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Antagonistic activity of *Trichoderma* spp. against *Scytalidium lignicola* CMM 1098 and antioxidant enzymatic activity in cassava

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Abstract *Trichoderma* spp. are used as antagonists against different pathogens. Despite many possibilities of using *Trichoderma* as an antagonist, there are gaps in the knowledge of the interaction between *Trichoderma*, cassava and *Scytalidium lignicola*. This fungus causes cassava black root rot and is an inhabitant of the soil, so it is difficult to control. Antagonists may contribute to the possible induction of resistance of plants because, when exposed to such pathosystems, plants respond by producing antioxidative enzymes. The test for potential inhibition of growth of *S. lignicola* CMM 1098 *in vitro* was performed in potato-dextrose-agar with two *Trichoderma* strains *T. harzianum* URM3086 and *T. aureoviride* URM 5158. We evaluated the effect of the two selected *Trichoderma* to reduce the severity of cassava black root rot and shoots. Subsequently, the production of enzymes (ascorbate peroxidase, catalase, peroxidase and polyphenol oxidase) was evaluated in cassava plants. All two *Trichoderma* strains show an inhibition of the growth of *S. lignicola* CMM 1098. The most efficient was *T. harzianum* URM 3086, with 80.78% of mycelial growth inhibition. *T. aureoviride* URM 5158 was considered the best chitinase producer. All treatments were effective in reducing severity, especially treatments using *Trichoderma*. Cassava plants treated with *T. aureoviride* URM 5158 had the highest enzyme activity, especially peroxidase and ascorbate peroxidase. *Trichoderma harzianum* URM3086 and *Trichoderma aureoviride* URM 5158 were effective in reducing the severity of cassava black root rot caused by *S. lignicola* CMM 1098.

Keywords *Manihot esculenta* · Cassava black root rot · Chitinase · Peroxidase · Ascorbate peroxidase

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Introduction

Trichoderma is a filamentous fungus with a fast growth. It intensively produces spores under adverse soil

conditions (Singh et al. 2010) and It is an endosymbiont of plants which produces spores under adverse soil conditions (Carreras-Villaseñor et al. 2012). This fungus exerts biocontrol action against plant pathogenic fungi through defensive mechanisms, antibiosis (Salas-Marina et al. 2011), competition for niche and nutrients (Hermosa et al. 2012), changes in environmental conditions (Steyaert et al. 2010), plant growth stimulation (Bogumił et al. 2013), and mycoparasitism (López-Mondéjar et al. 2011). *Trichoderma* spp. are capable of cleaving the hyphae of pathogens by secreting enzymes such as amylase (Anita et al. 2012), glucanases and chitinases (Marcello et al. 2010; Shoresh et al. 2010).

There are several studies evaluating the antagonistic potential of *Trichoderma* against plant pathogens (Doley et al. 2014; El-Gali 2015; Kumar et al. 2015). However, there is a gap of information about plant biochemical response when using these fungi as a biocontrol for soil-borne pathogen (Kipngeno et al. 2015; Srivastava et al. 2014; Tapwal et al. 2015), particularly in cassava (Sobowale et al. 2010).

The efficiency of *Trichoderma* against several pathogens was demonstrated for a number of crops such *T. viride* against root rot and white mold diseases in bean plants (Mohamed et al. 2010), *Trichoderma* spp. against *Pythium* isolated from *Lycopersicon esculentum* –Mill root rot infection, (Patil et al. 2012), *T. harzianum* for controlling peanut crown rot (Abdel-Kader et al. 2013) and *T. harzianum* in melons (Galletti et al. 2015).

Cassava suffers high losses because of diseases, especially a difficult to manage disease, cassava black root rot, caused by a soil-inhabiting pathogen, *Scytalidium lignicola* (Silva et al. 2013). The integrated disease management incorporating cultural and biocontrol agent and the reduction of chemical inputs have been promising as alternative for disease management in this crop. There are no agrochemicals registered for cassava root disease. Therefore, the use of *Trichoderma* as a biocontrol of pathogens is emerging among the alternatives for this crop (Buensanteai and Athinuwat 2012; Harman et al. 2012).

There are several studies using *Trichoderma* as a biocontrol against plant diseases. However, there are gaps in knowledge about the enzymatic action of such antagonists and the physiological response of cassava when infected by to *Scytalidium lignicola*. In the present study, we aimed to evaluate the antagonist from two *in vitro Trichoderma* strains against *Scytalidium lignicola* CMM1098 and compare to the resistance inducer for the management of cassava black root rot and determine antioxidant enzymatic responses in cassava plants.

Materials and methods

Trichoderma and pathogen isolates

T. harzianum URM 3086 and *T. aureoviride* URM 5158 were obtained from the URM (https://www.ufpe.br/micoteca/) collection of culture. They were grown in potato dextrose Agar (PDA) medium.

Scytalidium lignicola CMM 1098 was obtained from the collection of pathogenic fungi isolated from cassava roots that showed symptoms of cassava black root rot at commercial farms in Pernambuco State, Brazil (Notaro et al. 2013).

In vitro antagonistic potential of *Trichoderma* spp. against *S. lignicola* CMM1098

Experimental design was completely randomized with 3 treatments: T1 = *T. aureoviride* URM 5158, T2 = *T. harzianum* URM3086 and T3 = control (inoculated only with *S. lignicola* CMM 1098) and incubated at 26 °C ± 2 °C.

The antagonism of *Trichoderma* isolates against *S. lignicola* CMM1098 was evaluated following the dual cultures method according to Daayf et al. (2003). Petri dishes (15 cm diameter) with potato dextrose agar (PDA) were inoculated with a 5 mm diameter disc containing 8-days-old mycelial growth of *S. lignicola* CMM1098 on one edge of the plate; in the other edge, a 5 mm diameter disc with *Trichoderma* isolates was inoculated. The pathogen was inoculated first (24 h interval) due to its slow growth. We observed mycelia growth for 24 h, up to the time when the pathogen grew, covering the surface of the plate in the control treatment.

The variable analyzed was growth inhibition, calculated by the formula (1):

$$\%\text{growth inhibition} = [(C–T)/C] \times 100 \quad (1)$$

where C is radial growth of *S. lignicola* CMM 1098 in the control treatment and T is radial growth of *S. lignicola* CMM 1098 in the treatment using the *Trichoderma* isolate. The experiments were repeated and the data were subject to ANOVA. The means were

compared by Tukey tests; P values ≤0.05 were considered significant.

In vivo antagonist activity of *Trichoderma* spp. against *S. lignicola* CMM 1098

The best *Trichoderma* antagonist selected *in vitro*, the best chitinase producer, *Trichoderma aureoviride* URM 5158, obtained in previous study (Silva et al. 2016), and the pathogen *S. lignicola* CMM 1098 were cultivated in 250 ml Erlenmeyer flasks containing 50 ml of PD liquid. These plates were incubated at 26 °C ± 2 °C for 8 days.

For the experiment, we collected Regolithic Neosols (pH (H_2O 1:2.5) = 4.5, P (16.6 mg Kg^{-1}), Mg (0.8 $cmol_c$ dm^{-3}), Ca (0.8 $cmol_c$ dm^{-3}), Al (0.15 $cmol_c$ dm^{-3}), Na (0.28 $cmol_c$ Kg^{-1}), K (0.15 $cmol_c$ kg^{-1}) and H + Al (1.8 $cmol_c$ dm^{-3}) in dry season according to Silva et al. (2013). This soil was considered sandy, because it had 880 g kg^{-1} of sand, 40 g kg^{-1} of clay and 80 g kg^{-1} of silt.

In each pot (capacity of 4 L), we cultivated two cassava cuttings ("Pai Antônio", which is susceptible to *S. lignicola*) 15–20 cm long grown for 90 days. Subsequently, we inoculated the pathogen. The density of the inoculum was 1×10^6 propagules ml^{-1} according to Abo-Elyousr et al. (2014). Then, the cassava plants were covered with bags to keep the moisture for 48 h. After this period, the bags were removed.

The two *Trichoderma* (the best antagonist and the best chitinase producer) were selected according to the method by Abo-Elyousr et al. (2014). Each pot was sprayed with 100 ml of *Trichoderma* (1×10^6 conidia ml^{-1}) 48 h before and after the inoculation of the pathogen.

We used acibenzolar-S- methyl as a plant resistance inductor to compare the *Trichoderma* isolates and the two control treatments, with and without the inoculation of the pathogen *S. lignicola*. An application of an inductor (0.02 g l^{-1}) was performed by dispersion in the two soil applications, 48 h before and 48 h after the inoculation of the pathogen (Graham and Myers 2011).

The experimental design was completely randomized with five treatments: CWP (control without pathogen), IC (inoculated control), PRI (plant resistance inductor), THA (the best *in vitro* antagonist *Trichoderma harzianum* URM 3086), TAU (the best chitinase producer *Trichoderma aureoviridae* URM 5158).

The rot severity of cassava roots was based on the external symptoms shown by the plant, such as yellowing and wilting at 90 days after inoculation. The stem and roots were isolated in order to assess the internal symptoms evidenced by the dark coloration in the vascular tissue of the plant (Barros et al. 2014). A colonization by *S. lignicola* CMM 1098 was identified by morphological features. The disease severity assessment was recorded following the note scale described by Barros et al. (2014): 0 = no disease, 1 = plants with less than 10% to 25% of injuries, 2 = 25% to 50%, 3 = 50% to 75%, and 4 = 75% to 100% (dead plants).

Extraction and estimation of antioxidant enzymes in cassava plants

Five leaves from each plant were homogenized at 4 °C to establish enzyme activity. The sample was macerated in liquid N_2 and 4 mL of 50 mM potassium phosphate buffer (pH 7.0) to avoid phenol oxidative effects, and 0.05 g of polyvinylpyrrolidone (PVP) were added to it. The concentrates were centrifuged in a refrigerated centrifuge (4 °C) at 10,000 x g for 10 min. The supernatants were stored in microtubes at −20 °C.

The catalase (CAT, EC 1.11.1.6) activity was measured according to Havir and Mchale (1987). The ascorbate peroxidase (APX, EC 1.11.1.11) activity was measured according to the method described by Nakano and Asada (1981), which was modified according to Koshiba (1993). The peroxidase (POX, EC 1.11.1) activity was measured according to the method described by Urbanek et al. (1991) using guaiacol and H_2O_2 as substrates. The polyphenol oxidase (PPO, EC 1.10.3.1) activity was determined by pyrogallol oxidation (Kar and Mishra 1976). All enzyme activities were expressed in units of U min^{-1} mg^{-1}.

Statistical analysis

The data were analyzed by one-way ANOVA using the SPSS (version 19) software. Means and standard deviation were calculated for four replication (n = 4) values. Means were compared by Tukey test; P values ≤0.05 were considered significant. All experiments were repeated.

Results and discussion

In vitro antagonist potential of *Trichoderma* against *Scytalidium lignicola* CMM1098

Two *Trichoderma* strains showed a significant ($P \leq 0.05$) potential antagonism activity against *S. lignicola* CMM 1098 micelial growth. All two *Trichoderma* strains inhibited the mycelia growth of *S. lignicola* CMM 1098, which evidenced a direct antagonist action. The most efficient was *T. harzianum* URM 3086, with 80.78% of mycelial growth inhibition followed by *T. aureoviride* (5158) with 69.38% of mycelial growth inhibition. Reddy et al. (2014) tested *Trichoderma viride*, *T. harzianum*, *T. reesei*, *T. atroviride*, *T. pseudokoningii*, *T. koningii* and *T. virens* against *Fusarium oxysporum* f. sp. *Lycopersici*, *Alternaria solani*, *Aspergillus niger* and *Macrophomina phaseolina* and found that all antagonists inhibited the growth of pathogens, suggesting an inhibition mechanism such as mycoparasitism and the production of volatile and non-volatile metabolites.

T. harzianum URM 3086 showed a high antagonist capacity in PDA, and it led to the highest percentage of inhibition of *S. lignicola* CMM 1098. Therefore, the *T. harzianum* URM 3086 strain was selected because it was the best *in vitro* antagonist to be used in biological control experiments related to cassava black root rot caused by *S. lignicola* CMM 1098. In addition, Galletti et al. (2015) reported that this *Trichoderma* species showed a potential for the development of crops with an antagonist action against soilborne pathogens (Sundaramoorthy and Balabaskar 2013). Therefore, the *in vitro* antagonism is a direct effect interfering with the growth of mycelia of pathogens and very important to the selection of *Trichoderma* species (Khalili et al. 2012). In this case, we posit an efficient *in vitro* antagonism of *T. harzianum* URM 3086 and its mycoparasitic relationship against *S. lignicola* CMM 1098.

In a previous experiment, Silva et al. (2016) analyzed the potential of these 10 isolates of *Trichoderma* to produce chitinase and found that the *T. aureoviride* URM 5158 was the best chitinase producer (average of 6.70 U/ml). The production of chitinase may be considered a biocontrol factor (López-Mondéjar et al. 2011) because it degrades chitin, a polyssaccharide constituent of the cell wall of phytopathogenic fungi (De La Cruz et al. 1995).

Therefore, we selected *T. harzianum* URM 3086 as having the best antagonist effect and *T. aureoviride* URM 5158 as the best chitinase producer (Silva et al. 2016) for the biological control of cassava black root rot.

In vivo antagonist activity of *Trichoderma* spp. against *S. lignicola* CMM1098

All treatments reduced the severity of cassava black shoot and root (Table 1) (Fig. 1). In comparison to the control treatment with the pathogen, the inoculation of the best *in vitro Trichoderma* antagonist and the best chitinase producer resulted in a 52.32 and 40.22% reduction in the severity of disease in shoots, respectively, and 33.33 and 22.22% in roots (Fig. 1). The THA (*T. harzianum* URM 3086) was the most efficient, followed by TAU*T. aureoviride* URM 5158, which showed no differences regarding PRI treatment in roots.

T. harzianum URM 3086 was effective in controlling cassava black root rot caused by *S. lignicola* CMM 1098. This species has been studied as an antagonist against different plant diseases caused by soilborne pathogens by using different mechanisms as it grows faster than other fungi species and contributes to biological control because of its ability to compete for nutrients and space (Harman et al. 2012). The inoculation of this antagonist in a well-nourished environment contributes to crop resistance against pathogens (Abdel-Kader et al. 2013; El-Mohamady et al. 2014).

The antagonist against the *in vivo* pathogen contributes to future progresses in agriculture, providing more assurance to the decision-making regarding possible biocontrols against different diseases (Otadoh et al.

Table 1 Analysis of variance (ANOVA) showing the severity and enzymatic activities of cassava black root rot caused by *Scytalidium lignicola* CMM 1098 with the application of *Trichoderma harzianum* URM 3086 and *Trichoderma aureoviridae* URM 5158

	F	VC(%)	Pr > Fc
Severity (%) shoot	192.16	13.35	***
Severity (%) root	28.78	29.9	***
PXO	315.23	6.46	***
APX	156.73	7.06	***
CAT	63.83	13.39	***
PPO	96.5	9.99	***

***$p \leq 0.001$ significantly (F-test)

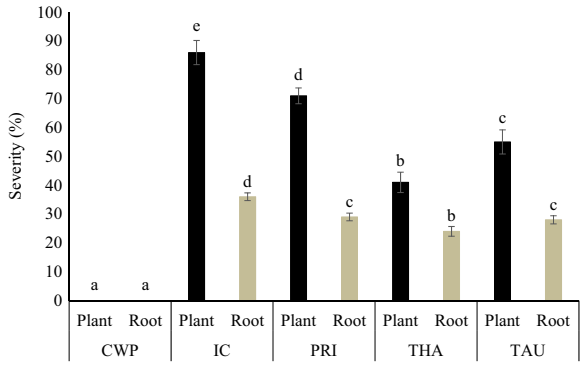

Fig. 1 Severity in shoots and roots (%) of cassava black root rot caused by *Scytalidium lignicola* CMM 1098 with the application of *Trichoderma*. CWP = control without pathogen, IC = inoculated control; PRI = plant resistance inductor; THA = the best *in vitro* antagonist (*Trichoderma harzianum* URM 3086); TAU = the best chitinase producer (*Trichoderma aureoviridae* URM 5158). Different *letters* indicate significant differences by Tukey test ($P \leq 0.05$)

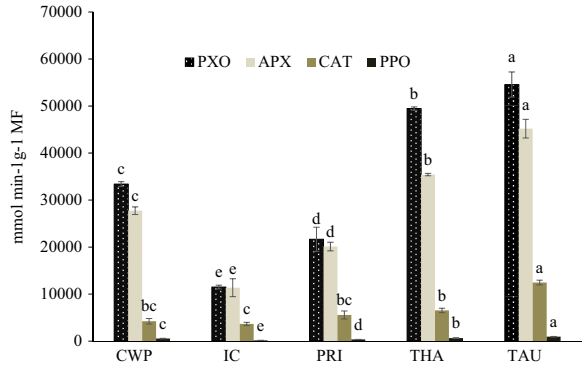

Fig. 2 Activity of peroxidase (POX), catalase (CAT), polyphenoloxidase (PPO) and ascorbate peroxidase (APX) in cassava plants inoculated with *Trichoderma* and *Scytalidium lignicola* CMM 1098. CWP = control without pathogen, IC = inoculated control; PRI = plant resistance inductor; THA = the best *in vitro* antagonist (*Trichoderma harzianum* URM 3086); TAU = the best chitinase producer (*Trichoderma aureoviridae* URM 5158). Different *letters* indicate significant differences by Tukey test ($P \leq 0.05$)

2011) and possible plant growth promoters (Joshi et al. 2010). Rinu et al. (2014) mentioned a study in which the use of *T. gamsii* in legumes and cereals did not show disease symptoms caused by *Alternaria alternata*, *Cladosporium oxysporum*, *Fusarium oxysporum*, *Fusarium pallidoroseum*, *Fusarium solani*, *Pythium afertile* and *Phomopsis archeri* in the field. Plant resistance inductors (PRI) have been an alternative to and a cultural practice in the management of diseases caused by root pathogens (Whan et al. 2008). However, in this study, the PRI treatment was less effective against the severity of *S. lignicola* CMM 1098, which may be explained by its time of operation since previous studies on the use of acibenzolar-S-methyl against soil pathogens were satisfactory after four weeks of application (Elmer 2006; Everts et al. 2014).

Extraction and estimation of antioxidant enzymes in cassava plants

Cassava plants responded to all tests of the group of reactive oxygen species enzymes (Table 1). The TAU treatment showed highest peroxidase (POX), ascorbate peroxidase (APX), catalase (CAT) and polyphenoloxidase (PPO) (Fig. 2).

All treatments with *Trichoderma* (THA and TAU) showed the highest values for all measured enzymes, especially TAU. Ojha and Chatterjee (2012) showed that *T. harzianum* potentiates the enzyme activity, especially peroxidase, against *Fusarium oxysporum* f. sp. *Lycopersicimedi* in tomatoes. This plant reacts to infection by maximizing the production of this enzyme and possibly stimulating the antagonist (Christopher et al. 2010). The activity of ascorbate peroxidase was the second highest because this enzyme belongs to the detoxification mechanism of peroxide (Tománková et al. 2006), helping to defend the plant.

In all treatments, CAT and PPO activities were lower compared to other enzymes. Gayatridevi et al. (2012) reported that a low or an inhibition of the CAT activity may be a result from the activation of systemic acquired resistance. Sánchez et al. (2000) explain the low or inhibition of PPO activity by the tension of pathogens in infected plants.

Plants are able to produce an immunization response according to the pathogen infection. This is known as systemic acquired resistance (SAR). Some fungi, in symbiosis with plants, helps them to induce the production of enzymes against pathogens of the genus *Trichoderma* (Jayalakshmi et al. 2009) in response to induced systemic resistance (ISR). This is a fact of high importance, but little studied in relation to the use of *Trichoderma* as a biocontrol (Silva et al. 2016).

Conclusions

Trichoderma harzianum URM 3086 and *Trichoderma aureoviride* URM 5158 were efficient antagonists against cassava black root rot caused by *Scytalidium*

lignicola CMM 1098 by different mechanisms such as *in vitro* antagonism, chitinase production, reduction of the severity of cassava black root rot in shoots and roots.

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