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**Identification of native soil-derived *Trichoderma* spp. isolates and analysis of their antagonist traits against *Lasiodiplodia theobromae* causing stem-end rot in papaya**

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# Identification of native soil-derived *Trichoderma* spp. isolates and analysis of their antagonist traits against *Lasiodiplodia theobromae* causing stem-end rot in papaya

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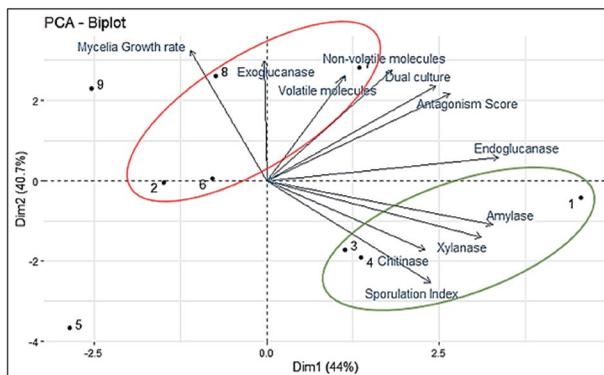
## ABSTRACT

The aim of the present research was to identify and analyse the biocontrol capacity of nine local *Trichoderma* spp. isolates against *Lasiodiplodia theobromae*. The isolates were identified as *T. asperellum* (3), *T. harzianum* (5) and *T. koningiospsis* (1). These fungi significantly slowed *L. theobromae* mycelial development *in vitro*, with maximum reductions of 75.4 and 64.1% reported with *T. asperellum* BRS-1 and *T. harzianum* BRS-7, respectively. The antagonistic fungi were able to inhibit the growth of *L. theobromae* through the production of mycotoxins. After 5 days of incubation, the fungi produced a large number of conidia and targeted lytic enzymes in solid-state fermentation (SSF). The principal component analysis (PCA) clustered *Trichoderma* strains according to their phylogenetical relationships and demonstrated a tight association between the species sub-group and antagonistic features. These findings suggest that local soil-derived *Trichoderma* spp. have the potential to be efficient biological agents against *L. theobromae*.

## KEYWORDS

Biocontrol;  
solid-state fermentation;  
mycotoxin compounds;  
lytic enzymes

## GRAPHICAL ABSTRACT



## Introduction

*Lasiodiplodia theobromae* (syn. *Botryodiplodia theobromae*) is a well-known fungus that causes significant crop losses in over 500 different plant species (Punithalingam 1980; Marquez et al. 2013). Geographically, it can be found almost everywhere around the world, with a higher frequency in tropical and subtropical areas, making it one of the most widely distributed Botryosphaeriaceae species (Phillips et al. 2013; Mehl et al. 2017). The fungus has been shown to cause pathogenic infection in papaya (Yuan et al. 2019), mango (Goudarzi et al. 2021), banana (Kulkarni et al. 2021), cocoa (Puig et al. 2021), cassava (Brito et al. 2020) and other tropical fruit plants (Karunanayake and Adikaram 2020). In general, *L. theobromae* infection causes diseases such as branch die-back, stem cankers, and seed and fruit decay (Netto et al. 2014). The utilization of chemical pesticides is the most popular method for protecting plants from this pathogenic fungi and reducing crop losses. However, the usage of such chemical compounds has a negative impact on the health of both humans and non-human biota (Carvalho 2017; Köhl et al. 2019). Furthermore, the expensive cost of researching, manufacturing and registering new synthetic pesticides, as well as the rapid emergence of pathogen resistance, have fueled interest in founding alternative disease management approaches (Matson et al. 2015). In this context, it is critical to develop promising practices and environmentally acceptable measures in order to lessen reliance on synthetic agrochemicals. Recent disease management trends have favored the use of biocontrol agents and/or their metabolites as an environmentally friendly strategy to decrease pesticide use and protect environmental and human health (Panth et al. 2020). Biocontrol using antagonistic microbes is a

solution with multiple benefits, including: crop yield enhancement, safety for humans and other non-target organisms, reduction of pesticide residues in food, water, and soil, and increase biodiversity (Mwamburi 2016; Azadi et al. 2016). Microbial biocontrol agents for plant disease are often bacterial or fungal strains obtained from the rhizosphere, phyllosphere or endosphere, and can play an important role in reducing plant-pathogenic organisms (Sood et al. 2020). They can prevent the invasion of the pathogenic agents, or their establishment in the host plant by a variety of mode of actions (Köhl et al. 2019; Thambulgala et al. 2020).

Fungi from the genus *Trichoderma* are one of those microorganisms that act as biocontrol agent. Fungal species from this genus are among the most frequently isolated soil-dwelling microorganisms and are abundant in the plant rhizosphere (Sood et al. 2020). These fast-growing fungi are opportunistic, avirulent plant mutualists with mode of actions that allow them to effectively defend plants from pathogenic fungi responsible for diseases (Vinale et al. 2008; Lorito et al. 2010). It has been considered that the principal mode of action implicated in biological disease management directly operates upon pathogens. These mode of action mechanisms principally include competition with other microbes for space and nutritive resources, mycoparasitism (in which the antagonist obtains some or all of its nutrients from the degradation of the fungal host) and antibiosis (where the antagonist inhibits the growth or kills the fungal pathogen through the production of inhibitory metabolites) (Zhang et al. 2017). Mycoparasitism is associated with production of lytic enzymes such as chitinases (endochitinases and exochitinases), cellulases (exoglucanases, endoglucanases, and  $\beta$ -1,3-glucanases) and proteases (Gajera et al. 2012). Microorganisms use these enzymes to breakdown the host cell wall and subsequently infiltrate the cell to get nutrients (Harman et al. 2004). These enzymes are not only essential for pest biocontrol, but they also play a significant role by increasing the rate of organic matter degradation in soil by *Trichoderma* spp. (Zin and Badaluddin 2020). Recent researches have shown that *Trichoderma* spp. can control and destroy a variety of phytopathogen pests (De la Cruz-Quiroz et al. 2018; Tchameni et al., 2020; Ferreira et al. 2020; Bedine et al. 2020). For example, De la Cruz-Quiroz et al. (2018) found that native Mexican *Trichoderma* strains reduced the growth of *Phytophthora capsici* and *Colletotrichum gloeosporioides* by up to 22.5% under *in vitro* conditions. Similarly, the effectiveness of *Trichoderma* isolates was shown to reduce the growth of 4 soil-borne pathogens that cause damping-off in common bean plants by 54.4–79.4% (Bedine et al. 2020). It is emphasized, however, that not all *Trichoderma* isolates were equally successful in suppressing pathogens across diverse ecological

niches. Consequently, accurate identification followed by systematic screening of local antagonist isolate(s) displaying potential biocontrol abilities for a particular disease or plant pathogen should be done prior to their application as fungal-based biopesticides. Furthermore, in order to provide large-scale availability of biocontrol agents in integrated pest management systems, beneficial fungal microorganisms must be responsive to mass multiplication in an easy, effective, low-cost, and economically advantageous manner (Naeimi et al. 2020). The use of solid-state fermentation (SSF) to produce a high spore concentration from filamentous fungi by using agroindustrial waste is an interesting biotechnological approach (Wijesinghe et al. 2011; De la Cruz-Quiroz et al. 2017; Hamrouni et al. 2019). The mass multiplication of fungi occurs in most cases through spore production. Fungal spores are resistant cell propagules that are routinely employed in the field. They have the particularity to possess long self-life and can withstand harsh environmental conditions (Lenz et al. 2004; Shah and Pell 2003).

The use of *Trichoderma* spp. as biocontrol agents against plant pathogenic fungi is well documented. These fungi are the most well-known and widely utilized biocontrol agents for a variety of plant infections, including soil-borne and airborne pathogens (Marraschi et al. 2019). However, few research have reported the biological control of *Lasiodiplodia* fungi, notably the efficiency of *Trichoderma* spp. for controlling *L. theobromae* which causes papaya stem-end rot. Therefore, in the present study, native isolates of fungi consisting of *Trichoderma* species were evaluated as biocontrol agents against *L. theobromae*. Thus, four assays were performed: (1) identification of nine indigenous *Trichoderma* isolates; (2) efficacy of identified *Trichoderma* spp. to suppress the growth of *L. theobromae* growth *in vitro*; (3) evaluation of *in vitro* mycotoxic effect of volatiles and non-volatiles metabolites from *Trichoderma* spp.; and (4) determination of spore production and enzyme activities in SSF. The antagonistic features observed were summarized using a principal component analysis (PCA) to highlight the major mode of actions used by *Trichoderma* species.

## **2. Materials and methods**

### **2.1. Origin of fungi**

The nine isolates of *Trichoderma* spp. used in this investigation were obtained from common bean soil (rhizosphere) in the Bafou subdivision (West, Cameroon) during the September/October 2019 growing season. The microorganisms were isolated using the serial dilution and plating method as mentioned by Dhingra and Sinclair (1995) and Aneja (2007).

The plant pathogen *L. theobromae* (BB-15) was obtained from the fungal core collection of the Department of Biochemistry, University of Douala, Cameroon. The fungus has previously been isolated from diseased papaya plants. Using the Koch's postulates, pathogenicity confirmed the microorganism as the causative agent of stem-end rot on *Carica papaya* (Fulton 1981). The fungal cultures were cultivated on potato dextrose agar (PDA) and incubated at 28°C for 7 days for standard cultivation. The fungal cultures were stored on the PDA slant at 4°C for long-term preservation.

## **2.2. Cultural and morphological characterization**

The cultural and morphological characteristics of the nine *Trichoderma* spp. isolates (BRS-1, BRS-2, BRS-3, BRS-4, BRS-5, BRS-6, BRS-7, BRS-8, and BRS-9) were examined from cultures grown on PDA. Based on macroscopic and microscopic observations, identification was performed using conventional classification keys (Gams et al. 1998; Samuels et al. 2006 and Siddiquee 2017). At 3 days incubation, macroscopic observations were conducted, including pigmentation, colony shape, growth and sporulation pattern, and characteristic odor. Microscopic observations were taken after 5 days of incubation, which included the branching patterns of conidia with conidiogenous cells, the organization arrangement of phialides, and the shapes and sizes of conidia. Lactophenol-cotton blue slide preparation was used for microscopic visualization. Conidia were measured using an Olympus BX 51 light microscope equipped with a digital camera (Evolt E330). Thirty measurements were made of the structures mounted.

## **2.3. Molecular identification**

### **2.3.1. Total genomic DNA extraction**

Fungal isolates for DNA extraction were transferred onto sterilized cellophane overlaid on PDA Petri dishes and cultured for 7 days at 28°C. Fresh mycelia (50–200 mg) grown on cellophane were collected and incubated overnight in a microcentrifuge tube containing 1 mL ethanol 95%. The genomic DNA was extracted and purified using the NucleoSpin® Plant II Genomic DNA isolation Kit (Macherey-Nagel, Germany) following the manufacturer's instructions.

### **2.3.2. PCR amplification and sequencing analyses**

The internal transcribed spacer (ITS) sections of ribosomal DNA (rDNA), including 5.8S, were amplified using the universal primers ITS1-F (5' - CTTGGTCATTTAGAGGAAGTAA - 3') and ITS4

(5'-TCCTCCGCTTATTGATATGC-3') (Gardes and Bruns 1993; White et al. 1990). Polymerase chain reaction (PCR) was conducted in a 20  $\mu$ L reaction volume containing 4  $\mu$ L of reaction buffer (5X), 8  $\mu$ L of ultrapure water, 3.2  $\mu$ L of dNTPs (200  $\mu$ M), 0.5  $\mu$ L of each primer (250 nM), 1.6  $\mu$ L of MgCl<sub>2</sub> (2 mM), 0.10  $\mu$ L of Promega GoTaq<sup>®</sup> DNA polymerase (0.75 U), 0.1  $\mu$ L BSA of BSA (0.4  $\mu$ g/mL) and 2  $\mu$ L template DNA. PCRs were run in a thermocycler (Eppendorf Mastercycler<sup>®</sup>Flexlid, France) under the following conditions: 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 50 °C for 30 s, 72 °C for 40 s, and finally 72 °C for 5 min. PCR products were then subjected to an ethidium bromide-stained 1% agarose gel, along with a 1 kb DNA smartladder (Eurogentec) to estimate the size of the amplified bands. Electrophoresis was performed in 0.5X Tris-acetate EDTA (TAE) buffer at 70 V for 30 min. An external service (GATC Biotech, Ebersberg, Germany) sequenced the obtained products in both directions, using the same primers.

### **2.3.3. Phylogenetic analysis**

The ITS rRNA gene sequences were submitted to BLASTn search in the GenBank database (<http://www.ncbi.nlm.nih.gov/BLAST/>) to retrieve the most similar hits. Those highly similar, homologous sequences were download from GenBank and aligned with our ITS sample sequences using the multiple sequence alignment algorithm MUSCLE from the Molecular Evolutionary Genetics Analysis (MEGAX) software (Kumar et al. 2018). The evolutionary distances were calculated by employing the unweighted pair-group method with arithmetic mean (UPGMA) method (Sneath and Sokal 1973). The percentage of replicate trees, in which the associated taxa clustered together was estimated by using 1000 replications of the bootstrap procedure (Felsenstein 1985). The plant pathogenic fungus *L. theobromae* was used as an outgroup to root the tree.

## **2.4. Growth rate and sporulation index of *Trichoderma* strains**

### **2.4.1. Growth rate**

The growth rate was determined by following the apical growth of microbes on PDA Petri plates (18 cm diameter). PDA plates holding 40 mL of culture media were centrally inoculated with 0.5 cm diameter agar plugs from a 5 day-old culture. The inoculated dishes were kept in an incubator at 28 °C. The growth rate (mm.h<sup>-1</sup>) of the colonies was recorded daily using a caliper in two perpendicular directions until the plates were fully colonized. The following equation was used to compute the growth rate:

$$\text{MGR} = \frac{(G_1 - G_0) + (G_2 - G_1) + \dots + (G_n - G_{n-1})}{T_1 + T_2 + \dots + T_n}$$

in which **MGR** = mycelial growth rate in mm.h<sup>-1</sup>, **G**<sub>0</sub> = apical growth at 0h referring to 0 mm; **G**<sub>1</sub> = apical growth obtained after 24 h; **G**<sub>2</sub> = apical growth obtained after 48 h; **G**<sub>n</sub> = apical growth obtained after n hours; **T**<sub>1</sub> = 24 h; **T**<sub>2</sub> = 48 h and **T**<sub>n</sub> = n hours

#### 2.4.2. Sporulation index

The sporulation index was assayed by growing the fungi in 250 mL Erlenmeyer flasks containing 21 mL PDA culture medium. A conidial suspension was prepared in water by scraping the surface of a 7 day-old cultures with a needle. The obtained suspension was adjusted to 2 × 10<sup>5</sup> conidia/mL using a Malassez counting chamber. Fifty microliters of the prepared suspension were incorporated into PDA medium maintained at 50 °C. The flasks were homogenized by gentle stirring and incubated at 30 °C. Five days after incubation, 100 mL of sterile water containing 0.01% (v/v) Tween 80 was introduced into the flasks, and the spores were dislodged from the hyphae using a magnetic stirrer. The number of conidia obtained in each culture was determined using a Malassez counting chamber and the results were expressed in spores per gram of carbon source.

### 2.5. Antagonistic activity of *Trichoderma* strains

#### 2.5.1. In vitro dual confrontation

A dual culture technique was used to assess the antagonistic activity of the nine *Trichoderma* strains against *L. theobromae* BB-15. Following the method outlined by Bell et al. (1982), each *Trichoderma* strain was paired with the plant pathogen in Petri dishes. The growing edge of five day-old *Trichoderma* and pathogen cultures was used to obtain a 5 mm agar plug. The *Trichoderma* and pathogenic agent agar pieces were put 6 cm apart on opposing ends of the PDA Petri plate (90 mm diameter). The paired cultures were incubated for 3, 6 and 9 days at 28 ± 2 °C. The growth of microorganisms was monitored and quantified at each incubation period. Following the formula described by Filizola et al. (2019), the percentage of colonization (%C) of each *Trichoderma* isolate was calculated:

$$\%C = (DT / DE) \times 100,$$

where **DT** is the pathogen growth distance after incubation time and **DE** is the initial distance between the mycelial discs of the pathogen

**Table 1.** Rating scale of Bell et al. (1982) used to classify *Trichoderma* strains according to their antagonism behaviors during co-culture with the plant pathogen.

| Colonization pattern   | Antagonism rating scale |
|--|-------------------------|
| <i>Trichoderma</i> strain grows completely over <i>L. theobromae</i> covering the entire surface of the culture medium.                          | 1                       |
| <i>Trichoderma</i> strain grow to at least about 2/3 of the surface of the culture medium.   | 2                       |
| <i>Trichoderma</i> strain and <i>L. theobromae</i> colonize approximately half the surface of the culture medium and neither appears dominating. | 3                       |
| <i>L. theobromae</i> colonizes at least 2/3 of the surface of the culture medium and exhibits resistance to the <i>Trichoderma</i> strain.       | 4                       |
| <i>L. theobromae</i> grows completely on the <i>Trichoderma</i> strain and occupies the entire surface of the surface of the culture medium.     | 5                       |

and *Trichoderma*. The inhibition index of the plant pathogen in relation to *Trichoderma* was calculated using the equation  $I = 100 - \%C$ . In addition to the inhibition index, each *Trichoderma* was categorized based on its according to its degree of antagonism (Table 1) using the scale described by Bell et al. (1982).

### 2.5.2. Scanning electron microscopy (SEM)

Scanning electronic photomicrographs showing the direct effect of the potent *Trichoderma* strains on the morphological development of the pathogen were obtained from 9 day-old dual cultures. *Trichoderma* and plant pathogen were grown on for 3 days at 28 °C on PDA plates overlapped with the cellophane membrane. After incubation, the cellophane membrane was removed from the plate, and small pieces (2 × 2 mm) were obtained from the intermingling zone of co-cultured fungi. The samples were fixed in a modified Karnovsky solution (2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.4) for 2 h at 4 °C. Glutaraldehyde-treated samples were immediately washed in the same buffer 3 times for 15 min before being postfixed for 2 h at room temperature in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer at pH 7.2. The samples were rinsed 3 times using demineralized water before being dehydrated in an ethanol gradient and dried with HDMS. Dried samples were sputter-coated (Au/Pd) 50 Å, before being imaged with a Gemini SEM 500 70-04 operating at 10 KV under high vacuum (carl Zeiss Microscopy GmbH).

### 2.5.3. Evaluation of the effect of volatile and non-volatile compounds

The procedure described by Dennis and Webster (1971a) was used to determine the influence of volatile compounds generated by *Trichoderma* spp. on the development of *L. theobromae*. A 5 mm mycelial disc of 3 days preculture of each *Trichoderma* strain was separately inoculated on the middle of a 90 mm PDA plate. The cover of each inoculated

Petri dish was replaced with dishes centrally inoculated with a 5 mm mycelial disc of *L. theobromae* 3 days preculture. The two plates (antagonist and pathogen) were sealed with paraffin tap, and the entire plate was incubated at 28 °C for 3 days. The antagonist was replaced with an agar disc on control plate. The method outlined by Dennis and Webster (1971b) was used to evaluate the effect of non-volatile compounds on the proliferation of the plant pathogen. Each *Trichoderma* strain was cultivated for 48 h on a sterile cellophane disc placed on a 90 mm PDA plate. The antagonist microorganisms were replaced with an agar disc on the control plate. The cellophane with the fungal colony was removed after incubation, and *L. theobromae* was placed on prepared plates. The plates were then subsequently incubated for 6 days at 28 °C. In both trials, each treatment included five replications, and the tests were performed twice. The colony diameter of *L. theobromae* was measured at the end of incubation, and the suppression of mycelial growth was assessed.

## **2.6. Determination of enzyme activities**

### **2.6.1. Substrate and culture conditions**

The SSF was carried out in 250 mL Erlenmeyer flasks containing 30 g of substrate. The fermentation medium was composed of a dry weight mixture of the following: sugarcane bagasse (25%), wheat bran (25%), vine shoots (20%), olive pomace (10%), chitin (10%), potato flour (10%), and olive oil (10%). The substrate moisture was achieved at 66% with the addition of deionized water and sterilized at 121 °C for 30 min. After cooling the substrate was inoculated with 10 mL spore suspension of *Trichoderma* containing  $2 \times 10^7$  spores/mL to reach a final moisture of 75% (Roussos et al. 1991). After adjusting the pH to 5.6 with 1.0 M HCl, the flasks were kept at 29 °C for 5 days to assess the sporulation index and measure enzyme activities.

### **2.6.2. Sporulation index**

Spores were collected after fermentation by combining 50 mL of sterile deionized water containing Tween 80 (0.01% v/v) with one gram of fermented matter in an Erlenmeyer flask (250 mL). Spores were released from the solid matter by swirling the flask (200 rpm) for 20 min using a magnetic stirrer. A Whatman n°1 paper filter was used to filter the homogenized suspension. The spores generated were measured using a Neubauer cell chamber, and the results were represented as spores per gram of dry weight (spores.gdw<sup>-1</sup>).

### **2.6.3. Determination of enzyme activities**

The enzymatic cocktail was extracted from medium fermented by SSF using the methodology described by De la Cruz-Quiroz et al. (2019). In a Falcon® tube, one gram of fermented material was mixed with 20 mL of distilled water and then homogenized for 1 min using an Ultra-Turrax dispersion machine. The resulting suspension was filtered using a Whatman n°1 paper filter to remove solid particles, and the cell-free supernatant was used to measure enzymatic activities.

Amylolytic activity was determined according to Singh et al. (2014) by mixing 1 mL of a 1% (w/v) starch solution prepared in sodium citrate buffer (50 mM, pH 5.9) with 1 mL of enzyme extract. The reaction mixture was incubated for 15 min at 50 °C. Endoglucanase activity was determined according to Nava-Cruz et al. (2016) by mixing 1 mL of a 1% (w/v) solution of carboxymethyl cellulose prepared in sodium citrate buffer (50 mM, pH 4.8) with 1 mL of enzyme extract. The reaction mixture was maintained at 50 °C for 30 min before being boiled to end the reaction. Exoglucanase activity was determined according to Nava-Cruz et al. (2016) by using Whatman No.1 filter paper (1 cm × 5 cm) in sodium citrate buffer (50 mM, pH 4.8). The reaction was carried out by introducing filter paper into 1 mL of crude enzyme extract. The reaction mixture was incubated for 60 min at 50 °C. Xylanase activity was determined by mixing 1 mL of xylan solution (1%) prepared in phosphate buffer (100 mM, pH 7.0) with 1 mL of crude extract (Tuncer et al. 1999). The enzymatic reaction was left to take place for 10 min at 50 °C. Chitinolytic activity was determined following the procedure reported by De la Cruz-Quiroz et al. (2017) with some modifications. A 5% (v/v) chitosan chlorhydrate (Kitostim, France) in sodium acetate buffer (50 mM, pH 5.0) was used as the substrate. One milliliter of this solution was added to 1 mL of enzyme extract. The reaction mixture was incubated in a water bath at 50 °C and maintained for 30 min.

The amount of reducing sugar levels obtained after enzymatic reactions was quantified by the dinitrosalicylic acid (DNS) method of Miller (1959) after the reactions were stopped by boiling. Except for xylanase, in which xylose was used as a standard, a glucose standard curve (0–1 mg ml<sup>-1</sup>) was used for all enzyme activities. All activities were expressed as units per gram of dry weight (U gdw<sup>-1</sup>). One unit of enzyme activity (U) was defined as the amount of enzyme required to release 1 μmol of reducing sugar per minute.

### **2.7. Statistical analysis**

All the treatments were completely randomized using block design (CRD), and the results were given as the mean ± standard deviation.

The collected observations were examined for significance using one-way ANOVA, and means were compared using Duncan's multiple range test at a significance threshold of 5%. The link between the collected data was described using principal component analysis (PCA). A score biplot of PCA was used for the distribution of the samples and to observe the importance and interactions of the variables. The first two principal components were extracted, and the scatter plot was created by graphing PC1 versus PC2. Statistical analyses were performed with the software R language version 3.2.4 (R Foundation for Statistical Computing, Vienna, Australia), in which the MissMDA package version 1.18, FactoMineR package version 2.4 and Factoextra package version 1.0.7 were used to plot the PCA biplot.

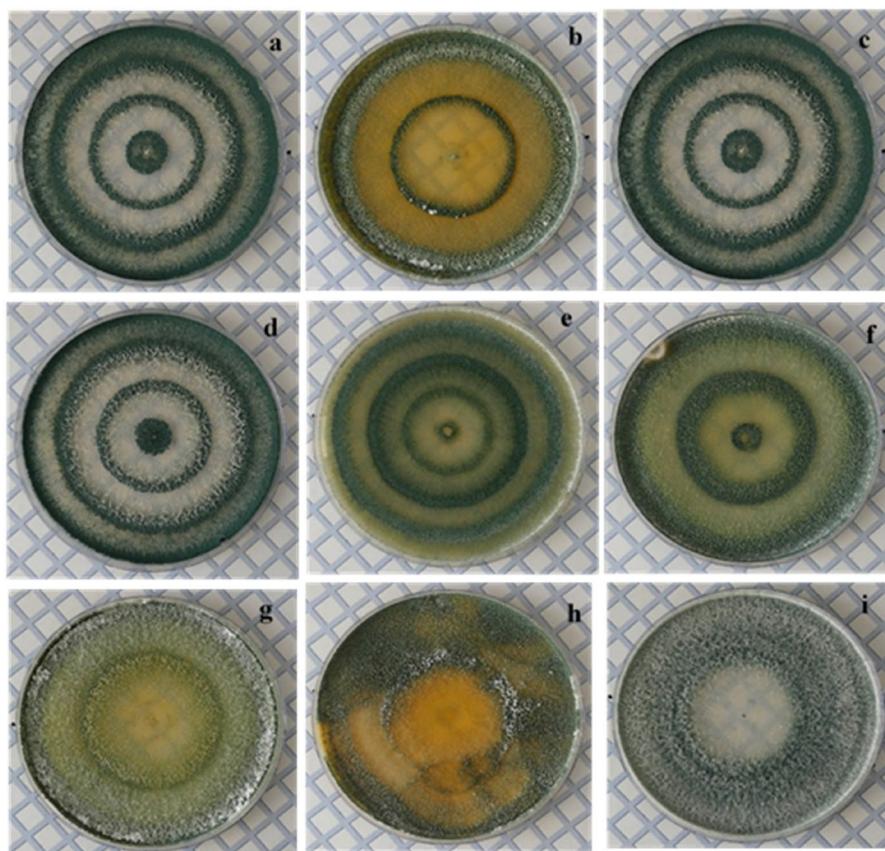
### **3. Results**

#### ***3.1. Morphological characterization of Trichoderma isolates***

The isolates were compatible with the general description for the genus *Trichoderma* and were labeled BRS-1 to BRS-9, respectively (Figure 1). Macroscopic and microscopic observation patterns of the fungi revealed substantial variances and similarities. On the PDA plates, macroscopic morphology revealed colonies with rapid growth rates, concentric halos, and floccose or compact surfaces that resembled tufts. Due to intense conidiation, mycelium initially had a white color that change to a green or yellow tone. The color of the colony's reverse differed greatly amongst *Trichoderma* isolates, ranging from yellow to colorless. Microscopically, abundant production of smooth or rough appearing conidia with green to dark green color was observed. The fruiting bodies originated from branched and irregularly verticillated conidiophores. Conidiogenic cells (phialides) were generally fusiform or ampliform and arranged in clusters. The presence of globose or subglobose chlamydospores was also noticed after 7 days of incubation in BRS-1, BRS-3, BRS-4, BRS-7, BRS-8 and BRS-9 isolates.

#### ***3.2. Molecular identification and phylogenetic analysis***

A product of approximately 560–600 bp was obtained from all the PCR amplifications with primers ITS1 and ITS4. The BLAST analysis established that the potential antagonistic strains belonged to three different species: *T. asperellum*, *T. harzianum*, and *T. koningiopsis* (Table 2). Five out of nine antagonistic strains: BRS-2, BRS-5, BRS-6, BRS-7, and BRS-8, were 99% to 100% identical to *T. harzianum* ITS sequences (accession nos. MF871563, KY644130, MK499449, KC139308 and KC139308,

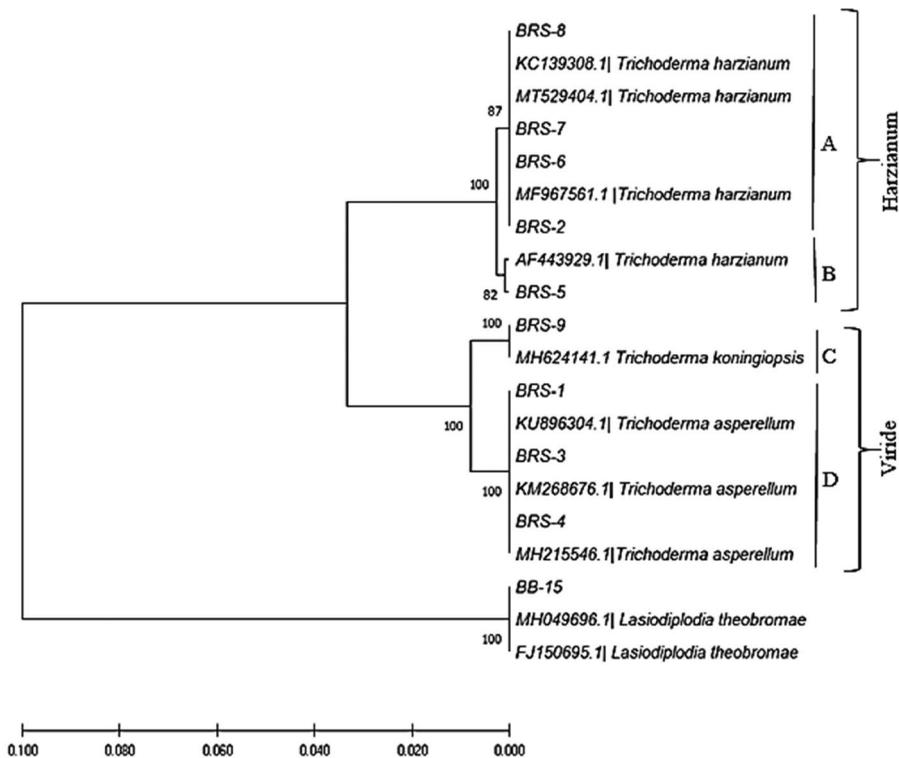


**Figure 1.** Colony appearance of *Trichoderma* species grown on PDA for 7 days.

**Table 2.** Closet matches between native *Trichoderma* strains and *Lasiodiplodia theobromae* with reference strains.

| Fungi species          | Isolate codes | Similarity | GenBank accession no. |
|------------------------|---------------|------------|-----------------------|
| <i>T. asperellum</i>   | BRS-1         | 100%       | MK204287              |
| <i>T. harzianum</i>    | BRS-2         | 100%       | MF871563              |
| <i>T. asperellum</i>   | BRS-3         | 100%       | MF074069              |
| <i>T. asperellum</i>   | BRS-4         | 99%        | KM268676              |
| <i>T. harzianum</i>    | BRS-5         | 99%        | KY644130              |
| <i>T. harzianum</i>    | BRS-6         | 100%       | MK499449              |
| <i>T. harzianum</i>    | BRS-7         | 100%       | KP009260              |
| <i>T. harzianum</i>    | BRS-8         | 100%       | KC139308              |
| <i>T. koningiopsis</i> | BRS-9         | 100%       | MH624141              |
| <i>L. theobromae</i>   | BB-15         | 100%       | MK166048              |

respectively), whereas three of them: BRS-1, BRS-3 and BRS-4 were identical to *T. asperellum* (accession nos. MK204287, MF074069 and KM268676, respectively) with percent similarity varying from 99 to 100%. The BRS-9 isolate was identified as 100% identical to Genbank ITS sequence of *T. koningiopsis* having accession no. MH624141. The ITS sequence of the pathogen BB-15 was 100% similar with the reference strain *L. theobromae* (accession no. MK166048).



**Figure 2.** UPGMA phylogenetic relationships of the 9 strains of *Trichoderma* spp. and their closest GenBank matches, inferred by analysis of ITS sequences. Bootstrap support values (1000 replicates) are provided at the corresponding nodes. Evolutionary analyses were conducted in MAGA X package.

The phylogenetic tree inferred from the ITS region of our biocontrol strains is represented in [Figure 2](#). The tree revealed that the nine strains of *Trichoderma* were divided into 2 distinct clades, both supported by a bootstrap value of 100%. The BRS-2, BRS-5, BRS-6, BRS-7 and BRS-8 strains belonged to the clade Harzianum, whereas the BRS-1, BRS-3 and BRS-4 and BRS-9 strains were located in the clade Viride. The clade Harzianum contains two subclades (A and B) supported by bootstrap values of 87 and 82%, respectively. All strains matched with *T. harzianum* species, but strain BRS-5 appeared slightly divergent. The clade Viride also contains two subclades (C and D), supported by bootstrap values of 100%. The first subclade comprises BRS-9, closely related to *T. koningiopsis*, whereas the second subclade comprises BRS-1, BRS-3, and BRS-4 strains, which are clustered with *T. asperellum* species.

### **3.3. Mycelial growth rate and sporulation**

The results of mycelial growth rate and sporulation index of *Trichoderma* strains growing on PDA are presented in [Table 3](#). In this assay, the

**Table 3.** Mycelial growth rate and sporulation index of *Trichoderma* strains obtained in PDA culture Petri plate and Erlenmeyer flask, respectively.

| <i>Trichoderma</i> strains  | MGR (mm h <sup>-1</sup> ) | Sporulation index (10 <sup>9</sup> spores.gCS <sup>-1</sup> *) |
|-----------------------------|---------------------------|--|
| <i>T. asperellum</i> BRS-1  | 0.9 ± 0.2 <sup>c</sup>    | 10.1 ± 1.8 <sup>a</sup>  |
| <i>T. harzianum</i> BRS-2   | 1.3 ± 0.2 <sup>b</sup>    | 3.4 ± 0.7 <sup>d</sup>   |
| <i>T. asperellum</i> BRS-3  | 0.9 ± 0.3 <sup>c</sup>    | 10.1 ± 2.3 <sup>a</sup>  |
| <i>T. asperellum</i> BRS-4  | 0.9 ± 0.2 <sup>c</sup>    | 8.7 ± 1.8 <sup>b</sup>   |
| <i>T. harzianum</i> BRS-5   | 0.8 ± 0.2 <sup>c</sup>    | 3.0 ± 0.5 <sup>d</sup>   |
| <i>T. harzianum</i> BRS-6   | 1.3 ± 0.4 <sup>b</sup>    | 8.8 ± 1.2 <sup>b</sup>   |
| <i>T. harzianum</i> BRS-7   | 1.4 ± 0.2 <sup>b</sup>    | 6.4 ± 1.7 <sup>c</sup>   |
| <i>T. harzianum</i> BRS-8   | 1.4 ± 0.2 <sup>b</sup>    | 4.4 ± 0.4 <sup>d</sup>   |
| <i>T. koningiopsis</i> BRS9 | 1.4 ± 0.4 <sup>b</sup>    | 9.2 ± 1.3 <sup>b</sup>   |
| <i>L. theobromae</i> BB-15  | 1.6 ± 0.5 <sup>a</sup>    | –  |

MGR: mycelial growth rate; gCS<sup>-1</sup>: gram per carbon source; –: No sporulation observed

Data are expressed as the mean of five different replicates ± SD, analyzed by two-way analysis of variance.

Values bearing the same letter(s) within each column are not significantly different as determined by Duncan's multiple range test at the 5% level of significance.

pathogen was used for comparison purposes. All the antagonistic microbes tested were fast growing fungi. The average growth rate ranged from 0.7 to 1.2 mm h<sup>-1</sup>. *T. harzianum* BRS-2, *T. harzianum* BRS-6, *T. harzianum* BRS-7, *T. harzianum* BRS-8 and *T. koningiopsis* BRS-9 showed the maximum mycelial growth rate, with no significant difference observed between the 5 strains. However, the growth rate (1.6 mm h<sup>-1</sup>) of *L. theobromae* was found to be significantly higher than those of all the antagonistic fungi. In parallel, by comparing the production of conidia by strains, it was observed that all the species were able to produce significant amounts of conidia. The sporulation index varied from 3.0 × 10<sup>9</sup> spores.gCS<sup>-1</sup> to 10.1 × 10<sup>9</sup> spores.gCS<sup>-1</sup>. *T. asperellum* BRS-1 and *T. asperellum* BRS-3 showed the highest conidial production (10.1 × 10<sup>9</sup> spores.gCS<sup>-1</sup>), followed by *T. koningiopsis* BRS-9 (9.2 × 10<sup>9</sup> spores.gCS<sup>-1</sup>), *T. asperellum* BRS-4 (8.7 × 10<sup>9</sup> spores.gCS<sup>-1</sup>) and *T. harzianum* BRS-6 (8.8 × 10<sup>9</sup> spores.gCS<sup>-1</sup>). In our study, *L. theobromae* was not able to produce spores after five days of incubation on PDA.

### 3.4. Antagonistic ability of the *Trichoderma* strain

#### 3.4.1. Dual culture and SEM observations

Antagonistic tests were performed to investigate the ability of *Trichoderma* strains to inhibit the growth of *L. theobromae* *in vitro*. The obtained results are shown in Table 4. All antagonistic fungi had the ability to inhibit the mycelial growth of the pathogen. The fungal strains showed the ability to reduce the mycelial growth of *L. theobromae* in dual culture in a range of 21.5–44.4%, 21.5–55.6% and 21.5–75.4% after 3, 6 and 9 days after incubation, respectively. *T. asperellum* BRS-1 and *T.*

**Table 4.** Antagonistic effect of *Trichoderma* strains through mycelial growth inhibition and mycoparasitism of *Lasiodiplodia theobromae* observed *in vitro* in paired cultures.

| Strains                      | Inhibition index (%)    |                         |                         | Bell scale* |
|------------------------------|-------------------------|-------------------------|-------------------------|-------------|
|                              | 3 days                  | 6 days                  | 9 days                  |             |
| <i>T. asperellum</i> BRS-1   | 44.4 ± 3.2 <sup>a</sup> | 55.6 ± 2.6 <sup>a</sup> | 75.4 ± 3.5 <sup>a</sup> | 1.0         |
| <i>T. harzianum</i> BRS-2    | 31.8 ± 5.0 <sup>c</sup> | 42.3 ± 3.0 <sup>e</sup> | 51.2 ± 4.0 <sup>f</sup> | 2.0         |
| <i>T. asperellum</i> BRS-3   | 25.3 ± 2.4 <sup>d</sup> | 35.3 ± 3.1 <sup>f</sup> | 53.1 ± 6.0 <sup>e</sup> | 2.0         |
| <i>T. asperellum</i> BRS-4   | 25.9 ± 1.5 <sup>d</sup> | 35.8 ± 4.0 <sup>f</sup> | 49.3 ± 2.8 <sup>f</sup> | 1.7         |
| <i>T. harzianum</i> BRS-5    | 21.5 ± 2.0 <sup>e</sup> | 21.5 ± 2.2 <sup>g</sup> | 21.5 ± 4.4 <sup>g</sup> | 3.7         |
| <i>T. harzianum</i> BRS-6    | 30.6 ± 2.5 <sup>c</sup> | 40.1 ± 2.8 <sup>e</sup> | 50.8 ± 2.5 <sup>f</sup> | 2.3         |
| <i>T. harzianum</i> BRS-7    | 44.1 ± 3.1 <sup>a</sup> | 52.8 ± 4.0 <sup>b</sup> | 64.1 ± 2.4 <sup>b</sup> | 1.0         |
| <i>T. harzianum</i> BRS-8    | 35.8 ± 2.0 <sup>b</sup> | 47.5 ± 3.1 <sup>c</sup> | 60.0 ± 3.1 <sup>c</sup> | 1.3         |
| <i>T. koningiopsis</i> BRS-9 | 34.9 ± 4.5 <sup>b</sup> | 44.9 ± 6.2 <sup>d</sup> | 58.1 ± 6.6 <sup>d</sup> | 2.3         |

\*Bell et al. (1982) rating scale for classification of degree of antagonism was obtained 9 days after incubation.

Data are expressed as the mean of five different replicates ± SD, analyzed by two-way analysis of variance.

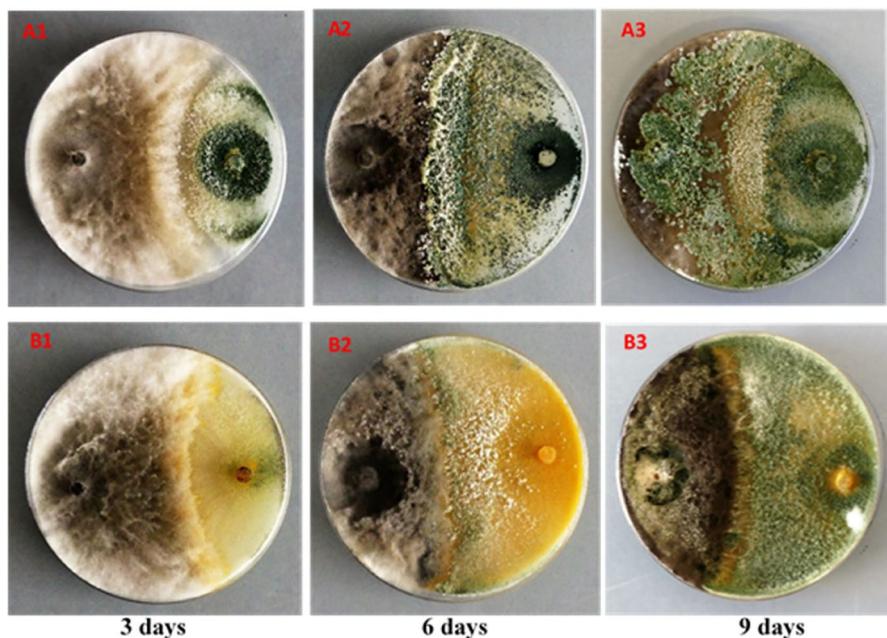
Values bearing the same letter(s) within each column are not significantly different as determined by Duncan's multiple range test at the 5% level of significance.

*harzianum*BRS-7 showed the strongest antagonistic properties against the plant pathogenic fungi. *T. harzianum* BRS-5 showed the lowest antagonistic properties. The percent inhibition of mycelial growth of the pathogen strain in paired cultures with *T. asperellum* BRS-1 ranged from 44.4 to 75.4% and from 44.1 to 64.1% with *T. harzianum* BRS-7. Nine days after incubation, the aforementioned strains with strong inhibitory activity were able to completely overgrow *L. theobromae* colonies by covering the entire surface of the culture medium and sporulating on pathogen. Therefore, the sporulation observed was more active in the case of *T. asperellum* BRS-1 than in *T. harzianum* BRS-7 (Figure 3). These two *Trichoderma* strains were then classified as Class I antagonistic fungi according to the Bell scale rating.

SEM examinations of the morphological structure and distribution of the hyphae of *L. theobromae* in pure culture, and the changes that these structures experienced when the plant pathogen was co-cultured with *T. asperellum* BRS-1 are presented in Figure 4. The electron micrographs of the interaction zone revealed that parasitic hyphae of the antagonistic fungi grew by surrounding and wrapping the hyphae of pathogenic microbe with profuse conidiation. It appears evident that *Trichoderma* strains, particularly *T. asperellum* BRS-1, induced changes in the morphological pattern of *L. theobromae*. The changes were materialized by the swelling and the depletion of hyphae, which showed differences when compared to pure culture.

### 3.4.2. Antibiosis

The results obtained show that all nine antagonistic fungi produced volatile and non-volatile toxic secondary metabolites that showed significant ( $P < 0.05$ ) inhibitory effects on the mycelial growth of the



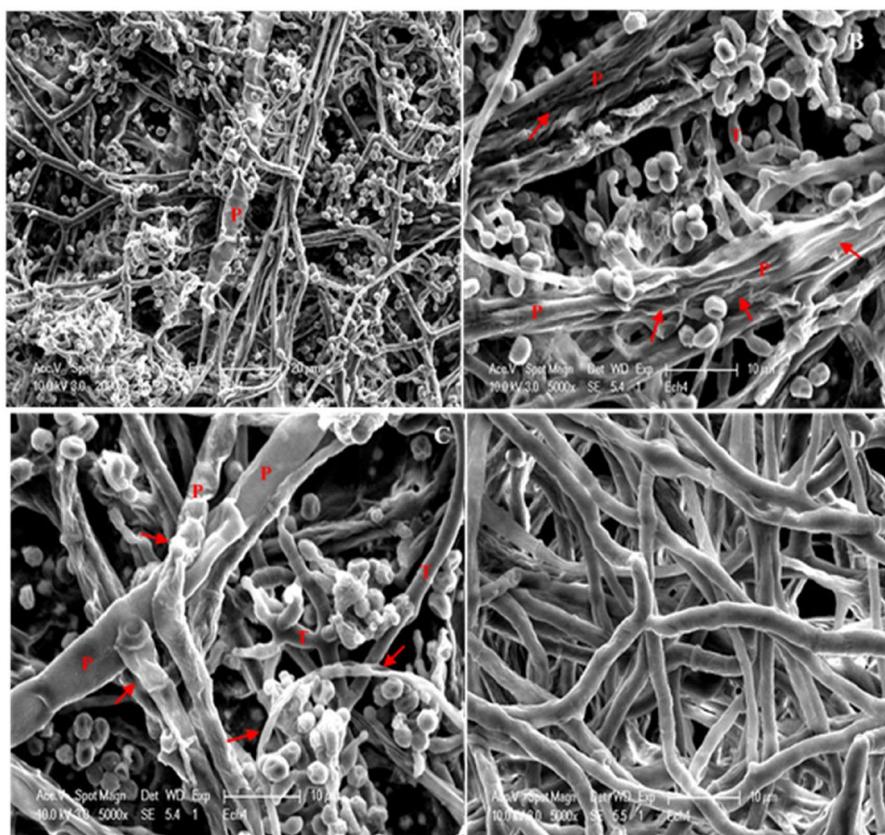
**Figure 3.** Antagonistic effect observed during co-cultures of fungi in PDA Petri plates.

pathogenic fungi (Table 5). The inhibition rate initiated by volatile compounds ranged between 21.4 and 68.2%, with the highest mycelial reduction produced by *T. harzianum* BRS-7 (68.2%) and *T. koningiopsis* BRS-9 (68.2%). However, the percent inhibition mediated by non-volatile compounds varied from 27.5 to 53.5% with *T. harzianum* BRS-8 (53.5%) and *T. asperellum* BRS-1 (53.4%) showing the highest inhibitory rate.

### **3.5. Spore production and enzyme activities in SSF**

The fungi were able to grow over the substrate and initiate visible conidiogenesis after 2 days of incubation. The sporulation index obtained after the fermentation process varied significantly among the strains tested (Figure 5). The maximum sporulation index was obtained with *T. asperellum* BRS-1 ( $5.8 \times 10^9$  spores.gdw<sup>-1</sup>), followed by *T. asperellum* BRS-3 and *T. asperellum* BRS-4 which showed  $4.1 \times 10^9$  spores.gdw<sup>-1</sup> and  $4.3 \times 10^9$  spores.gdw<sup>-1</sup>, respectively. A slight increase in pH was observed in all cultures.

All nine *Trichoderma* strains were able to produce targeted enzymes in SSF after 5 days of incubation as shown by enzymatic activities obtained (Figure 6). The production of extracellular amylase, endoglucanase, exoglucanase, chitinase and xylanase was found to vary significantly between the different strains of *Trichoderma*. The highest amylase activity was observed in *T. asperellum* BRS-1 (23.2 U.gdw<sup>-1</sup>), followed



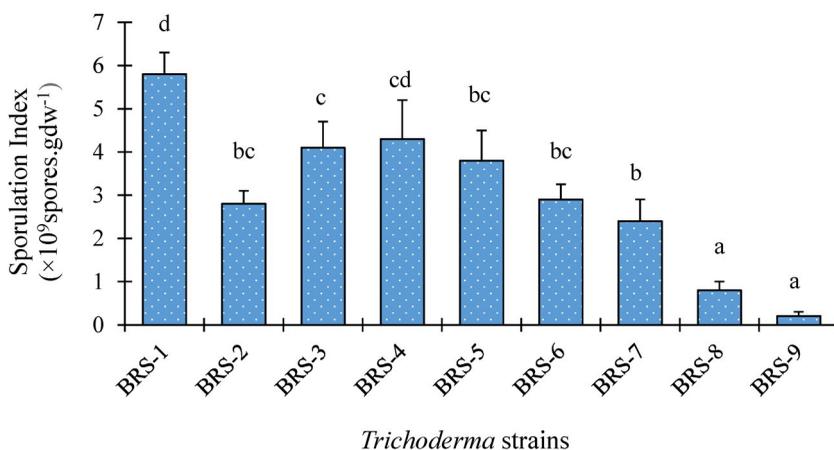
**Figure 4.** Scanning electron microscopy (SEM) micrographs displaying the antagonistic activity of *T. asperellum* BRS-1 (T) in the intermingling growth with *L. theobromae* (P) after 9 days incubation.

**Table 5.** *In vitro* growth inhibition of *Lasiodiplodia theobromae* by volatile and non-volatile metabolites released by *Trichoderma* strains.

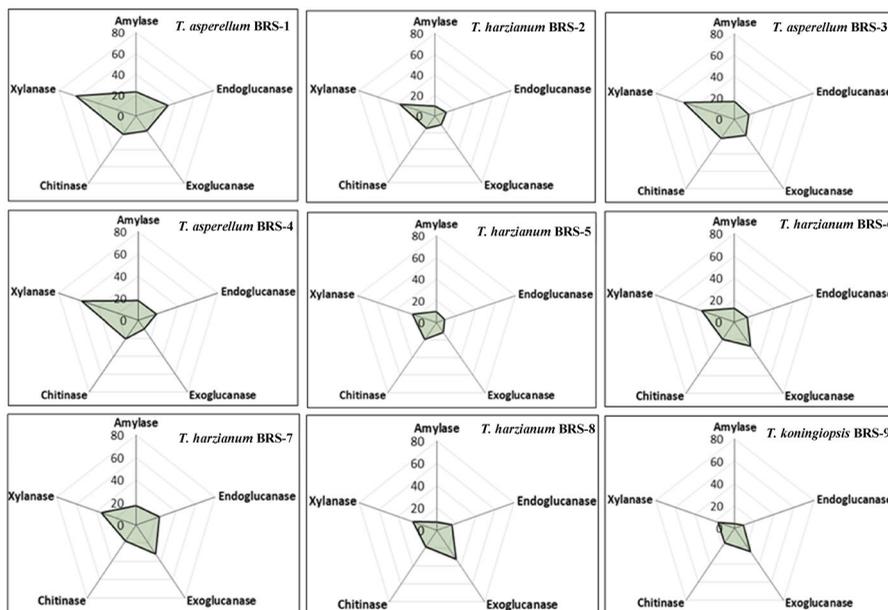
| Strains                      | Mycelial growth inhibition (%) |                          |
|------------------------------|--------------------------------|--------------------------|
|                              | Volatile                       | Non-volatile             |
| <i>T. asperellum</i> BRS-1   | 46.1 ± 5.4 <sup>bc</sup>       | 53.4 ± 3.9 <sup>c</sup>  |
| <i>T. harzianum</i> BRS-2    | 50.3 ± 7.2 <sup>c</sup>        | 32.8 ± 8.6 <sup>ab</sup> |
| <i>T. asperellum</i> BRS-3   | 45.4 ± 3.1 <sup>bc</sup>       | 37.4 ± 6.1 <sup>ac</sup> |
| <i>T. asperellum</i> BRS-4   | 43.1 ± 5.5 <sup>bc</sup>       | 37.0 ± 5.4 <sup>ac</sup> |
| <i>T. harzianum</i> BRS-5    | 21.4 ± 6.4 <sup>a</sup>        | 27.5 ± 8.7 <sup>a</sup>  |
| <i>T. harzianum</i> BRS-6    | 31.4 ± 6.5 <sup>ab</sup>       | 43.7 ± 9.5 <sup>ac</sup> |
| <i>T. harzianum</i> BRS-7    | 68.2 ± 5.1 <sup>d</sup>        | 51.1 ± 6.5 <sup>bc</sup> |
| <i>T. harzianum</i> BRS-8    | 44.4 ± 3.1 <sup>bc</sup>       | 53.5 ± 8.1 <sup>c</sup>  |
| <i>T. koningiopsis</i> BRS-9 | 54.8 ± 6.9 <sup>cd</sup>       | 45.2 ± 7.8 <sup>ac</sup> |

Data are expressed as the mean of five different replicates ± SD, analyzed by two-way analysis of variance. Values bearing the same letter(s) within each column are not significantly different as determined by Duncan's multiple range test at the 5% level of significance.

by *T. asperellum* BRS-4 and *T. harzianum* BRS-7, with 18 U.gdw<sup>-1</sup> and 17.2 U.gdw<sup>-1</sup>, respectively. *T. asperellum* BRS-1 also resulted in important-doglycanase activity (32.8 U.gdw<sup>-1</sup>), followed by *T. harzianum* BRS-7

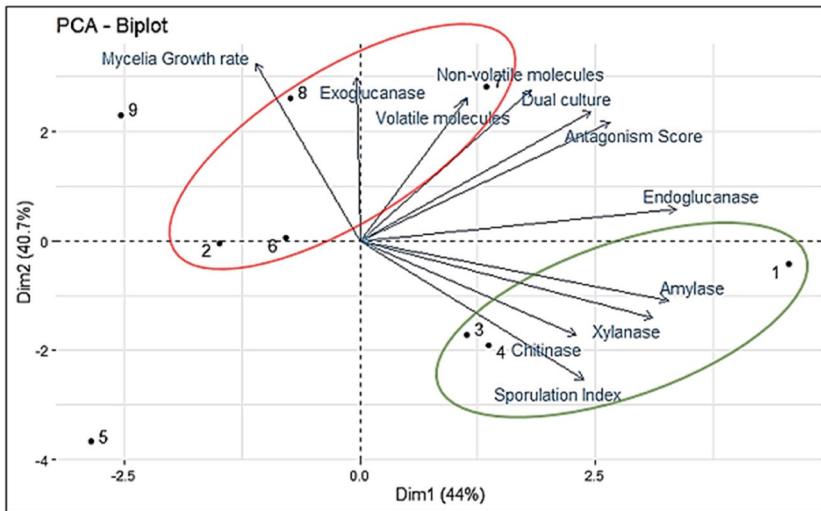


**Figure 5.** Assessment of sporulation production in *Trichoderma* strains grown under solid-state fermentation.



**Figure 6.** Enzymatic activity of *Trichoderma* strains obtained 7 days after incubation in solid-state fermentation. Results are expressed in U.gdw<sup>-1</sup> (Units per gram of dry weight).

and *T. asperellum* BRS-4, with 23.6 and 18.4 U.gdw<sup>-1</sup>, respectively. In the case of exoglucanase activities, the highest values were recorded with *T. harzianum* BRS-8 (32.4 U.gdw<sup>-1</sup>) and *T. harzianum* BRS-7 (32.0 U.gdw<sup>-1</sup>), followed by *T. harzianum* BRS-6 (26.8 U.gdw<sup>-1</sup>). The strains *T. harzianum* BRS-7, *T. aspererellum* BRS-1 and *T. asperellum* BRS-4 exhibited the highest chitinase activities, with values of 11.6, 10.8 and 10.4 U.



**Figure 7.** Principal component analysis (PCA)-Biplot showing relationship existing between antagonistic traits and *Trichoderma* strains obtained from rhizosphere.

$\text{gdw}^{-1}$ , respectively. The maximum xylanase activities were shown by *T. aspererellum* BRS-1, with a value of  $62.4 \text{ U.gdw}^{-1}$ , followed by *T. aspererellum* BRS-4 ( $56.8 \text{ U.gdw}^{-1}$ ) and *T. aspererellum* BRS-3 ( $51.6 \text{ U.gdw}^{-1}$ ).

### 3.6. Relationships between biocontrol features

PCA (Figure 7) was performed to assess the relationship between the antagonist activity and the activity of each enzyme as well as the spore production in solid-state fermentation. The analysis revealed that the first two components explained 84.7% (44.02% for PC1 and 40.7% for PC2) of the total variance. The rotated component matrix (Varimax rotation method) is shown in Table 6. The association between each component and each variable included in this in this investigation is described in this matrix. The first component, which accounts for 44.02% of the overall information, is mostly connected to sporulation, amylase, chitinase and xylanase and endoglucanase activities. The second component, which accounts for 40.7% of the total variance, is associated with antagonism level, mycelia growth inhibition, exoglucanase activities, and mycelial growth rate. The mycelia growth rate was negatively correlated with chitinase and xylanase activities as well as with sporulation. Such a correlation suggests that strains that growth faster in Petri plates have lower xylanase and chitinase activities, as well as lower spore production in solid-state fermentation. The species belonging to the Viride section were well segregated into two sub-groups, with *T. aspererellum* strains gathered together (red ellipse) and *T. koningiopsis* isolated from

**Table 6.** Correlation matrix of variables obtained related to biocontrol trait of *Trichoderma* strains and dimensions of principal component.

| Variables               | Components   |               |        |
|-------------------------|--------------|---------------|--------|
|                         | 1            | 2             | 3      |
| Mycelia Growth Rate     | -0.309       | <b>0.917</b>  | -0.097 |
| Sporulation Index       | 0.667        | <b>-0.712</b> | -0.066 |
| Antagonism Score        | <b>0.745</b> | 0.611         | -0.137 |
| Amylase                 | <b>0.918</b> | -0.304        | -0.048 |
| Endoglucanase           | <b>0.943</b> | 0.163         | 0.012  |
| Exoglucanase            | -0.008       | <b>0.835</b>  | 0.494  |
| Chitinase               | 0.644        | -0.481        | 0.539  |
| Xylanase                | <b>0.872</b> | -0.392        | -0.201 |
| Non-volatiles molecules | 0.511        | <b>0.771</b>  | 0.344  |
| Volatile molecules      | 0.321        | <b>0.733</b>  | -0.476 |
| Dual culture (MGI)      | 0.688        | 0.664         | -0.061 |
| Eigenvalues             | 4.840        | 4.481         | 0.949  |
| Percent variance        | 44.025       | 40.71         | 7.699  |
| Cumulative variance     | 44.025       | 84.735        | 92.434 |

Values in bold presented a significant *P*-value ( $P < 0.05$ ).

MGR: Mycelia Growth Inhibition.

this sub-group. The 5 species from section *Harzianum* were likewise divided into two groups with isolates BRS-2, BRS-6, BRS-7 and BRS-8 of *T. harzianum* strains grouped together (green ellipse) and isolate BRS-5 of *T. harzianum* strains separated from this sub-group.

#### 4. Discussion

*Trichoderma* spp. have acquired notorious importance as a viable alternative for agrochemical pesticides because they can be employed as a safe, environmentally friendly and effective biocontrol agent for a variety of crop species, ranking these fungi among the most studied microorganisms (Lorito et al. 2010; Fraceto et al. 2018; Zin and Badaluddin 2020; Sood et al. 2020). However, only a few papers highlight the biological management of *Lasiodiplodia* spp., including the potential of *Trichoderma* spp. for suppressing *L. theobromae*: a causative agent of stem-end rot in papaya. In this work, nine indigenous *Trichoderma* spp. obtained from the common bean rhizosphere were identified, and their antagonistic properties against *L. theobromae* were investigated.

The correct identification of pathogenic agents and their antagonists is fundamental for implementing disease prevention and management in economically valuable crops. Because identifying *Trichoderma* species just based on appearance is challenging, fungal identification was accomplished by a combination of morphological and phylogenetical analyses based on rRNA internal transcribed spacers (ITS) amplification. Previous studies have demonstrated that using ITS sequences is efficient for identifying *Trichoderma* isolates (Torres-De la Cruz et al. 2015; Feitosa et al. 2019). In the present study, the obtained results enabled the

identification and separation of isolates in three different taxonomic groups: *T. asperellum* (BRS-1, BRS-3 and BRS-4), *T. harzianum* (BRS-2, BRS-5, BRS-6, BRS-7 and BRS-8), and *T. koningiopsis* (BRS-9). These findings are closed to those obtained by others authors, including Islam et al. (2021), who identified 7 isolates of *Trichoderma* spp. obtained from agricultural soils collected from Sabah, Malaysia, using a combination of morphological and molecular data from the ITS 1-5.8S-ITS 2 regions of the rDNA. The phylogenetic tree of ITS 1 and 2 regions revealed two separate clades containing *T. harzianum* strains on the one hand, and *T. koningiopsis*/*T. asperellum* strains on the other hand, classed under the section of Harzianum and Viride, respectively.

All identified strains of *Trichoderma* showed a similar *in vitro* growth pattern, with an average growth rate of  $0.1144 \text{ cm h}^{-1}$ , as found by Filizola et al. (2019), who obtained an average growth rate of  $0.1207 \text{ cm h}^{-1}$  from 11 *Trichoderma* isolates. However, in this study, the growth rate of *L. theobromae* ( $0.16 \text{ cm.h}^{-1}$ ) was found to be significantly greater ( $P < 0.05$ ) than that of antagonistic microbes. By comparing the production of conidia, it was observed that all the *Trichoderma* strains were capable of producing significant amounts of conidia on agar plates. The sporulation index ranged from  $3.0 \times 10^9$  spores.gCS<sup>-1</sup> to  $10.1 \times 10^9$  spores.gCS<sup>-1</sup> with *T. asperellum* BRS-1 showing the highest conidial production. *Trichoderma* are well-known as invasive and rapid colonizing fungi with strong conidia production. Such advantageous features make them adapted to harsh conditions and colonize different type of environments (Al-Ani 2018; Prakash and Basu 2020; Carro-Huerta et al. 2021). These characteristics inherent to *Trichoderma* species play a pivotal role during interactions with some disadvantageous microorganisms allowing them the dominant advantage of competing for space and nutrients (Sarrocchio 2016; Amira et al. 2017; Mukhopadhyay and Kumar 2020). The results of this investigation demonstrate that antagonistic microbes were unable to proliferate faster than the plant pathogen when growth rate was measured in pure culture. In paired cultures, however, the antagonistic fungi were able to hinder the plant pathogen's radial development. The findings obtained during the *in vitro* co-culture show that, the native *Trichoderma* isolates tested had a remarkable antagonistic effect against the pathogenic microorganism. After 9 days of incubation, the percent reduction of *L. theobromae* growth ranged from 21.5 to 75.4%, with substantial differences in efficiency across antagonistic strains. Several authors noticed similar variations in the effectiveness of *Trichoderma* against *L. theobromae*, including Marraschi et al. (2019) who evaluated *Trichoderma* strains against trunk diseases induced by *L. theobromae* in grapevine.

The damages made on the different fungal structures of the pathogenic fungus paired with *Trichoderma* strains were analyzed by visualizing co-culture photomicrographs using a scanning electron microscope. Because it has been found to be the most effective among the antagonist microbes, preparations of *T. asperellum* BRS-1 strain were selected. Following the confrontation experiment in dual culture, *T. asperellum* BRS-1 hyphae grew on *L. Lasiodiplodia* hyphae, leading morphological deformations and disorganization in the structure of the cell wall expressed by swelling and hyphae depletion. These morphologic alterations in hyphal structure were most likely caused by the release of antifungal metabolites (Burhanah et al. 2019; Wonglom et al. 2020; Rajani et al. 2021). These findings show that the antagonist microorganism has a specific antifungal capacity, which allows it to eventually impede the growth of the plant pathogen. *T. asperellum* generated morphological alterations in the structure of phytopathogenic fungi that were comparable to those reported for other phytopathogenic fungi (Mazrou et al. 2020; Rajani et al. 2021). Jiang et al. (2016) described some mycoparasitism episodes involving *T. asperellum* against *P. capsici*. Mazrou et al. (2020) observed hyphae of *Rhizoctonia solani* and *Pythium ultimum* encircled by *Trichoderma harzianum* in SEM photomicrographs and highlighted the importance of mycotoxic secondary metabolites and lytic enzymes in plant pathogens inhibition.

Our investigation highlighted that our antagonist microorganisms were also able to limit the development of *L. theobromae* without any physical contact. These results show that all nine strains of *Trichoderma* produce diffusible volatile and non-volatile compounds that have significant inhibitory effects on the development of pathogenic fungi. The maximum growth inhibition was achieved by *T. harzianum* BRS-7 in the case of volatiles secondary molecules. Non-volatile chemicals produced by *T. asperellum* BRS-1 and *T. harzianum* BRS-8, on the other hand, induced the greatest suppression of plant pathogenic fungal development. *Trichoderma* species have been reported to produce a wide range of mycotoxic and diffusible organic metabolites, including ketones, alcohols, furans, alkanes, and mono- and sesquiterpenes, in order to hinder the growth of phytopathogens (Vinale et al. 2006; Xuan et al. 2014; Li et al. 2019; Stracquadanio et al. 2020). For instance, the 6-pentyl- $\alpha$ -pyrone (6PP) a flavoring molecule responsible for the scent of coconut in some *Trichoderma* species has been reported to have antifungal properties (Vinale et al. 2008). Dennis and Webster (1971b) demonstrated that 6PP inhibited the growth of phytopathogenic fungi such as *Botrytis cinerea*, *R. solani* and *Fusarium* species. Vinale et al. (2012) demonstrated the strong antifungal activity of harzianic acid. They showed that this non-volatile chemical produced by the *T. harzianum* M10 strain is

capable for reducing the development of different plant pathogenic fungi, such as *Sclerotinia sclerotiorum*, *Pythium irregulare*, and *R. solani*. The chemical composition of secondary inhibitory molecules depends on the strains, and they can be classified as volatile, water-soluble or water-insoluble compounds (Vinale et al. 2006).

*Trichoderma* are also known to secrete extracellular lytic enzymes in addition to producing secondary metabolites, as demonstrated in this study during the SSF assay. Such enzymes may act by dissolving the plant pathogen cell walls, which in turn stimulates the production of more enzymes and, as a result, a series of physiological changes that promote the rapid and direct growth of *Trichoderma* spp. (Troian et al. 2014). Several studies have recently well demonstrated the functions of extracellular enzymes, such as amylolytic enzymes (Abdulaal 2018; Hamrouni et al. 2019),  $\beta$ -1,3-glucanase (Mercado et al. 2015; Kandasamy et al. 2016),  $\beta$ -1,6-glucanases (Montero et al. 2007),  $\alpha$ -1,3-glucanases (Nava-Cruz et al. 2016), chitinase (De la Cruz-Quiroz et al. 2017; Dinesh et al. 2017) and proteases (Jun-Jin et al. 2018), which are considered key factors in pathogen cell wall lysis during mycoparasitism (De Lima et al. 2016).

The quantitative-based biplot resulting from PCA analysis clustered the antagonistic *Trichoderma* strains according to their phylogenetical relationships. The isolate BRS-5 of *T. harzianum* strains was segregated from the other species of *T. harzianum*, indicating the existence of differences in terms of antagonistic features caused by an interspecific genetic variability. Aside from the depiction of grouping patterns, the qualitative-based biplot demonstrated a tight association between the species sub-group and antagonistic features. This correlation suggests that depending on the species, *Trichoderma* may be specialized in particular antagonistic mode of action. The principal component analysis also revealed that the control of the plant pathogen *L. theobromae* appears to be driven by a combination of different modes of action, including: extracellular lytic enzymes secretion, mycoparasitism, competition for space and nutrients, and antimicrobial compound production (antibiosis). During the biocontrol process, all of these mechanisms operate sequentially or/and continuously (Vinale et al. 2008, Zhang et al. 2014; Deketelaere et al. 2017). A combination of such synergistic mechanisms also called direct biocontrol mechanisms has been reported (Sood et al. 2020).

## 5. Conclusion

The current study gives preliminary data on the biological management of *L. theobroma*, the pathogen that causes stem-end rot in papaya. Through a combination of several mode of actions, the *Trichoderma* strains

examined demonstrated considerable antagonistic activity against plant pathogenic fungi. It would be interesting to identify the antifungal compounds produced by these antagonistic fungi in future studies. The encouraging results indicate that the investigated antagonistic microorganisms might be employed as bioactive components in novel biopesticides to reduce stem-end rot in papaya. However, further *in vivo* studies are required to validate the promise of these antagonistic strains as an alternative to synthetic fungicides for the treatment of stem-end rot disease.

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