollution Research, *29*(47), 70686–70705. https://doi.org/10.1007/s11356-022-22705-6

Illescas, M., Rubio, M. B., Hernández-Ruiz, V., Morán-Diez, M. E., Martínez de Alba, A. E., Nicolás, C., Monte, E., & Hermosa, R. (2020). Effect of Inorganic N Top Dressing and Trichoderma harzianum Seed-Inoculation on Crop Yield and the Shaping of Root Microbial Communities of Wheat Plants Cultivated Under High Basal N Fertilization. *Frontiers in Plant Science*, *11*. https://www.frontiersin.org/articles/10.3389/fpls.2020.575861

Lawrence, D., & Vandecar, K. (2015). Effects of tropical deforestation on climate and agriculture. *Nature Climate Change*, *5*(1), 27–36. https://doi.org/10.1038/nclimate2430

Li, J.-X., Zhang, F., Li, J., Zhang, Z., Bai, F.-W., Chen, J., & Zhao, X.-Q. (2019). Rapid production of lignocellulolytic enzymes by Trichoderma harzianum LZ117 isolated from Tibet for biomass degradation. *Bioresource Technology*, *292*, 122063. https://doi.org/10.1016/j.biortech.2019.122063

Mendoza-Mendoza, A., Zaid, R., Lawry, R., Hermosa, R., Monte, E., Horwitz, B. A., & Mukherjee, P. K. (2018). Molecular dialogues between Trichoderma and roots: Role of the fungal secretome. *Fungal Biology Reviews*, *32*(2), 62–85. https://doi.org/10.1016/j.fbr.2017.12.001

Mona, S. A., Hashem, A., Abd_Allah, E. F., Alqarawi, A. A., Soliman, D. W. K., Wirth, S., & Egamberdieva, D. (2017). Increased resistance of drought by Trichoderma harzianum fungal treatment correlates with increased secondary metabolites and proline content. *Journal of Integrative Agriculture*, *16*(8), 1751–1757. https://doi.org/10.1016/S2095-3119(17)61695-2

Monteiro, V. N., do Nascimento Silva, R., Steindorff, A. S., Costa, F. T., Noronha, E. F., Ricart, C. A. O., de Sousa, M. V., Vainstein, M. H., & Ulhoa, C. J. (2010). New Insights in Trichoderma harzianum Antagonism of Fungal Plant Pathogens by Secreted Protein Analysis. *Current Microbiology*, *61*(4), 298–305. https://doi.org/10.1007/s00284-010-9611-8

Oliveira, H. (2012). Chromium as an Environmental Pollutant: Insights on Induced Plant Toxicity. *Journal of Botany*, *2012*, e375843. https://doi.org/10.1155/2012/375843

Pascale, A., Vinale, F., Manganiello, G., Nigro, M., Lanzuise, S., Ruocco, M., Marra, R., Lombardi, N., Woo, S. L., & Lorito, M. (2017). Trichoderma and its secondary metabolites improve yield and quality of grapes. *Crop Protection*, *92*, 176–181. https://doi.org/10.1016/j.cropro.2016.11.010

Pérez-Escamilla, R. (2017). Food Security and the 2015–2030 Sustainable Development Goals: From Human to Planetary Health. *Current Developments in Nutrition*, *1*(7). https://doi.org/10.3945/cdn.117.000513

Qin, F., Gao, Y. X., Guo, B. Y., Xu, P., Li, J. Z., & Wang, H. L. (2014). Environmental behavior of benalaxyl and furalaxyl enantiomers in agricultural soils. *Journal of Environmental Science and Health, Part B*, *49*(10), 738–746. https://doi.org/10.1080/03601234.2014.929482

Santos, C. A., Ferreira-Filho, J. A., O'Donovan, A., Gupta, V. K., Tuohy, M. G., & Souza, A. P. (2017). Production of a recombinant swollenin from Trichoderma harzianum in Escherichia coli and its potential synergistic role in biomass degradation. *Microbial Cell Factories*, *16*(1), 83. https://doi.org/10.1186/s12934-017-0697-6

Sawa, R., Mori, Y., Iinuma, H., Naganawa, H., Hamada, M., Yoshida, S., Furutani, H., Kajimura, Y., Fuwa, T., & Takeuchi, T. (1994). HARZIANIC ACID, A NEW ANTIMICROBIAL ANTIBIOTIC FROM A FUNGUS. *The Journal of Antibiotics*, *47*(6), 731–732. https://doi.org/10.7164/antibiotics.47.731

Shah, A. A., Shah, A. N., Bilal Tahir, M., Abbas, A., Javad, S., Ali, S., Rizwan, M., Alotaibi, S. S., Kalaji, H. M., Telesinski, A., Javed, T., & AbdElgawad, H. (2022). Harzianopyridone Supplementation Reduced Chromium Uptake and Enhanced Activity of Antioxidant Enzymes in Vigna radiata Seedlings Exposed to Chromium Toxicity. *Frontiers in Plant Science*, *13*. https://www.frontiersin.org/articles/10.3389/fpls.2022.881561

Silva, R. N., Monteiro, V. N., Steindorff, A. S., Gomes, E. V., Noronha, E. F., & Ulhoa, C. J. (2019). Trichoderma/pathogen/plant interaction in pre-harvest food security. *Fungal Biology*, *123*(8), 565–583. https://doi.org/10.1016/j.funbio.2019.06.010

Singh, D., Sharma, N. L., Singh, C. K., Yerramilli, V., Narayan, R., Sarkar, S. K., & Singh, I. (2021). Chromium (VI)-Induced Alterations in Physio-Chemical Parameters, Yield, and Yield Characteristics in Two Cultivars of Mungbean (Vigna radiata L.). *Frontiers in Plant Science*, *12*. https://www.frontiersin.org/articles/10.3389/fpls.2021.735129

Taylor, C. M., Belušić, D., Guichard, F., Parker, D. J., Vischel, T., Bock, O., Harris, P. P., Janicot, S., Klein, C., & Panthou, G. (2017). Frequency of extreme Sahelian storms tripled since 1982 in satellite observations. *Nature*, *544*(7651), 475–478. https://doi.org/10.1038/nature22069

The World Population Prospects: 2015 Revision. (2015). [Report]. world-population-prospects-2015-revision.html

Vieira, A. A., Vianna, G. R., Carrijo, J., Aragão, F. J. L., & Vieira, P. M. (2021). Generation of Trichoderma harzianum with pyr4 auxotrophic marker by using the CRISPR/Cas9 system. *Scientific Reports*, *11*(1), Article 1. https://doi.org/10.1038/s41598-020-80186-4

Walsh, K. J. E., McBride, J. L., Klotzbach, P. J., Balachandran, S., Camargo, S. J., Holland, G., Knutson, T. R., Kossin, J. P., Lee, T., Sobel, A., & Sugi, M. (2016). Tropical cyclones and climate change. *Wiley Interdisciplinary Reviews: Climate Change*, *7*(1), 65–89. https://doi.org/10.1002/wcc.371

Webb, N. P., Marshall, N. A., Stringer, L. C., Reed, M. S., Chappell, A., & Herrick, J. E. (2017). Land degradation and climate change: Building climate resilience in agriculture. *Frontiers in Ecology and the Environment*, *15*(8), 450–459. https://doi.org/10.1002/fee.1530

Yang, C.-A., Cheng, C.-H., Liu, S.-Y., Lo, C.-T., Lee, J.-W., & Peng, K.-C. (2011). Identification of antibacterial mechanism of l-amino acid oxidase derived from Trichoderma harzianum ETS 323. *The FEBS Journal*, *278*(18), 3381–3394. https://doi.org/10.1111/j.1742-4658.2011.08262.x

Yang, C.-A., Cheng, C.-H., Lo, C.-T., Liu, S.-Y., Lee, J.-W., & Peng, K.-C. (2011). A Novel l-Amino Acid Oxidase from Trichoderma harzianum ETS 323 Associated with Antagonism of Rhizoctonia solani. *Journal of Agricultural and Food Chemistry*, *59*(9), 4519–4526. https://doi.org/10.1021/jf104603w

Yang, L., Yang, Q., Sun, K., Tian, Y., & Li, H. (2011). Agrobacterium tumefaciens Mediated Transformation of ChiV Gene to Trichoderma harzianum. *Applied Biochemistry and Biotechnology*, *163*(8), 937–945. https://doi.org/10.1007/s12010-010-9097-7

Yedidia, I., Benhamou, N., & Chet, I. (1999). Induction of Defense Responses in Cucumber Plants (Cucumis sativus L.) by the Biocontrol AgentTrichoderma harzianum. *Applied and Environmental Microbiology*, *65*(3), 1061–1070. https://doi.org/10.1128/AEM.65.3.1061-1070.1999

Zhang, Y., Yang, J., Luo, L., Wang, E., Wang, R., Liu, L., Liu, J., & Yuan, H. (2020). Low-Cost Cellulase-Hemicellulase Mixture Secreted by Trichoderma harzianum EM0925 with Complete Saccharification Efficacy of Lignocellulose. *International Journal of Molecular Sciences*, *21*(2), Article 2. https://doi.org/10.3390/ijms21020371

9 Appendix

App. 1

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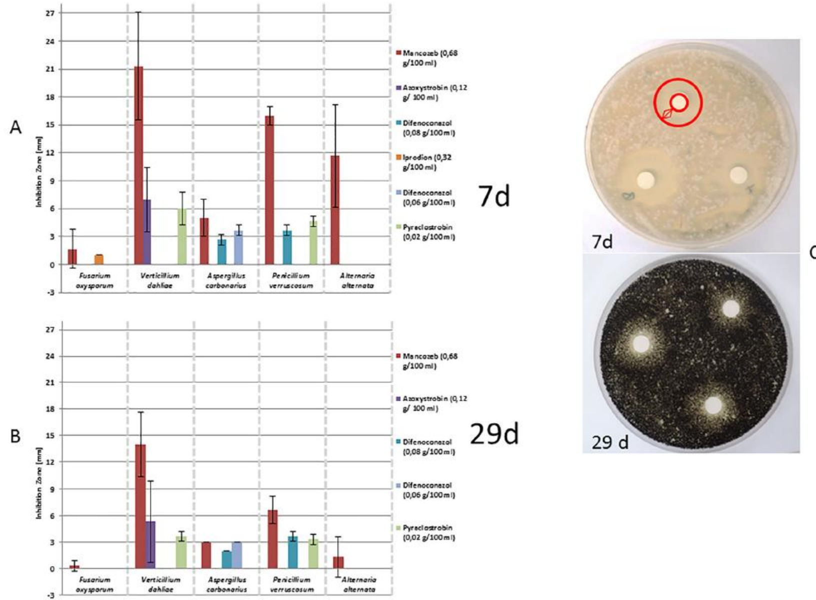


Fig. 2. Fungicide inhibition test: Quantitative assessment of the inhibition zone around the fungicide containing cellulose platelets after 7 days of growth on YES medium in the dark at 25 °C (A) and after prolonged incubation of 29 days (B). C shows the visualization of the diameter of the inhibition zone after 67 days of growth surrounding the cellulose platelets containing the respective fungicide solution (picture above) and the development of resistance against the applied fungicides after prolonged incubation of 29 days, exemplarily in case of *A. carbonarius* (picture beyond).

Source: (Braun et al., 2018)

App. 2

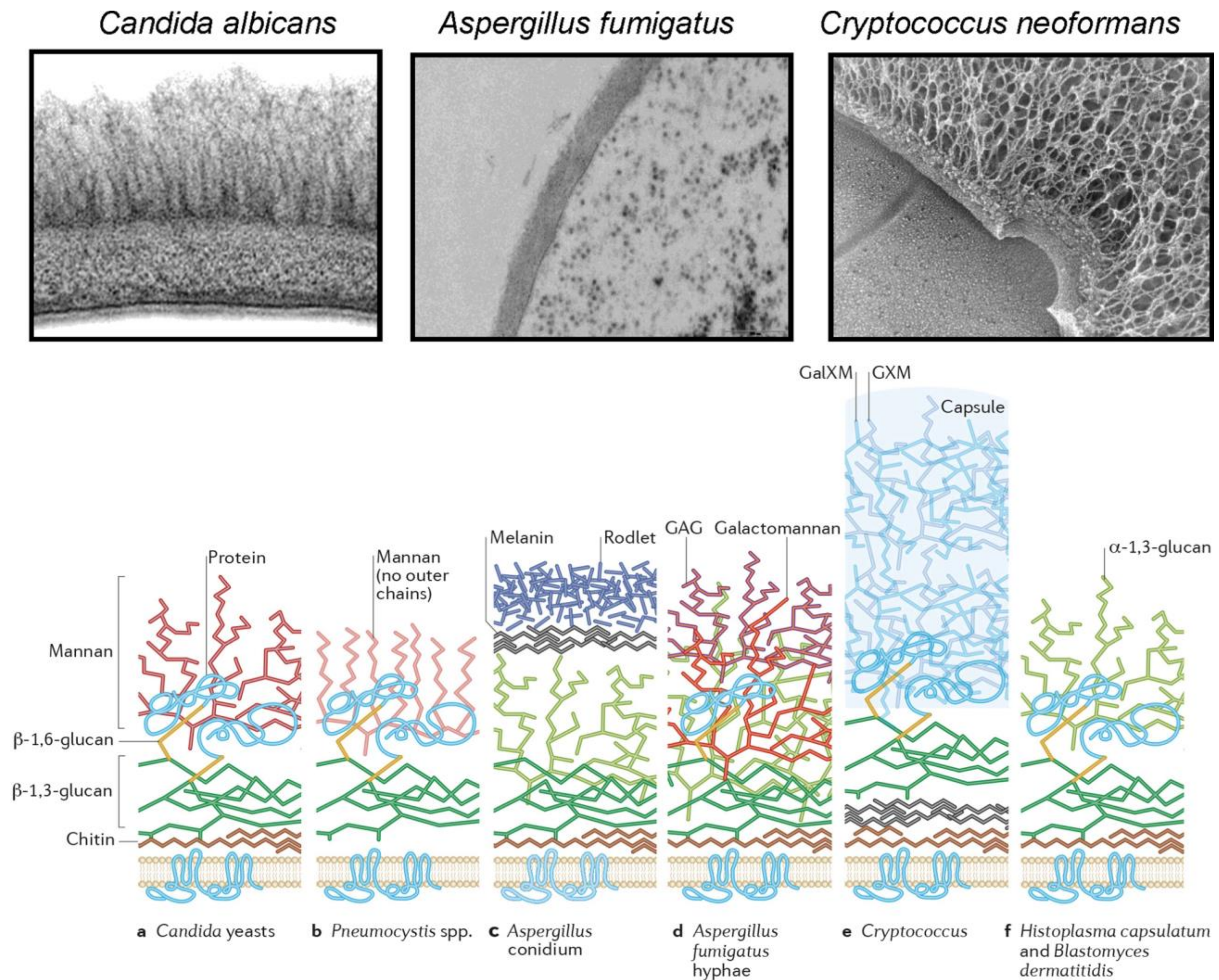


FIGURE 1 Structural organization of the cell walls of fungal pathogens. The upper panels show transmission electron micrograph sections of the cell walls, revealing mannoprotein fibrils in the outer walls of *C. albicans*, the fibril-free cell wall of an *A. fumigatus* hypha, and the elaborate capsule of *C. neoformans*. The cartoons (below) show the major components of the wall and current hypotheses about their interconnections. Most fungi have a common alkali-insoluble core of branched β-(1,3) glucan, β-(1,6) glucan, and chitin but differ substantially in the components that are attached to this. In *C. albicans*, the outer wall is heavily enriched with highly mannosylated proteins that are mostly attached via glycosylphosphatidylinositol remnants to β-(1,6) glucan and to the β-(1,3) glucan-chitin core. In *A. fumigatus*, typical of many filamentous fungi, mannan chains are of lower molecular weight and are modified with β-(1,5) galactofuran. These mannans are not components of glycoproteins but are attached directly to the cell wall core. The cell wall core polysaccharides of *A. fumigatus* are β-(1,3)-β-(1,4) glucans and are attached to an outer layer of alkali-soluble linear α-(1,3)(1,4) glucan. Conidial walls of *Aspergillus* have an outer hydrophobin rodlet layer of highly hydrophobic portions (hydrophobins) and a melanin layer; hyphae of *Aspergillus* have α-(1,3) glucan GM, and galactosaminoglycan (GAG) in the outer cell wall and limited glycosylated proteins. In *C. neoformans*, an outer capsule is composed of glucuronoxylomannan (GXM) and lesser amounts of galactoxylomannan (GalXM). The capsule is attached to α-(1,3) glucan in the underlying wall, although peptides or other glycans may also be required for anchoring the capsule to the cell wall. The inner wall has a β-(1,3) glucan-β-(1,6) glucan-chitin core, but most of the chitin is deacetylated to chitosan, and some of the chitosan/chitin may be located further from the membrane. *C. neoformans* also has a layer of melanin whose precise location is not known, but it may be incorporated into several cell wall polysaccharides and may assemble close to the chitin/chitosan layer. *Pneumocystis* cell walls may lack chitin and the outer chain *N*-mannans but retain core *N*-mannan and *O*-mannan modified proteins (56). Hyphae of *H. capsulatum* and *Blastomyces dermatitidis* have an outer cell wall layer of α-(1,3) glucan that prevents efficient immune recognition of β-(1,3) glucan in the inner cell wall. (From reference 7, with permission.)

Source: (Gow et al., 2017)

App. 3

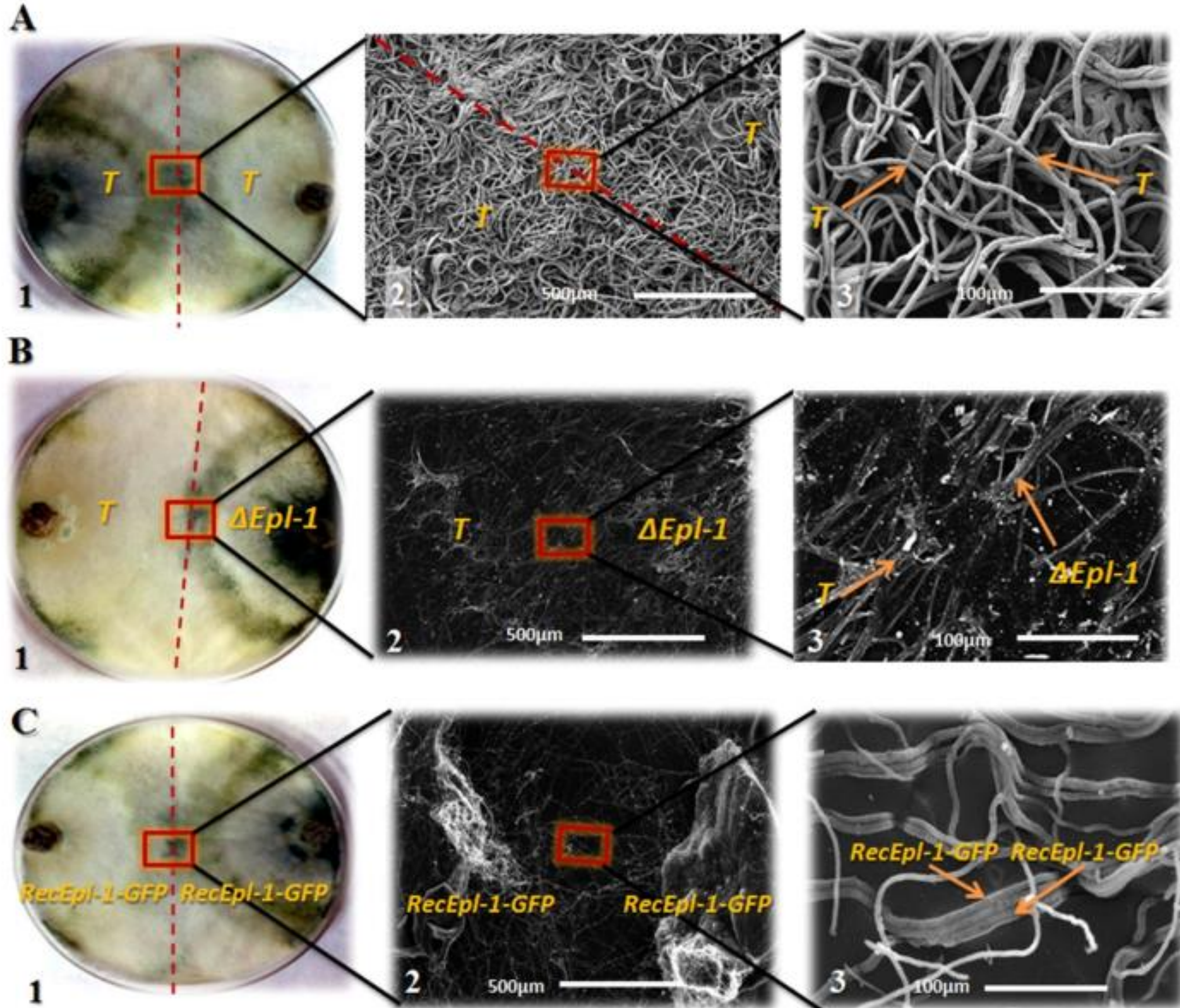


Figure 3. Scanning electron microscopy analysis (SEM). (**A**) - SEM analysis of the interaction between *T. harzianum* wild type (*T*) strains. **1** – *T. harzianum* wild type dual culture plate. **2** – SEM analysis of *T. harzianum* wild type strains interaction. 2.5 kV acceleration voltage, magnification 250×. **3** – SEM analysis of *T. harzianum* wild type strains. 2.5 kV acceleration voltage, magnification 500×. (**B**) - SEM analysis of the interaction between *T. harzianum* wild type (*T*) and *T. harzianum* Δ*epl-1* (Δ*epl-1*) strains. **1** – *T. harzianum* wild type and mutant Δ*epl-1* strains dual culture plate. **2** – SEM analysis of *T. harzianum* wild type and mutant Δ*epl-1* interaction. 30 kV acceleration voltage, magnification 350×. **3** – SEM analysis of *T. harzianum* wild type and mutant Δ*epl-1* interaction. 30 kV acceleration voltage, magnification 500×. (**C**) - SEM analysis of the interaction between *T. harzianum* RecEpl-1-GFP strains (RecEpl-1-GFP). **1** – *T. harzianum* RecEpl-1-GFP strains dual culture plate. **2** – SEM analysis of *T. harzianum* RecEpl-1-GFP strains interaction. 30 kV acceleration voltage, magnification 350×. **3** – SEM analysis of *T. harzianum* RecEpl-1-GFP strains interaction. 30 kV acceleration voltage, magnification 500×.

Source:(Gomes et al., 2015)

App. 4

Table 1. Antifungal activity[a] (EC_{50} µg mL^{-1}).

Compounds	*R. solani*	*S. rolfsii*	*M. phaseolina*	*F. oxysporum*
1	498.9	563.9	623.1	578.8
2	1515.4	1126.5	1093.1	1619.8
3	64.6	38.8	90.6	74.3
4	124.3	237.4	193.8	154.8
5	683	521.8	695.8	635.8
6	886.8	779.4	490.4	589.0
7	655.7	542.8	363.6	727.6
8	70.6	122.2	141.4	124.1
9	434.2	464.5	449.6	531.7
10	35.9	42.2	60.4	50.2
11	529.7	468.8	314.5	356.1
Bavistin	10.7	14.8	13.5	15.8

Tested compounds: palmitic acid (1), 1,8-dihydroxy-3-methylanthraquinone (2), 6-pentyl-2H-pyran-2-one (3), 2(5H)-furanone (4), stigmasterol (5) and b-sitosterol (6), 1-hydroxy-3-methylanthraquinone (7), d-decanolactone (8), ergosterol (9), harzianopyridone (10) and 6-methyl-1,3,8-trihydroxyanthraquinone (11) and Bavistin. Target fungi: Macrophomina phaseolina, Rhizoctonia solani, Sclerotium rolfsii and Fusarium oxysporum;

Source:(Ahluwalia et al., 2015)

App. 5

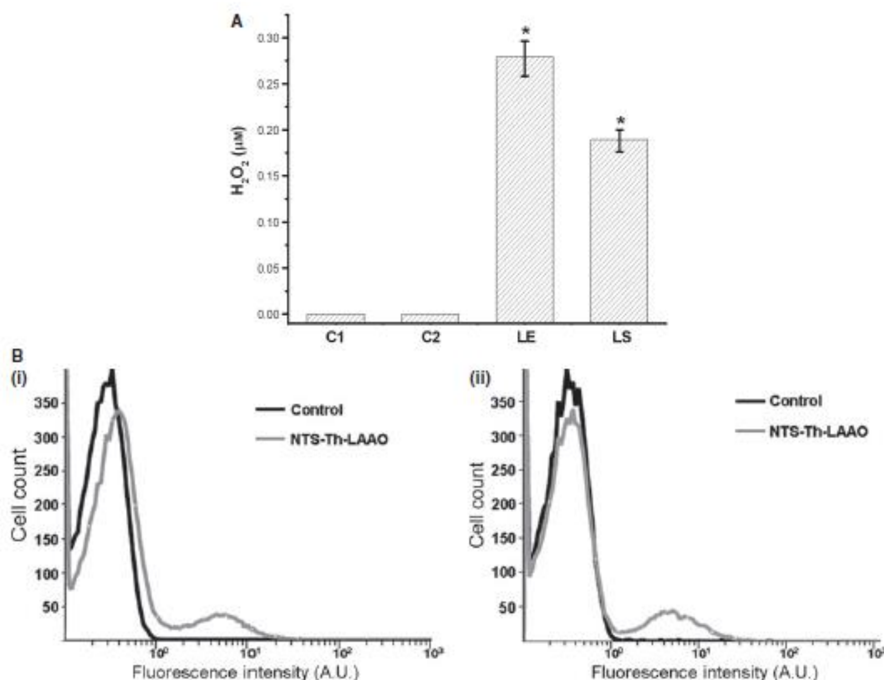


Fig. 9. (A) Exogenous H_2O_2 production after *E. coli* and *S. aureus* exposure to Th-LAAO. C1 and C2 are *E. coli* and *S. aureus* only, respectively. LE and LS are *E. coli* and *S. aureus* exposed to Th-LAAO for 10 h, respectively. All H_2O_2 concentrations were estimated by subtracting the background variations from the broth medium for different experimental conditions. Data are expressed as the mean ± SD. *Significantly different from control group ($P < 0.01$). (B) Membrane permeability measurement of (i) *E. coli* and (ii) *S. aureus* after exposure with the truncated N-terminal sequence of Th-LAAO (NTS-Th-LAAO; amino acids 1–21). Bacteria were incubated with (grey line) or without (control, black line) truncated peptide.

Source: (C.-A. Yang, Cheng, Liu, et al., 2011)

App. 6

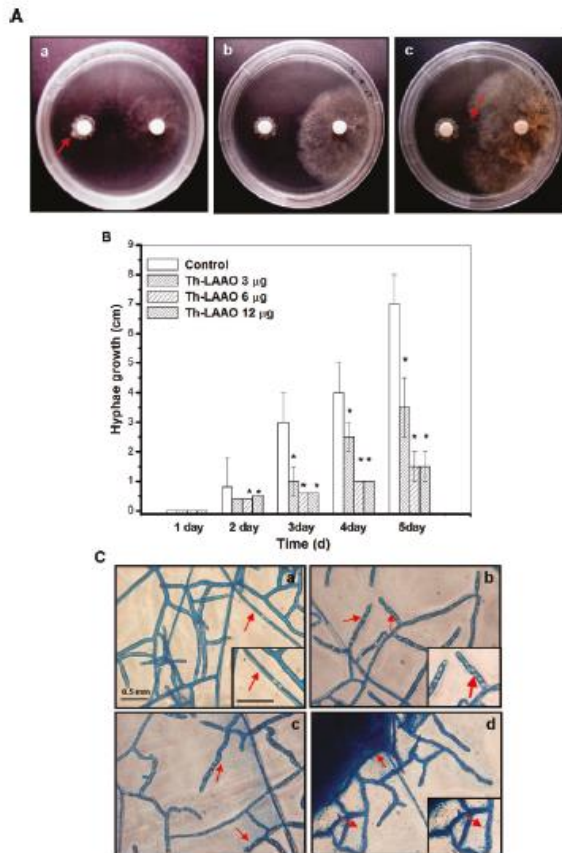


Figure 5. Effect of Th-LAAO on the hyphal growth of *R. solani*. (A) Paper disk method: growth of *R. solani* on PDA media with (left) or without (right) Th-LAAO (6 μg disk^{-1}) on PDA plates were photographed on the 4th (a), 5th (b), and 6th (c) days. The arrow in panel (a) indicates a large amount of hyphal lysis. In panel c, the arrow indicates the antifungal zone of Th-LAAO against *R. solani*. The asterisk in plot B indicates hyphal lysis. (C) Microscopy observations: all panels are presented in the same magnification. Panel (a) and its inset indicate the control treatment after the 5th day of incubation. The arrows in panel (b) and its inset indicate the malformed and vacuolated hyphae of *R. solani* after the 5th day of incubation with Th-LAAO. The arrows in panel (c) indicate the lysis of malformed hyphae after vacuolation. In panel (d) and its inset, the arrow indicates large amounts of hyphal lysis. Scale bar for inset panels = 0.5 mm, and all inset panels are presented in the same magnification.

Source: (C.-A. Yang, Cheng, Liu, et al., 2011)

App. 7

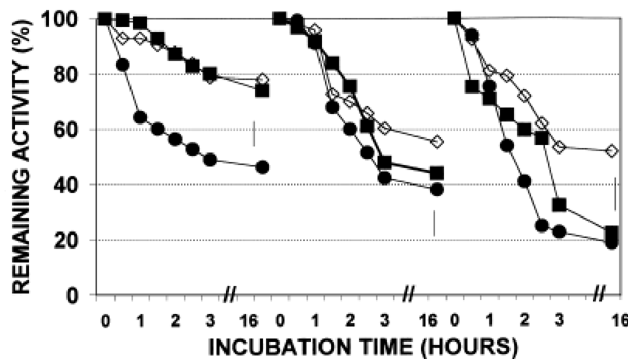
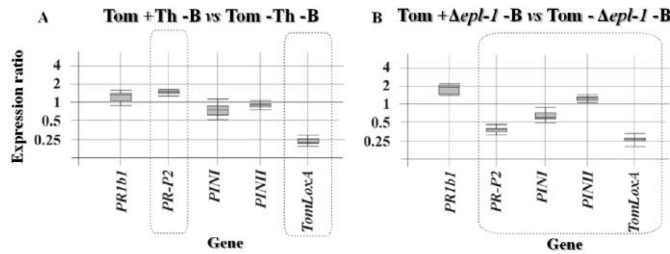


Figure 4. Effect of crude protease from cultures of *Trichoderma harzianum* T39 (■) and NCIM1185 (●) on the residual activity of the enzymes, carboxymethyl cellulase (left), endo-polygalacturonase (center) and exo-polygalacturonase (right) of *Botrytis cinerea*. ◊ = Control. Bar = LSD.

Source: (Elad & Kapat, 1999)

App. 8



Supplementary Figure S3 – Relative expression levels of genes belonging to the SA and JA defense-related pathways in 4-week-old tomato plants not infected with the pathogen *B. cinerea* (-B). **A.** Expression ratios in plants inoculated with *T. harzianum* wild type strain (Th) *versus* expression levels in non-inoculated (-Th), non-infected (-B) plants. **B.** Expression levels in tomato plants inoculated with *T. harzianum* mutant Δ*epl-1* strain (*Δepl-1*) *versus* non-inoculted (-*Δepl-1*), non-infected (-B) plants. qPCR comparative calculations and representations were carried out as indicated in the legend of Supplementary Fig. 1. Numeric values are included in Supplementary Table S2b.

Adapted from: (Gomes et al., 2017)

App. 9

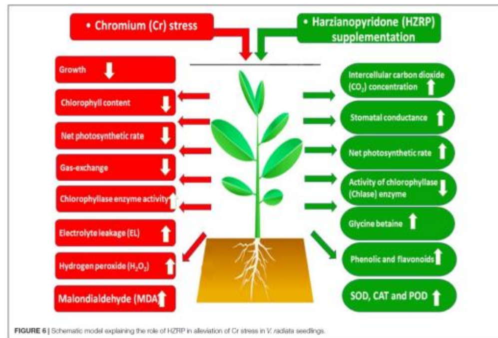


FIGURE 6 | Schematic model explaining the role of HZRP in alleviation of Cr stress in V. radiata seedlings.

Source: (Shah et al., 2022)

App. 10

Table 1 Shoot and root length, shoot and root dry weight, and leaf area in drought stressed and *Trichoderma harzianum* inoculated *Solanum lycopersicum* plants

Treatments[1)]	Shoot length (cm)	Root length (cm)	Shoot dry weight (g plant^{-1})	Root dry weight (g plant^{-1})	Leaf area (cm^2 plant^{-1})
Irrigated (Tr–)	27.12 b	13.78 b	2.43 b	3.11 b	21.87 b
Irrigated (Tr+)	35.47 a	15.16 a	3.18 a	4.21 a	38.19 a
Drought (Tr–)	13.01 d	8.75 d	1.25 d	1.68 d	14.75 d
Drought (Tr+)	20.14 c	10.83 bc	1.76 c	2.79 bc	18.96 c

[1)] Tr–, absence of *T. harzianum* inoculated; Tr+, presence of *T. harzianum* inoculated.
Data presented are means of five replicates. Different letters indicate significant differences based on Turkey's honest significant difference (HSD) test at *P*<0.05.

Table 2 Photosynthetic pigments in drought stressed and *Trichoderma harzianum* inoculated *Solanum lycopersicum* plants

Treatments[1)]	Photosynthetic pigments (mg g^{-1} FW)[2)]					
	Chlorophyll *a*	Chlorophyll *b*	Chlorophyll (*a*/*b*)	Chlorophyll (*a*+*b*)	Carotenoids	Total pigments
Irrigated (Tr–)	1.42 b	0.39 b	3.64 b	1.81 b	0.28 d	2.09 c
Irrigated (Tr+)	1.68 a	0.43 b	3.90 a	2.11 a	0.35 c	2.46 ab
Drought (Tr–)	0.81 d	0.21 c	3.85 a	1.02 c	0.46 b	1.48 d
Drought (Tr+)	1.28 c	0.67 a	1.91 c	1.95 b	0.67 a	2.62 a

[1)] Tr–, absence of *T. harzianum* inoculated; Tr+, presence of *T. harzianum* inoculated.
[2)] FW, fresh weight.
Data presented are means of five replicates. Different letters indicate significant differences based on Turkey's honest significant difference (HSD) test at *P*<0.05.

Adapted from: (Mona et al., 2017)

App. 11

TABLE 1 | Effect on plant height (cm), root length (cm) and dry weight (g plant^{-1}) under NaCl stress in presence and absence of *Trichoderma harzianum* (*TH*) in *Brassica juncea* seedlings.

Treatments	Plant height (cm)	Root length (cm)	Dry weight (g plant^{-1})
C	51.72 ± 1.57^{a}	21.11 ± 1.00^{a}	15.39 ± 0.88^{a}
C + *TH*	53.19 ± 1.59^{a}	23.72 ± 1.04^{a}	16.61 ± 0.95^{b}
100 mM	40.19 ± 1.31^{b}	15.29 ± 0.94^{b}	12.87 ± 0.73^{c}
100 mM + *TH*	47.22 ± 1.42^{c}	20.04 ± 0.98^{c}	15.73 ± 0.90^{a}
200 mM	34.24 ± 1.11^{d}	11.82 ± 0.86^{d}	10.08 ± 0.64^{d}
200 mM + *TH*	39.73 ± 1.22^{e}	14.81 ± 0.90^{e}	12.11 ± 0.70^{c}

Values are means ± S.E (n = 5), superscript letters indicate significant difference between means at $p < 0.05$.

Source: (Ahmad et al., 2015)

App. 12

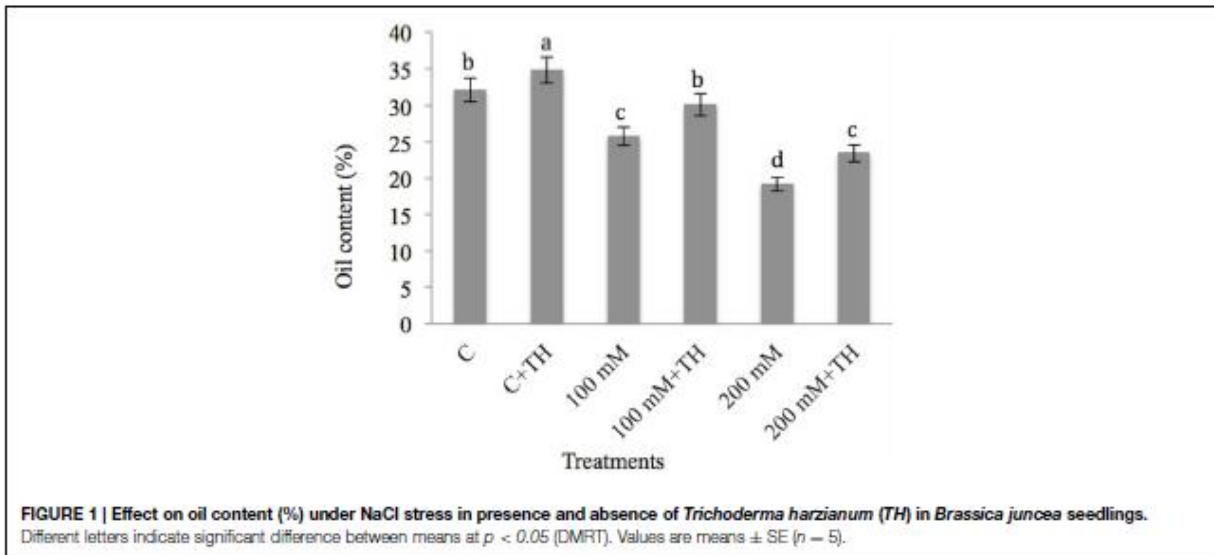


FIGURE 1 | Effect on oil content (%) under NaCl stress in presence and absence of *Trichoderma harzianum* (*TH*) in *Brassica juncea* seedlings. Different letters indicate significant difference between means at $p < 0.05$ (DMRT). Values are means ± SE ($n = 5$).

Source: (Ahmad et al., 2015)