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Full Length Article

Antifungal Potential of *Trichoderma afroharzianum* Metabolites

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Abstract

Trichoderma is a fungal genus of undeniable importance for agriculture, whose species being used as biofungicides or even as biofertilizers. The objective of this work was to evaluate the *in vitro* production of volatile and non-volatile metabolites by five *Trichoderma afroharzianum* strains active against the phytopathogenic fungi *Phaeocytostroma sacchari*, *Macrophomina phaseolina*, *Sclerotium rolfsii*, *Sclerotinia sclerotiorum* and *Fusarium verticillioides*. In the investigation of volatile metabolites production, the technique of overlapping Petri dishes was used while in the non-volatile metabolites bioassay, the technique of incorporation of the filtrates in the potato-dextrose-agar medium was used. For evaluation antifungal effects, the radial mycelial growth of the fungi and the mycelial growth inhibition index (MGI) were used as parameters. All *Trichoderma* isolates produced volatile metabolites capable of inhibiting, to some degree, the growth of the phytopathogens in question. Inhibition by volatile metabolites of *M. phaseolina* ranged from 20.02 to 29.32%; *S. sclerotiorum* between 28.46 and 51.19%; *S. rolfsii* from 40.0 to 51.47%; *P. sacchari* from 51.29 to 56.91% and for *F. verticillioides* between 26.77 and 40.92%. Regarding non-volatile metabolites did not inhibit the growth of *M. phaseolina*, while for *S. sclerotiorum* the MGI varied from 11.76 to 52.94%, *S. rolfsii* between 7.84 and 62.74%, *P. sacchari*, from 0.0 to 33.72% and for *F. verticillioides* between 16.47 and 31.37%. The present study presents a better understanding of the mechanism of action of *Trichoderma* isolates against plant pathogenic fungi, observing an isolate-specific inhibitory activity.

Keywords: Antagonistic fungi; Culture filtrates; Hyperparasitism; Phytopathogens; Overlapping plates; Volatile organic compounds

Introduction

Trichoderma is a soil-borne ascomycete fungus known for almost 200 years (Singh *et al*. 2020). With great utility in agriculture, it has been used as a biofuncide or biofertilizer. In this way, this biological control agent (BCA) has gained more space as an ally in the integrated management of diseases, and improving the quantity and quality of agricultural products (Ali *et al.* 2020; Asad 2022). As a BCA, several mechanisms or benefits regarding the positive influence of *Trichoderma* on plants have been suggested (Jaiswa and Khadk 2020; Khan and Javaid 2020). These antagonistic fungi are able to interact with plants, awakening latent mechanisms of resistance (resistance induction), in addition to competing for nutrients and space (competition), modulating growth conditions of phytopathogens and plants (growth promotion). They also hyperparasite phytopathogenic fungi and produce antibiotics compounds (antibiosis). Understanding the mode of action of these BCAs is paramount to achieving the desired control of plant diseases (Köhl *et al.* 2019; Khan *et al.* 2021). The selection of antagonists like *Trichoderma* spp. is a routine process in many research centers in order to search for more effective isolates in the control of different phytopathogens (Marques *et al*. 2016; Javaid *et al.* 2018, 2021). Thus, the study of its mechanisms of action involves *in vitro* techniques such as the filtrate of cultures and overlapping plates, was carried out more than 50 years ago, which aimed to evaluate the production of volatile metabolites and non-volatile by such BCAs (Dennis and Webster 1971a, b).

Fungi produce a wide variety of secondary metabolites, *i.e.,* low molecular weight compounds associated with potentially useful biological activities (Keller *et al*. 2005; Khan and Javaid 2021, 2022a, b). Such compounds are not directly involved in essential metabolic processes of growth and energy generation of the fungus but exhibit a series of biological activities that contribute to the survival of the producing microorganism in the ecological niche in which it occupies (Dias *et al.* 2012). Therefore, these metabolites are characterized and applied in the

medicinal, pharmaceutical, and agricultural industries (Daley *et al.* 2017; Javaid *et al.* 2022). Patil *et al*. (2016) reaffirmed that information on secondary metabolism, mechanism of action and their applications are useful for biologists, chemists and farmers for better integrated pest and disease management.

Trichoderma-derived secondary metabolites encompass non-ribosomal peptides such as peptaibols, siderophores and gliotoxin and gliovirin, polyketides, terpenes, pyrones, and isocyanine metabolites (Frisvad *et al*. 2018). However, it is worth mentioning that the production of these substances is dependent on the species and even the strain, and not the entire repertoire will be biosynthesized by a particular fungal isolate *in vitro*, as specific stimuli may be required (Zeilinger *et al*. 2016). Several studies have demonstrated the effectiveness of secondary metabolites produced by *Trichoderma* species in inhibiting plant pathogenic fungi, such as those carried out for *Macrophomina phaseolina* (Choudhary *et al*. 2021; Khan *et al.* 2021), *Fusarium verticillioides* (Kumar *et al*. 2021; Yassin *et al*. 2021), *Sclerotium rolfsii* (Marques *et al.* 2018; Blanco *et al*. 2021) and *Sclerotinia sclerotiorum* (Marques *et al.* 2018; Carvalho *et al*. 2019; Silva *et al*. 2021). Keeping in view the importance of secondary metabolites, the objective of the present work was to evaluate the production of volatile and non-volatile by *Trichoderma afroharzianum* against five plant pathogenic fungi.

Materials and Methods

Place of testing and origin of fungal isolates

Trichoderma isolates were obtained from Núcleo de Pesquisa em Fitopatologia (NPF), Department of Agronomy, Universidade Federal de Goiás – UFG (Table 1). They were stored in cryovials in 10% glycerol and were recovered in commercial potato dextrose agar medium (PDA).

Concerning phytopathogenic fungi, they belonged to the collection of the NPF (UFG) namely: *M. phaseolina* (common bean – *Phaseolus vulgaris* L.), *Fusarium verticillioides* (sugarcane – *Saccharum officinarum* L.), *Phaeocytostroma sacchari* (sugarcane), *S sclerotiorum* (common bean) and *S. rolfsii* (host not known).

Test of non-volatile metabolite

In evaluating the potential of non-volatile metabolites produced by the *Trichoderma* isolates against the phytopathogens in question, the methodology described by Dennis and Webster (1971a) was used. The multiplication of both antagonists and pathogens was carried out in Petri dishes containing the medium of PDA and kept in a BOD (biochemical oxygen demand, Fanem, mod. 347) at 25ºC, with a photoperiod of 12 h, for seven days. To obtain the liquid phase, the fungus was cultivated in PD medium (potato dextrose) in an orbital shaker at 150 rpm, at 25°C, in the absence of light, for seven days. After this period, the liquid part was collected by filtering through filter paper (Millipore Qualy®) and centrifuged to remove the fungal spores that could make membrane sterilization difficult. The liquid phase was sterilized in a 0.45 μm cellulose membrane (Merck S/A) and incorporated into the melting PDA medium (~ 50ºC), in the proportion of 25% (v/v). For this bioassay, three replicates were prepared with agar disks (5 mm in diameter) taken from cultures of the pathogens. Mycelium discs were deposited in the center of each Petri dish containing PDA medium, supplemented with the respective antagonist filtrates. The control was each pathogen cultured in PDA medium plus sterile distilled water. The test was performed twice.

Test of volatile metabolites

The inhibitory effect of volatile metabolites was tested as described by Dennis and Webster (1971b), by means of the overlapping plate method, where two 90 mm diameter Petri dishes containing PDA culture medium received, individually, discs (5 mm of diameter) of the pathogen and antagonist cultures. After 6 h, the bases of the plates containing antagonist and pathogen were overlapped and carefully sealed with PVC film, the plate with *Trichoderma* was on the bottom. As a control, plates containing only the pathogen were used. The plates were incubated under the same conditions mentioned in the previous item. The test was performed twice.

Experimental design and statistical analysis

Each treatment consisted of three replications, in a completely randomized design, in a 5 × 5 factorial arrangement: 5 antagonist fungi × 5 phytopathogenic fungi. The test data were submitted to analysis of variance (ANOVA) using the SISVAR 5.6 Program (Ferreira 2014). The average values of the mycelial growth were compared by the Scott-Knott test, at 5% probability.

Results

Test of non-volatile metabolite

Based on the results of the bioassays (Fig. 1a–f), variation was observed in the inhibitory potential of the non-volatile metabolites evaluated between and among the evaluated isolates and phytopathogens. The mycelial growth of the phytopathogenic fungus *M. phaseolina* was not inhibited by the tested metabolites. On the other hand, *S. rolfsii* inhibition ranged from 11.76 to 59.94%. A similar variation was observed for *S. rolfsii*, where the inhibition varied from 7.84 to 62.95%. The sugarcane fungus *P. sacchari* had its growth inhibited between 0 and 33.73%. In respect of the *F. verticillioides* fungus, the amplitude of the MGI ranged

Table 1: Description of the antagonistic fungi used in this study

Species	Strain	Origin	Source
Trichoderma afroharzianum	Tricho 1 (T1)	Brazil	Lemongrass rhizosphere
Trichoderma afroharzianum	Tricho 2 (T2)	Brazil	Lemongrass rhizosphere
Trichoderma afroharzianum	Tricho 3 (T3)	Brazil	Citronella rhizosphere
Trichoderma afroharzianum	Tricho 4 (T4)	Brazil	Citronella rhizosphere
Trichoderma afroharzianum	Tricho 5 (T5)	Brazil	Citronella rhizosphere

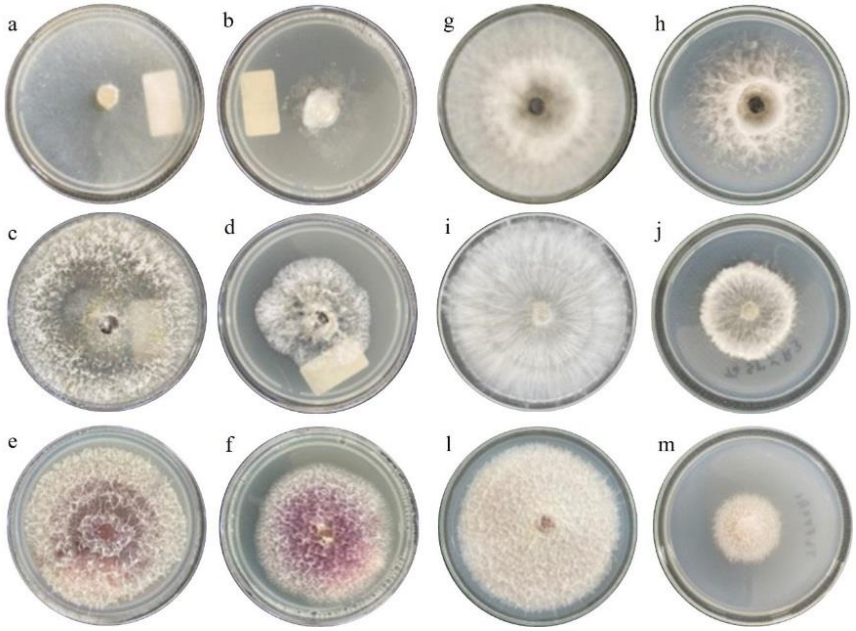

Fig. 1: Some results of the bioassay of *T. afroharzianum* metabolites inhibiting the growth of phytopathogenic fungi, where non-volatile metabolites: **a**)- Control of *S. sclerotiorum*, **b**)- Tricho 5 x *S. sclerotiorum*; **c**)- Control of *P. sacchari*, **d**)- Tricho 2 x *P. sacchari* and **e**)- Control of *F. verticillioides* and **f**)- Tricho 4 x *F. verticillioides*; and volatile metabolites: **g**)- Control of *P. sacchari*, **h**) Tricho 5 x *P. sacchari*, **i**)- Control of *S. rolfsii*, **j**)- Tricho 4 x *S. rolfsii*, **l**)- Control of *F. verticillioides*, and **m**)- Tricho 3 x *F. verticillioides*

from 16.47 to 31.37%.

Now observing the statistical analysis (Fig. 2) of the mycelial growth of the fungi when confronted with the non-volatile metabolites with positive results, for *S. sclerotiorum* (Fig. 2a) the isolate T5 stood out with a significant difference from the others, followed by T2 and the other treatments did not differ from the witness. Concerning *S. rolfsii* (Fig. 2b), the T2 and T4 isolates stood out significantly with the lowest growth averages, the T1 and T3 isolates did not differ from each other and the T5 isolate did not differ from the control. T1, T2, T4 and T5 were the non-volatile metabolites that most inhibited the fungus *P. sacchari* (Fig. 2c), although they did not differ significantly from the control. As for the *F. verticillioides* fungus (Fig. 2d), isolates T3, T4 and T5 stood out significantly, with the lowest averages of mycelial growth, followed by isolates T1 and T2.

Test of volatile metabolites

Based on the results of the bioassay (Fig. 1g–m) with volatile metabolites, it was observed that for *M. phaseolina* the inhibition ranged between 20.02 and 29.32%. The MGI of *Ss* varied between 28.46 and 51.19%. The soil fungus *S rolfsii* had its mycelial growth inhibited between 40 and 51.47%. On the other hand, for *P. sacchari*, an inhibition ranging from 51.29 to 56.91% was observed. As for the *F. verticillioides* fungus, the inhibition fluctuated between 26.77 and 40.92%.

Statistical analysis of mycelial growth (in cm) revealed that there was no significant difference between treatments with volatile metabolites for *M. phaseolina* (Fig. 3a), *S. rolfsii* and *P. sacchari* (Fig. 3d) fungi, despite that they differed from the control. However, for *S. sclerotiorum* (Fig. 3b) the treatments with T1, T2 and T3 differed significantly from the others and from the control, with the lowest values of mycelial growth. For *F. verticillioides* (Fig. 3e), the same was observed, although now the treatments with T1 and T3 differed from the others.

Discussion

In the present study, a variation in the antifungal potential of volatile and non-volatile metabolites produced by *T. afroharzianum* isolates was observed. Such variation

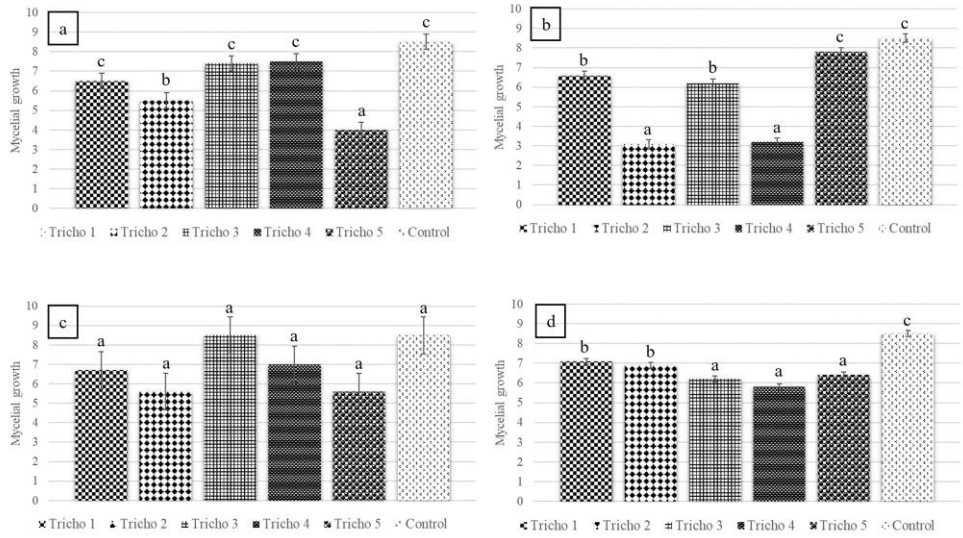

Fig. 2: Means of radial mycelial growth (cm, Y axis) of phytopathogenic fungi challenged in a bioassay with non-volatile metabolites (volatile metabolitesnon-volatile metabolites) of *T. afroharzianum* isolates (X axis), where **a**) *S. sclerotiorum*, **b**) *S. rolfsii*, **c**) *P. sacchari* and **d**) *F. verticillioides*. Means followed by the same letter do not differ significantly by the Scott-Knott test ($P \leq 0.05$)

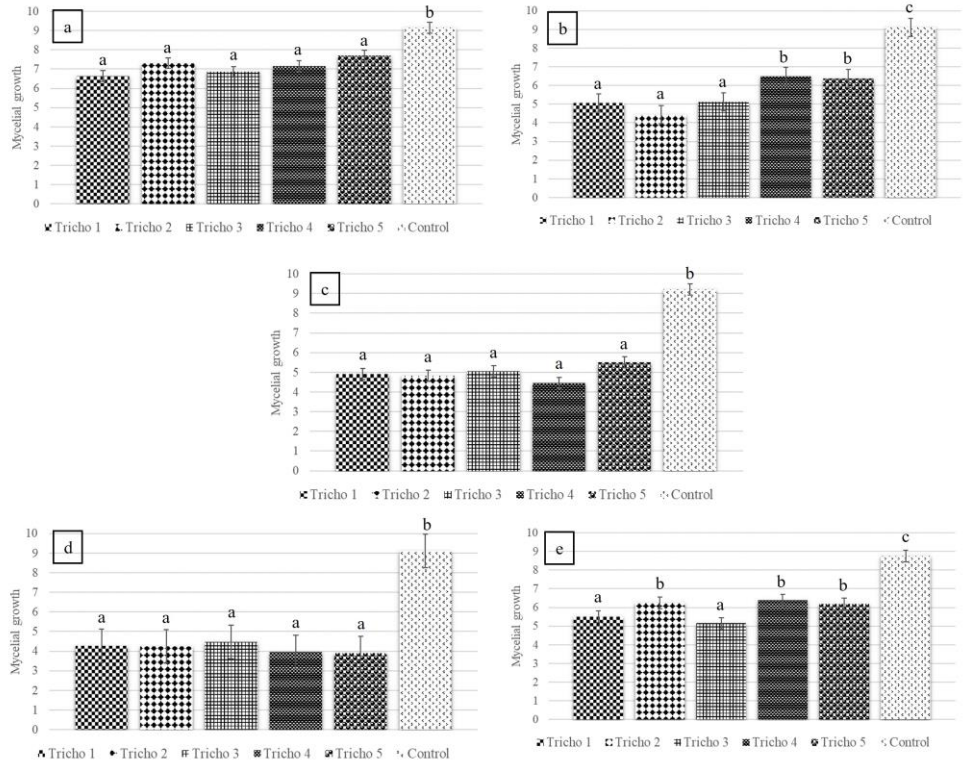

Fig. 3: Means of radial mycelial growth (cm, Y axis) of phytopathogenic fungi confronted in a bioassay with volatile metabolites (volatile metabolites) of *Trichoderma afroharzianum* isolates (X axis), where: **a**) *Macrophomina phaseolina*, **b**) *Sclerotinia sclerotiorum*, **c**) *Sclerotium rolfsii*, **d**) *Phaeocytostroma sacchari* and **e**) *Fusarium verticillioides*. Means followed by the same letter do not differ significantly by the Scott-Knott test ($P \leq 0.05$)

occurred both within and between antagonist isolates and also phytopathogens. It is worth mentioning again that the production of metabolites, whether by antibiosis or volatile organic compounds (VOCs), are not the only mechanisms of antagonistic action of *Trichoderma* species. As mentioned above, the production of such compounds may

vary between isolates of the biocontrol agent or even by the conditions imposed on them (Zeilinger *et al*. 2016; Marques *et al*. 2018), corroborating our results. The important point in these studies is to understand the mode of action of these BCAs to achieve the desired control of plant diseases (Patil *et al*. 2016; Köhl *et al*. 2019).

The present work shows a low inhibition of the soil fungus *M. phaseolina*, only by volatile metabolites (< 29.32%). Choudhary *et al*. (2021) observed an inhibition varying between 49 and 78% for volatile metabolites and between 28 and 63% for non-volatile metabolites, with emphasis also on *T. harzianum*.

The *S. sclerotiorum* inhibition levels here were the highest overall by both metabolites evaluated (volatile metabolites < 51.19% and non-volatile metabolites < 59.94%). According to Marques *et al*. (2018), the mycelial growth of this fungus was inhibited between 64 and 77% per non-volatile metabolites, the highest results was observed in the treatment with *T. harzianum*. Carvalho *et al*. (2019) reported inhibition ranging between 4.7 and 99.8% for non-volatile metabolites and 38.2 and 85.8% for volatile metabolites, both belonging to *T. harzianum*. Later, Silva *et al*. (2021) described inhibition above 80% for this pathogen by VOCs of *T. azevedoi*, *T. koningiopsis* and *T. asperelloides* isolates.

For *S. rolfsii*, our results show a median inhibition (< 51.47% for volatile metabolites and < 62.75% for non-volatile metabolites). Marques *et al.* (2018) observed variation in non-volatile metabolites inhibition between 0 and 73%, with better performance of *T. brevicompactum*. Blanco *et al*. (2021) reported an average reduction in growth of 57% for non-volatile metabolites and 40% for volatile metabolites of a *T. asperellum* isolate.

With respect to the description of *Trichoderma* metabolites active against *F. verticillioides*, Kumar *et al*. (2021) reported variation between 20.27 and 36.12% of inhibition for volatile metabolites, with significance on a strain of *T. harzianum*; and non-volatile metabolites between 66.15 and 76.92%, especially *T. viride*. Yassin *et al*. (2021) describe MGI of 56.7 and 44.09 non-volatile metabolites of *T. viride* and *T. harzianum*, respectively. Inhibitions below 40.92% corroborate the findings of the present work.

Finally, *P. sacchari* is considered an emerging fungus in sugarcane crops, causing stalk rot, therefore, there are few studies on it and none of secondary metabolites. The dual culture test performed by this research group as well as the present metabolite assessment are considered pioneers for this pathogen.

Conclusion

It was concluded that the antifungal activity of the volatile and non-volatile metabolites produced by *T. afroharzianum*, against the five plant pathogenic fungi, was isolate-specific. These results show that the action of these antagonists also occurs through the production of secondary metabolites, in addition to the mycoparasitism already observed in previous studies in confrontation of cultures, demonstrating the importance of such metabolites in the multifunctional action of the biocontrol agent. The potential of such metabolites will be evaluated in the induction of resistance and plant growth promotion.

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Author Contributions

EM and MGC planned the experiments, wrote up the findings, and statistically analyzed and interpreted the results; VPA, MRS, KHMC, CMLSC and ACA performed the experiments, wrote up the findings and statistically analyzed the results.

Conflicts of Interest

All authors declare no conflicts of interest

Data Availability

Data presented in this study will be available on a fair request to the corresponding author

Ethics Approval

Not applicable in this paper

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