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Pathogen genetic diversity shapes the in vitro antagonistic responses of native Trichoderma spp. against Bipolaris sorokiniana

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Abstract

Spot blotch caused by *Bipolaris sorokiniana* is a major threat to barley production worldwide. Although the genus *Trichoderma* is widely recognized for its biocontrol potential, the extent to which genetic diversity among distinct pathogen isolates influences antagonistic responses remains poorly understood. This study screened eight native *Trichoderma* isolates from threatened Araucaria Forest remnants against six *B. sorokiniana* field isolates using exclusively *in vitro* assays. Pathogen genetic diversity was assessed using inter-simple sequence repeat (ISSR) markers, while biocontrol potential was evaluated through dual culture and volatile/non-volatile metabolite production. Nei's genetic diversity (h = 0.444) and Shannon's information index (I = 0.634) revealed high genetic diversity among *B. sorokiniana* isolates, with no clear geographic association. Our results showed that the antagonistic efficacy of *Trichoderma* varied according to the pathogen genotype. While all *Trichoderma* isolates inhibited pathogen growth, their performance was highly isolate-dependent: *T. harzianum* LMA A14 and *T. azevedoi* LMA A22 exhibited the highest inhibition in dual culture (up to 82%), whereas *T. asperelloides* (LMA A20 and LMA A24) and *T. longibrachiatum* LMA A21 excelled differentially in volatile and non-volatile metabolite production, showing distinct suppression profiles across different *B. sorokiniana* genotypes. This study suggests that microbial biocontrol is a dynamic interaction in which pathogen genetic variability may influence antagonist performance, highlighting the importance of evaluating genetically diverse pathogen isolates when screening broad-spectrum native biocontrol candidates for sustainable disease management.

Introduction

Bipolaris sorokiniana is a necrotrophic pathogen and the causal agent of spot blotch in barley, a polycyclic disease characterized by rapid conidial production and dissemination via wind, water, and seeds (Basak et al. 2024; Gupta et al. 2018). The infection process involves the release of toxins that induce severe necrosis and chlorosis, potentially leading to premature defoliation and reduced grain yield (Al-Sadi 2021). The ability of the pathogen to persist in crop residues and soil organic matter facilitates survival between growing seasons (Bessadat et al. 2023). Although conventional control relies on triazoles, strobilurins, and carboxamides, the intensive use of these agrochemicals has selected resistant isolates, underscoring the need for sustainable management alternatives (Leng et al. 2020).

Addressing these challenges requires an integrated disease management approach, combining genetic resistance, crop rotation, and cultural practices with chemical and biological control (Singh and Mehta 2019). Biological control, which reduces pathogen inoculum or activity through antagonistic organisms (Stenberg et al. 2021), has emerged as a key strategy. These microorganisms suppress pathogens through rapid substrate colonization and other diverse antagonistic mechanisms, effectively inhibiting fungal development (Guzmán-Guzmán et al. 2023).

The filamentous fungal genus *Trichoderma* has been extensively used as a biological control agent. Its effectiveness is attributed to the broad array of secondary metabolites and hydrolytic enzymes involved

in communication, natural defense, and environmental competition. The beneficial traits of *Trichoderma* have led to its use as a biofungicide, biopesticide, biofertilizer, biostimulant, and resistance inducer (Saldaña-Mendoza et al. 2023). Differences among *Trichoderma* isolates result in variable antagonistic efficiency and environmental adaptability, making the screening of local isolates better adapted to local ecological and climatic conditions highly recommended (Moya et al. 2020).

Previous studies have demonstrated the potential of *Trichoderma* spp. to control *B. sorokiniana* isolates from wheat and barley *in vitro* (Villa-Rodríguez et al. 2019; Shpanev and Denisyuk 2020; Momtaz et al. 2022; Metwally et al. 2024), as foliar biofungicides (Kumar et al. 2019), and as resistance inducers in wheat plants challenged with the pathogen (Singh and Malviya 2019; Tiwari et al. 2022). However, most of these studies have evaluated antagonism against a single pathogen isolate, leaving insufficiently explored the extent to which intraspecific genetic variability within *B. sorokiniana* influences the outcome of these interactions. Because microbial interactions are governed by a dynamic, bi-directional cross-talk, distinct genotypes of a target pathogen may possess different physiological sensitivities, cell wall structures, or detoxification mechanisms, potentially influencing antagonist performance and the relative contribution of different antagonistic mechanisms. Therefore, evaluating antagonistic activity across multiple genetically characterized pathogen strains is essential for identifying resilient and broad-spectrum antagonists.

The Araucaria Forest, a distinct ecosystem within the Atlantic Forest biome, is recognized as a global biodiversity hotspot (Myers et al. 2000). Despite its ecological importance, this forest is currently one of the most threatened in Brazil, with only 4.3% of its original cover remaining, and just 13.5% of these remnants located within protected areas (Zorek et al. 2024), highlighting the vulnerability of this landscape.

Nonetheless, the microbial diversity harboring within these Araucaria Forest remnants remains largely underexplored, representing a valuable and untapped source of microorganisms with biotechnological potential.

Considering this vast biological heritage, we hypothesized that native *Trichoderma* isolates from Araucaria Forest remnants possess diverse antagonistic mechanisms and that their biocontrol efficacy may vary according to the genetic profiles of the target pathogen strain. Therefore, this study aimed to evaluate the in vitro antagonistic potential of eight native *Trichoderma* isolates against six *Bipolaris sorokiniana* field isolates. Using inter-simple sequence repeat (ISSR) markers to characterize pathogen variability together with dual culture and volatile and non-volatile metabolite assays, we assessed whether pathogen genetic background influences the performance of these native biocontrol candidates.

Material and Methods

Eight *Trichoderma* isolates previously recovered from soil samples collected in Araucaria Forest remnants in the Guarapuava region, Paraná, Brazil, were evaluated for their antagonistic potential against

B. sorokiniana. Their molecular identities had been established in a previous barcoding study using the internal transcribed spacer (ITS), translation elongation factor 1-alpha (*tef1*-α), and RNA polymerase II second largest subunit (*rpb2*) regions (Selestrino 2024) as *T. harzianum* (LMA 14 and LMA A19), *T. asperelloides* (LMA A20 and LMA A24), *T. longibrachiatum* (LMA A21), *T. azevedoi* (LMA A22), *T. lentiforme* (LMA A26), and *T. virens* (LMA A27). The resulting sequences were deposited in the National Center for Biotechnology Information (NCBI) GenBank database and the accession numbers for the ITS, *tef1*, and *rpb2* sequences of each *Trichoderma* isolate are provided in Table S1 (Supplementary Material). These publicly available sequences ensure the traceability and independent verification of isolate identities. Cultures were maintained in the laboratory through periodic subculturing on potato dextrose agar (PDA) and preserved using the Castellani method (Castellani 1939).

Isolation, cultivation, and maintenance of B. sorokiniana

Isolates of *B. sorokiniana* (BS1–BS6) were obtained from leaf fragments of barley plants showing typical spot blotch symptoms, collected in rural areas of the municipalities of Guarapuava (25°23'36" S, 51°27'19" W), and Ponta Grossa (25°05'42" S, 50°09'43" W), Paraná, Brazil. Fragments were surface disinfected in 0.5% sodium hypochlorite (NaClO) for 30 s, rinsed in sterile distilled water, dried on sterile paper towels, and placed onto PDA plates. Cultures were incubated in the dark for 4 days at 25 ± 2°C. Successive subcultures were performed until pure cultures were obtained. All isolates were preserved using the Castellani method (Castellani 1939). The pathogenicity of all isolates was confirmed on barley seedlings following Koch's postulates prior to the *in vitro* experiments.

Morphological identification and characterization of B. sorokiniana

Species identification was performed using a polyphasic approach based on cultural, morphological, and molecular characteristics. For morphological characterization, pure cultures were grown on potato dextrose agar (PDA) at 25 ± 2°C for 7 days. Colony characteristics, including color, texture, pigmentation, exudate production, margin characteristics, and growth pattern, were recorded according to Gupta et al. (2018), Debnath et al. (2022), Sharmin et al. (2022), and Yadav et al. (2022). Microscopic characteristics, including conidial shape, pigmentation, and septation, were examined under a light microscope.

Molecular identification of B. sorokiniana

Pathogen isolates were grown on PDA for 7 days at 25 ± 2°C. Harvested mycelia were ground to a fine powder in liquid nitrogen and immediately transferred into sterile 1.5 mL microtubes. Total genomic DNA was then extracted following the cetyltrimethylammonium bromide (CTAB)-based method of Doyle and Doyle (1987). DNA quality was assessed on 1% agarose gels, and DNA concentration was estimated using a NanoDrop 2000/2000c spectrophotometer (Thermo Scientific, CA, USA).

Identification of *B. sorokiniana* isolates was based on sequencing of the ITS region, using primers ITS4 (5’-CTTGGTCATTTAGAGGAAGTAA-3’) and ITS5 (5’-CTCACTAAGCCATTCAATCGG-3’) (White et al. 1990). Amplification reactions were performed in a Veriti thermocycler (Applied Biosystems) in a volume of 50

µL using 25 ng of DNA, 1× polymerase chain reaction (PCR) buffer with 1.0 mM $MgCl_2$, 0.25 mM of each deoxynucleoside triphosphate (dNTP), 1 U Taq DNA polymerase, 0.4 µM of each primer (final concentration), and ultrapure water. The PCR program consisted of initial step to DNA denaturation at 95°C for 3 min, followed by 35 cycles of DNA denaturation at 95°C for 15 s, primers annealing at 53°C for 15 s, and extension at 72°C for 1 min, with a final extension at 72°C for 10 min.

Amplification of the expected 600–700 base pair (bp) fragment was confirmed by electrophoresis using 10 µL of the PCR product on a 1.8% agarose gel stained with ethidium bromide (0.5 µg mL^{-1}). Following confirmation of amplification, the remaining 40 µL of the PCR products were purified with absolute ethanol, quantified using NanoDrop 2000/2000c spectrophotometer (Thermo Scientific, CA, USA). The purified product was sent to the company ACTGene Análises Moleculares LTDA (Alvorada, RS, Brazil) for sequencing by Sanger method on an ABI 3500 Genetic Analyzer (Applied Biosystems). The sequences were inspected for quality using BioEdit v.7.2.5 (Hall 1999). Homology searches were performed using Basic Local Alignment Search Tool for nucleotides (BLASTn) (Altschul et al., 1997) in the NCBI database (GenBank Overview 2025). The ITS sequences were deposited in GenBank under the accession numbers PX735807–PX735812. Species identification was supported by the combination of morphological characteristics, pathogenicity, and ITS sequence similarity.

ISSR marker analysis

To perform the genetic similarity analysis between *B. sorokiniana* isolates, the Inter-Simple Sequence Repeat (ISSR) technique was used. Genomic DNA from each isolate was extracted as described in the molecular identification step and amplified using a set of 10 ISSR primers developed by the University of British Columbia (UBC), Vancouver, Canada (Table 1). The ISSR primers used were selected based on the number of amplified bands, percentage of polymorphism, polymorphism information content (PIC) and resolution power presented in the study of Fedrigo et al. (2016), and in the number of amplified bands and percentage of polymorphism presented by de Arruda et al. (2021).

Table 1
List of the 10 ISSR (inter-simple sequence repeat) primers used to assess the genetic variability of *B. sorokiniana* isolates. The annealing temperature (T_a, °C), total number of amplified fragments (NAF), and percentage of polymorphism (P) for each primer are provided

Primer	Sequence*	T_a (°C)	NAF	*P* (%)
UBC 807	$(AG)_8T$	52	10	0.00
UBC 808	$(AG)_8C$	50	16	81.25
UBC 809	$(AT)_8T$	55	15	93.33
UBC 811	$(GA)_8C$	53	12	66.66
UBC 827	$(AC)_8G$	53	10	10.00
UBC 835	$(AG)_8YC$	54	9	55.55
UBC 848	$(CA)_8RG$	55	9	0.00
UBC 861	$(ACC)_6$	52	0	0.00
UBC 868	$(GAA)_6$	50	11	54.54
UBC 873	$(GACA)_4$	50	8	12.50
*Y = (C, T); R = (A, G)				

PCR was performed in a final volume of 12.5 µL containing approximately 20 ng of genomic DNA, 0.2 µM of primer, 200 µM of each dNTP, 1.5 mM $MgCl_2$, 1 U Taq DNA polymerase, and 1× reaction buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl]. Amplifications were carried out in a Veriti thermocycler (Applied Biosystems) using the following cycling conditions: an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 45 s, primer-specific annealing temperature (Table 1) for 30 s, and extension at 72°C for 1 min. A final step at 72°C for 10 min was added to complete fragment extension.

PCR products were run on 1.8% agarose gels stained with ethidium bromide (0.5 µg mL^{-1}) and electrophoresed for approximately 4h at 110 V. Fragment sizes were estimated using a 100-bp DNA ladder (Invitrogen). Bands were visualized under UV light and photodocumented using the L-PIX EX digital imaging system (Loccus do Brasil).

Of the 10 primers initially used, seven that provided clear, intense, and distinct DNA bands were selected for the final analysis, while primers that failed to amplify or produced unscoreable profiles were completely discarded. To ensure high reproducibility and reliability in the genetic analysis, only clear and reproducible DNA bands were scored. The binary matrix (band present = 1, band absent = 0) obtained

from the amplification data of all primers was used in subsequent analyses. Genetic similarity among isolates was estimated using the Jaccard coefficient (Sneath and Sokal 1973) with NTSYSpc version 2.1 (Rohlf 2000). The dendrogram was constructed using the unweighted pair group method with arithmetic mean (UPGMA). Node support was evaluated by bootstrap analysis with 1,000 replicates. Genetic groups were defined based on a similarity threshold derived from the dendrogram. PopGene version 1.32 was used to calculate descriptive parameters, including Nei's gene diversity (*h*), Shannon's Information index (*I*), the number of polymorphic loci, and the percentage of polymorphism.

Assessment of B. sorokiniana antagonism by Trichoderma isolates in dual culture

Antagonistic activity in dual culture was evaluated following Dennis and Webster (1971). PDA plates (90 mm) were inoculated with 5-mm mycelial discs obtained from seven-day-old cultures of each *Trichoderma* isolate and *B. sorokiniana.* A commercial wettable powder formulation of *Trichoderma harzianum* (Ecotrich® WP, Ballagro Agro Tecnologia Ltda., Bom Jesus dos Perdões, SP, Brazil; strain ESALQ-1306, containing a minimum of 2 x 10^9 viable conidia g^{-1} was used as a standard reference treatment. The powder was spread onto PDA plates using a sterile inoculation loop, incubated for 7 days, and used to obtain active mycelial discs.

The discs were placed on opposite sides of the plate, 7 cm apart, and incubated at 25 ± 2°C for 7 days. After incubation, the pathogen colony diameter was measured along a single axis passing through the center of the colony, and the inhibition rate (%) was calculated as:

$$\text{Inhibition rate } (\%) = \frac{\text{R1-R2}}{\text{R1}} \times 100$$

Where R1 is the colony diameter of the control (pathogen alone), and R2 is the diameter in the presence of the antagonist.

Assessment of the inhibition effect of volatile secondary metabolites (VOCs) from Trichoderma isolates against B. sorokiniana

Volatile-mediated inhibition was evaluated according to Dennis and Webster (1971). PDA plates were inoculated with 5-mm mycelial discs of each *Trichoderma* isolate or *B. sorokiniana* and incubated for 24 h at 25 ± 2°C to allow initial establishment. For the control treatment, a PDA plate inoculated with *B. sorokiniana* was paired with a plate containing fresh, uninoculated PDA medium. For VOC assessment, the lids were removed, and the plate containing the pathogen was inverted over the base containing the antagonist. The paired plates were sealed together with Parafilm® and incubated at 25 ± 2°C for 7 days. After incubation, the pathogen colony diameter was measured, and the inhibition percentage was calculated as described above.

Assessment of the inhibition effect of non-volatile secondary metabolites (non-VOCs) from Trichoderma isolates against B. sorokiniana

The effect of non-volatile metabolites was assessed following Dennis and Webster (1971). Each *Trichoderma* isolate was grown in 250 mL Erlenmeyer flasks containing 125 mL of potato dextrose broth (PDB) and inoculated with five 5-mm mycelial discs from 7-day-old cultures. Flasks were incubated at 150 rpm and 25 ± 2°C for 5 days.

Cultures filtrates were obtaneid by vacuum filtered through filter paper and subsequently through sterile cellulose membranes (0.45 µm) and used immediately after filtration. The sterile filtrates were incorporated into PDA at 25% (v/v) by mixing with molten PDA cooled to approximately 45°C. Control plates consisted of PDA medium mixed with sterile uninoculated broth instead of the fungal filtrate. Plates were then inoculated with a single 5-mm disc of *B. sorokiniana* and incubated at 25 ± 2°C for 7 days. After incubation, colony diameters of *B. sorokiniana* were measured, and inhibition was quantified as previously described.

Statistical analyses

Each treatment consisted of three biological replicates (one Petri dish per replicate), and the entire experiment was conducted twice independently. Prior to the analysis of variance (ANOVA), the assumptions of normality of residuals and homogeneity of variances were checked and confirmed using Shapiro–Wilk and Levene's tests, respectively. Data were then subjected to analysis of variance (ANOVA) at $p \leq 0.05$, and means were compared using Tukey's test. Statistical analyses were performed using SISVAR version 5.6 (Ferreira, 2019).

Results

Morphological and molecular characterization of B. sorokiniana

Six isolates of *B. sorokiniana* (BS1–BS6) were recovered from barley leaves showing typical brown spot lesions. Isolates BS1, BS3, and BS6 originated from barley crops in the Guarapuava/PR region, whereas the remaining isolates came from Ponta Grossa/PR. Morphological characterization was based on colony (macroscopic) and microscopic features. The isolates exhibited marked phenotypic variability (Fig. 1). On PDA, colony coloration ranged from cottony white to dark melanized. BS1 and BS6 developed dark gray mycelia, whitish aerial tufts and circular margins (Fig. 1A and 1F, respectively).

BS2 showed brownish-gray, cottony colonies with irregular margins (Fig. 1B). BS3 displayed white-brown cottony mycelium with irregular margins (Fig. 1C), whereas BS4 formed velvety white-brown colonies with a darkened center (Fig. 1D). BS5 exhibited grayish, cottony mycelia with light, circular borders (Fig. 1E).

Micromorphological observations revealed simple, rarely branched, septate conidiophores. Conidia were produced singly at the apex of conidiogenous cells and were light brown to golden, curved, with multiple

transverse septa and tapered apices, morphological features characteristic of *B. sorokiniana*. Molecular identification was confirmed through ITS-rDNA sequencing. The obtained sequences showed > 99.82% similarity to *B. sorokiniana* reference sequences deposited in public databases, confirming the identity of all isolates.

Genetic diversity of B. sorokiniana based on ISSR markers

Of the 10 ISSR primers evaluated, seven exhibited clear banding profiles, yielding a total of 81 amplified bands, of which 48 were polymorphic (Table 1). Among these primers, polymorphism rates varied substantially, ranging from 10.00% (primer UBC 827) to 93.33% (primer UBC 809), with an average polymorphism rate of 59.26% across all loci. Overall, the ISSR marker profiles revealed a high degree of genetic diversity among the investigated *B. sorokiniana* isolates. The mean Nei's gene diversity (*h*) was 0.4444 (± 0.0743). Additionally, Shannon's Information Index (*I*) averaged 0.6342 (± 0.0811), indicating substantial genetic variation among the analyzed isolates. The average Jaccard genetic similarity among the isolates was 0.30, indicating high genetic diversity within the investigated set of isolates. Based on the UPGMA dendrogram, a similarity threshold of 0.33 was used to delimit the major genetic clusters.

The UPGMA dendrogram, supported by 1,000 bootstrap replicates, demonstrated high statistical confidence for the major cluster nodes (Fig. 2). The first cluster comprised isolates BS1, BS2, and BS3; within this group, BS1 and BS2 showed the highest similarity, indicating their close genetic relationship.

The second cluster included isolates BS5 and BS6, which formed a distinct group separated from the remaining genotypes. In contrast, isolate BS4 formed an independent branch and did not cluster with the remaining isolates at the adopted similarity threshold, indicating marked genetic divergence.

Regarding geographic origin, isolates BS1, BS3, and BS6 were obtained from Guarapuava, whereas BS2, BS4, and BS5 originated from Ponta Grossa, municipalities located approximately 160 km apart. No clear association between genetic clustering and geographic origin was observed within this sample set, as isolates from both municipalities occurred within the same genetic groups.

Antagonism in dual culture

Eight *Trichoderma* isolates were evaluated for their biocontrol activity against six isolates of *B. sorokiniana.* ANOVA revealed significant effects for the *Trichoderma* isolate effect ($F = 31.14$, $p < 0.01$), the pathogen isolate effect ($F = 3.07$, $p < 0.01$), and their interaction ($F = 3.23$, $p < 0.01$) (Table 2).

Table 2

In vitro mycelial growth inhibition of *B. sorokiniana* by *Trichoderma* isolates in dual culture assays after seven days at 25 ± 2°C. Inhibition values were calculated using the percentage inhibition of radial growth

***Trichoderma* isolates**	**Mycelial growth inhibition (%)**					
	***Bipolaris sorokiniana* isolates**					
	BS1	**BS2**	**BS3**	**BS4**	**BS5**	**BS6**
T. harzianum LMA A14	78.05 abA	82.51 abA	73.65 bcAB	75.96 abAB	65.71 bcB	72.82 abAB
T. harzianum LMA A19	79.56 aA	71.52 bcdAB	71.60 bcAB	69.70 bAB	74.74 abAB	67.48 bB
T. asperelloides LMA A20	75.16 abAB	65.97 dB	67.82 cdB	70.54 abAB	72.31 abAB	79.88 aA
T. longibrachiatum LMA A21	66.48 bcA	62.74 dA	51.92 eB	57.95 cAB	57.37 cAB	65.15 bA
T. azevedoi LMA A22	80.87 aA	73.68 bcdA	76.40 bcA	75.36 abA	78.14 aA	74.36 abA
T. asperelloides LMA A24	77.48 abAB	79.12 bcAB	82.25 bA	69.40 bcB	72.75 abAB	72.64 abAB
T. lentiforme LMA A26	61.00 cB	62.57 dB	58.33 deB	67.47 bcAB	73.72 abA	66.26 bAB
T. virens LMA A27	61.25 cAB	68.42 cdAB	57.88 deB	68.92 bcA	63.20 bcAB	65.28 bAB
Standard (T. harzianum)	85.66 aAB	91.35 aAB	94.49 aA	81.93 aB	83.33 aB	82.03 aB

Means followed by the same uppercase letter in rows (*B. sorokiniana* isolates) and same lowercase letter in columns (*Trichoderma* isolates) do not differ by Tukey's test ($p < 0.05$).

All *Trichoderma* isolates inhibited the mycelial growth of *B. sorokiniana*, with inhibition ranging from 51.92 to 82.51% (Table 2). The commercial standard (*T. harzianum*) inhibited all six pathogen isolates. Among the native isolates, *T. harzianum* LMA A14, *T. asperelloides* LMA A20, and *T. azevedoi* LMA A22 suppressed four pathogen isolates; *T. asperelloides* LMA A24 inhibited three; *T. harzianum* LMA A19 inhibited two; and *T. lentiforme* LMA A26 inhibited one.

Trichoderma longibrachiatum LMA A21 and *T. virens* LMA A27 displayed the lowest inhibition percentages (51.92–66.48% and 57.88–68.92%, respectively). During incubation, *Trichoderma* isolates rapidly colonized the culture medium and frequently overgrew the pathogen colonies (Fig. 3). Physical contact generally occurred only after substantial expansion of the antagonist colony, resulting in restricted radial growth of *B. sorokiniana*.

Visual assessment indicated that isolates such as *T. longibrachiatum* LMA A21, *T. lentiforme* LMA A26, *T. virens* LMA A27, and the commercial standard formed clear inhibition zones suggestive of antibiosis. In

contrast, isolates including *T. harzianum* LMA A14 and LMA A19, *T. asperelloides* LMA A20 and LMA A24, and *T. azevedoi* LMA A22 exhibited abundant sporulation over pathogen hyphae, a patter consistent with putative mycoparasitic behavior. Regarding pathogen response, isolates BS1, BS4, BS5, and BS6 were the most susceptible, being suppressed by seven of the eight *Trichoderma* isolates tested.

Volatile secondary metabolites

Significant differences were detected among *Trichoderma* isolates factor ($F = 31.05$, $p < 0.01$), among *B. sorokiniana* isolates factor ($F = 73.4$, $p < 0.01$), and their interaction ($F = 6.64$, $p < 0.01$). All *Trichoderma* isolates produced volatile compounds that reduced pathogen growth relative to the control (Table 3).

Table 3
In vitro inhibition of *B. sorokiniana* mycelial growth by volatile secondary metabolites (VOCs) produced by *Trichoderma* isolates. Plates were incubated at 25 ± 2°C for seven days

***Trichoderma* isolates**	**Mycelial growth inhibition (%)**					
	***Bipolaris sorokiniana* isolates**					
	BS1	**BS2**	**BS3**	**BS4**	**BS5**	**BS6**
T. harzianum LMA A14	39.66 bcBC	54.63 aAB	61.87 abA	30.10 aCD	40.09 bBC	14.04 cD
T. harzianum LMA A19	24.78 cdB	57.37 aA	55.49 abcA	0.00 cC	56.26 abA	21.17 bcB
T. asperelloides LM A20	60.7 aA	40.75 abBC	67.16 aA	26.30 abC	50.42 abAB	37.08 abBC
T. longibrachiatum LMA A21	51.1 abAB	52.99 aAB	52.57 abcAB	28.52 abC	65.35 aA	41.47 abBC
T. azevedoi LMA A22	20.61 cdBC	29.79 abAB	39.22 cdAB	0.00 cD	39.94 bA	3.30 cCD
T. asperelloides LMA A24	39.87 bcAB	41.08 abAB	51.24 abcA	29.20 aB	40.99 bAB	22.95 bcB
T. lentiforme LMA A26	0.00 eE	27.15 bcCD	44.06 bcdBC	8.62 bcDE	63.56 aA	51.93 aAB
T. virens LMA A27	13.98 deBC	13.79 bcBC	28.44 dB	0.00 cC	50.82 abA	6.84 cC
Standard (T. harzianum)	61.41 aAB	79.58 aA	54.90 abcB	61.63 aAB	50.37 abB	55.29 aB

Means followed by the same uppercase letter in rows (*B. sorokiniana* isolates) and same lowercase letter in columns (*Trichoderma* isolates) do not differ by Tukey's test ($p < 0.05$).

Trichoderma asperelloides LMA A20 and *T. longibrachiatum* LMA A21 were the most effective, showing inhibition ranges of 26.03–67.16% and 28.52–65.35%, respectively. Together with the commercial standard, these isolates significantly suppressed the mycelial growth of all six *B. sorokiniana* isolates.

Volatile metabolites from *T. harzianum* LMA A14 and LMA A19, and *T. asperelloides* LMA A24, significantly reduced the growth of three pathogen isolates. *T. lentiforme* LMA A26 inhibited two isolates, whereas *T. azevedoi* LMA A22 and *T. virens* LMA A27 suppressed one isolate each (Table 3, Fig. 4).

Isolate BS5 was the most susceptible to volatile metabolites, being suppressed by seven of the eight *Trichoderma* isolates. In contrast, BS4 was the least affected, consistently showing the lowest inhibition percentages across all antagonists tested.

Non-volatile secondary metabolites

Significant differences were observed among *Trichoderma* isolates factor ($F = 118.61$, $p < 0.01$), among *B. sorokiniana* isolates factor ($F = 50.40$, $p < 0.01$), and in their interaction ($F = 2.89$, $p < 0.01$). All *Trichoderma* isolates produced non-VOCs that significantly reduced pathogen growth compared with the control (Table 4).

Table 4
In vitro inhibition of *B. sorokiniana* mycelial growth by non-volatile secondary metabolites produced by *Trichoderma* isolates

***Trichoderma* isolates**	**Mycelial growth inhibition (%)**					
	***Bipolaris sorokiniana* isolates**					
	BS1	**BS2**	**BS3**	**BS4**	**BS5**	**BS6**
T. harzianum LMA A14	36.23 bcCD	59.82 abA	55.18 abAB	38.79 bBCD	41.69 bcBC	23.24 bD
T. harzianum LMA A19	15.58 deBC	16.08 dBC	43.74 bA	27.15 bcAB	22.07 deB	0.00 cC
T. asperelloides LMA A20	4.90 eBC	27.42 cdA	19.66 cAB	0.00 dC	6.44 eBC	0.00 cC
T. longibrachiatum LMA A21	66.02 aAB	74.77 aA	68.91 aA	73.86 aA	63.4 aAB	50.17 aB
T. azevedoi LMA A22	33.36 cdA	33.10 cdA	23.25 cAB	15.00 bcBC	24.8 cdAB	0.00 cC
T. asperelloides LMA A24	29.88 cdB	24.90 cdB	47.53 bA	17.12 cdBC	21.27 deB	0.00 cC
T. lentiforme LMA A26	16.50 deAB	29.04 cdA	22.51 cA	15.78 cdAB	16.27 deAB	0.00 cB
T. virens LMA A27	53.88 abA	52.70 bA	49.53 bA	36.68 bAB	49.28 abA	21.67 bB
Standard (T. harzianum)	42.59 bcB	42.83 bcB	57.75 abAB	66.27 aA	43.43 bB	8.41 bcC

Means followed by the same uppercase letter in rows (*B. sorokiniana* isolates) and same lowercase letter in columns (*Trichoderma* isolates) do not differ by Tukey's test ($p < 0.05$).

Among the native isolates, *T. longibrachiatum* LMA A21 was the most effective, inhibiting all six pathogen isolates with inhibition values ranging from 50.17% to 74.77%. *T. harzianum* LMA A14, *T. asperelloides* LMA A20, *T. virens* LMA A27, and the commercial standard inhibited only two pathogen isolates each (Fig. 5).

Inhibition profiles varied markedly among pathogen isolates. BS6 was the least susceptible, showing inhibition values between 0.00 to 50.17% and none of the antagonists completely inhibited its growth. In contrast, BS3 was the most susceptible, exhibiting inhibition levels ranging from 19.66 to 68.91% when exposed to all eight antagonists.

Discussion

In the present study, eight local *Trichoderma* isolates were evaluated for their antifungal potential against different isolates of the phytopathogen *B. sorokiniana*. Overall, all *Trichoderma* isolates suppressed pathogen growth *in vitro*, reinforcing the value of native strains adapted to local conditions as potential biocontrol agents.

In dual-culture assays, all isolates displayed antagonistic activity, although with variable efficiency. Such variability is expected, since the expression of key antagonistic traits, including enzyme production, secondary metabolite profiles, and competitive colonization, may differ substantially among *Trichoderma* isolates. Similar isolate-specific responses were reported by Rodríguez-Martínez et al. (2025) when evaluating antagonism against *Rhizoctonia solani*, *Phytophthora capsici*, and *Fusarium* spp., highlighting the importance of screening multiple candidates when identifying effective biocontrol agents. Our data also indicate that this interaction is not unidirectional: the pathogen genotype influenced the outcome, and the efficiency of each *Trichoderma* isolate varied significantly depending on the *B. sorokiniana* genotype encountered.

In addition to the genetic backgrounds of both antagonist and pathogen, the physiological traits of *Trichoderma* isolates may also have contributed to the observed differences in antagonistic performance. Variations in mycelial growth rate, resource acquisition, and the production and diffusion of antifungal metabolites have been reported to affect the outcome of fungal interactions *in vitro* (Kubiak et al. 2023; Hernández et al. 2024). Similarly, isolates with faster colony expansion may occupy the available substrate more rapidly, restricting pathogen growth before extensive metabolite-mediated inhibition occurs (Nguyen et al. 2025). Therefore, the antagonistic profiles observed here likely resulted from the combined influence of the genetic characteristics of both organisms and isolate-specific physiological traits.

Among the isolates evaluated, *T. harzianum* LMA A14, *T. asperelloides* LMA A20, and *T. azevedoi* LMA A22 showed the strongest inhibition in dual culture, followed by *T. asperelloides* LMA A24. These findings are consistent with previous reports demonstrating the high antagonistic potential of *T. harzianum*, including 90.12% inhibition of *Fusarium oxysporum* (Girma 2022) and inhibition rates above 76% and 90% against *B. sorokiniana* (Edermus and Katircioglu 2008; Villa-Rodríguez et al. 2022). Likewise, studies have shown that *T. harzianum* can achieve up to 86% inhibition of *B. sorokiniana* (Raj et al. 2024), supporting the robust performance observed for LMA A14 in our assay.

Intraspecific variation in phytopathogenic fungi can lead to differences in cell wall composition, toxin production, and enzymatic detoxification pathways capable of neutralizing *Trichoderma* secondary metabolites (Apoga et al. 2002; Ghazvini and Tekauz 2007; Gow et al. 2017). Consequently, a single *Trichoderma* isolate, such as LMA A14, may encounter different levels of physiological resistance depending on the pathogen strain. This bidirectional, isolate-dependent interaction explains why standardizing biocontrol evaluations against a single reference strain may overestimate or underestimate an antagonist's true field potential, underscoring the ecological complexity of these multi-genotype fungal interactions.

Several findings support the biocontrol patterns observed here. For instance, *T. azevedoi* and *T. asperelloides* strains have been documented as effective antagonists against a broad spectrum of phytopathogens, with inhibition rates matching or exceeding those reported in previous *in vitro* assessments (Silva et al. 2022; Ruangwong et al. 2021).

In our dual-culture assays, antagonism was driven mainly by a combination of antibiosis and aggressive mycoparasitism. Most *B. sorokiniana* colonies showed typical signs of parasitism, such as overgrowth and extensive sporulation by isolates LMA A20, LMA A14, LMA A19, LMA A22, LMA A24, and LMA A26 (El-Komy et al. 2022). This structural degradation and the efficiency of mycoparasitism are known to vary significantly among *Trichoderma* species (Wang and Zhuang 2019; Mukherjee et al. 2022). The process involves hyphal penetration mediated by the secretion of cell wall-degrading enzymes, particularly chitinases and β-1,3-glucanases (Xue et al. 2021; Singh et al. 2024). In this study, the rapid colonization and physical displacement of *B. sorokiniana* further support the crucial role of these hydrolytic pathways in the competitive success of native antagonists.

Genomic heterogeneity among *Trichoderma* species, particularly in the repertoire of genes encoding hydrolytic enzymes and secondary metabolites, may help explain the distinct and superior antagonistic levels observed for isolates LMA A14 and LMA A20 (Alfiky and Weisskopf 2021). Antibiosis also played a key role in suppressing *B. sorokiniana*. Clear inhibition zones lacking pathogen hyphae were produced by *T. virens* LMA A27, *T. longibrachiatum* LMA A21, and *T. lentiforme* LMA A26, a phenotype directly linked to the secretion of bioactive compounds such as peptaibols, polyketides, terpenes, and pyrones (Hou et al. 2021; Ma et al. 2023; Nguyen et al. 2025).

Interestingly, some *Trichoderma* isolates expressed multiple antagonistic mechanisms depending on the target pathogen genotype. For example, *T. lentiforme* LMA A26 combined rapid colony expansion, inhibition-zone formation, and mycoparasitism, reinforcing that *Trichoderma* spp. frequently employ complementary mechanisms that act simultaneously against phytopathogens (Paul et al. 2021; Villar et al. 2022; Poletto et al. 2024).

The volatile organic compounds (VOCs) released by *Trichoderma* isolates inhibited *B. sorokiniana* growth to varying degrees, reflecting the extensive metabolic diversity associated with volatile production in this genus. Rather than acting as universal inhibitors, VOC-mediated activity is highly isolate-specific and depends on unique chemical profiles (van Zijl de Jong et al. 2023). This inhibition is further modulated by bidirectional chemical cross-talk, as volatile compounds produced by the pathogen itself can interact with and influence *Trichoderma* metabolites (Momtaz et al. 2022). Functionally, the synthesis of these diverse volatile mixtures provides an ecological advantage by suppressing competitors, increasing resource availability, and facilitating rapid substrate colonization (Fishal et al. 2022; Abdenaceur et al. 2024).

In this work, *T. asperelloides* LMA A20 and *T. longibrachiatum* LMA A21 were the most effective volatile producers against *B. sorokiniana*. This performance is consistent with previous literature documenting the strong volatile activity of both *T. asperelloides* (Heflish et al. 2021)d *longibrachiatum* (Joo and

Hussein 2022) against broad-spectrum phytopathogens. Taken together, these findings indicate that the volatile antagonistic potential observed in LMA A20 and LMA A21 is a conserved and effective mechanism for suppressing fungal targets.

Among the antagonists evaluated, *T. longibrachiatum* LMA A21 was the most effective in suppressing *B. sorokiniana* growth through non-volatile metabolite production, a phenotype typical of inhibition mediated by highly diffusible bioactive compounds (Adnan et al. 2019; Sawant 2014; Stange et al. 2023). Conversely, *T. asperelloides* LMA A20 displayed limited activity in this specific assay, suggesting that its antagonistic profile relies more heavily on alternative mechanisms, such as VOC production or aggressive mycoparasitism (Go et al. 2023). This metabolic variability aligns with Sazonova et al. (2025), who noted that the capacity to secrete appreciable levels of non-volatile antifungal metabolites is not universally conserved among all *Trichoderma* strains. The pronounced suppressive effects of *T. longibrachiatum* non-volatile filtrates observed here are further supported by literature showing their fungistatic mode of action, which induces hyphal abnormalities and restricts mycelial expansion across diverse plant pathogens (Sridharan et al. 2020).

Furthermore, isolates belonging to the same *Trichoderma* species differed markedly in their antagonistic profiles (Izidoro-Morimitsu et al. 2026). Although *T. asperelloides* LMA A20 and LMA A24 displayed similar mechanisms, the two *T. harzianum* isolates differed substantially in volatile and non-volatile metabolite production, highlighting the pronounced intraspecific variability commonly observed within the genus.

The commercial *T. harzianum* product used as a standard treatment exhibited high inhibition levels *in vitro*, reinforcing the well-documented capacity of this species to suppress a wide range of phytopathogens (Swathy et al. 2024). This consistent performance across studies reflects the extensive validation *T. harzianum* has undergone for biocontrol applications, aligning with the inhibitory activity observed in the present work. Nevertheless, although dual-culture and metabolite assays provide robust evidence of antagonism, future studies incorporating specific enzymatic assays, advanced microscopy, and metabolomic characterization via GC-MS or LC-MS remain necessary to identify and quantify the precise physical and chemical mechanisms involved.

Our in vitro screening revealed that *B. sorokiniana* isolates showed distinct levels of susceptibility to the same *Trichoderma* antagonist, with certain pathogen genotypes consistently exhibiting low inhibition across multiple mechanisms. For instance, isolate BS6 was minimally affected by volatile metabolites, demonstrating that antifungal efficacy depends on the intrinsic characteristics of the target pathogen. This variable susceptibility indicates that genetic and biochemical diversity within *B. sorokiniana* populations directly influences their resistance to microbial defenses, likely due to isolate-specific differences in enzymatic profiles and detoxification pathways (Al-Sadi 2021).

Studies conducted worldwide indicate that *B. sorokiniana* isolates from barley and wheat exhibit intense genetic heterogeneity, accompanied by marked variation in morphology, pathogenicity, and virulence (Ghazvini and Tekauz 2008; Persson et al. 2009; Gamba et al. 2021; Alkan et al. 2022; Kang et al. 2023).

This high genetic variability likely drives the phenotypic and molecular plasticity observed here, where macromorphological traits occasionally fell outside established cultural groups (Gupta et al. 2018; Yadav et al. 2022). Our findings agree with Mann et al. (2014), who reported extensive morphological variability in monoconidial isolates, reinforcing the highly heterogeneous nature of this pathogen population.

Consistently, our ISSR analysis confirmed this high genetic variability, evidenced by a low average similarity coefficient (0.30) and supported by relatively high values of Nei's genetic diversity (h = 0.444) and Shannon's information index (I = 0.634). These metrics indicate a highly polymorphic genetic structure, and the UPGMA clustering failed to correlate with geographic origin. Notably, the dendrogram revealed that morphological resemblance does not mirror genetic relatedness; for instance, isolates BS1 and BS6 shared similar macromorphological traits but were assigned to distinct clades. Furthermore, the co-clustering of isolates from Guarapuava and Ponta Grossa suggests that geographic distance is not the main driver of genetic divergence in these regional populations.

This lack of spatial structuring may be attributed to the high dispersal capacity of *B. sorokiniana* via infected seeds, anemochory, and crop residues, which facilitates pathogen movement and maintains high genetic diversity across geographic regions. Furthermore, since the teleomorph (sexual stage) of this pathogen is rarely observed under natural conditions (Roy et al. 2023), this elevated genetic variability is likely sustained by a combination of mutation, parasexual recombination, and demographic factors (Sharma et al. 2022). Such multi-genotypic diversity has important implications for disease management, as shifting pathogen populations may display different levels of aggressiveness and unpredictable responses to control strategies.

In this context, the separation of isolate BS4 as an independent lineage and isolate BS6 into a distinct clade, despite their shared low susceptibility to certain *Trichoderma* metabolites, is noteworthy. This pattern suggests that reduced susceptibility may arise from distinct genetic backgrounds rather than from a single evolutionary origin. It also reinforces the need for future screening programs to include diverse isolates from different genetic clusters so that robust *Trichoderma* strains can be selected against the multifaceted defense strategies present across the pathogen's evolutionary spectrum.

Furthermore, information on the genetic variability of *B. sorokiniana* in Brazil remains limited, as existing studies have focused mainly on wheat-derived isolates (Müller and Germani 2005; Mann et al. 2014). Consequently, the diversity of populations infecting barley in the country remains largely unexplored. Advancing this knowledge is essential to ensure the consistency of disease-management strategies, given that pathogen genetic diversity directly influences susceptibility to biocontrol agents. However, considering our regional sample size, these genetic and geographic trends should be treated as preliminary; larger population-level samplings are required to map regional gene flow more comprehensively.

Finally, although our in vitro assays provide strong screening evidence, they do not replace validation under greenhouse or field conditions, where environmental fluctuations, soil-microbe interactions, and host physiological responses can strongly influence biocontrol performance. Future research should

therefore move toward controlled-environment and multi-location field trials to evaluate application methods, assess compatibility with the native microbiota, and determine the long-term stability and practical applicability of these native *Trichoderma* isolates.

Conclusions

The present study demonstrated that native *Trichoderma* isolates differ markedly in their ability to suppress genetically diverse *B. sorokiniana* isolates in vitro, indicating that biocontrol performance is influenced by the genetic variability of both the pathogen and the antagonist. ISSR analysis revealed high genetic diversity among *B. sorokiniana* isolates, with low genetic similarity and no clear geographic structure, supporting the inclusion of genetically diverse pathogen populations in future biocontrol screening programs.

The significant interaction between *Trichoderma* and *B. sorokiniana* across all antagonism assays confirmed that pathogen genotype is a key determinant of biocontrol outcome. Among the evaluated isolates, *T. harzianum* LMA A14, *T. azevedoi* LMA A22, *T. asperelloides* LMA A20 and LMA A24, and *T. longibrachiatum* LMA A21 showed the strongest antagonistic performance, albeit through different and complementary mechanisms of action. These findings suggest that screening antagonists against a single pathogen isolate may underestimate or overestimate their true biocontrol potential.

Overall, these results provide a solid basis for developing sustainable strategies to manage barley spot blotch. However, greenhouse and multilocation field trials are still needed to validate the stability and consistency of these antagonists under natural conditions. In addition, the microbial diversity of threatened ecosystems such as the Araucaria Forest may represent an important source of regionally adapted *Trichoderma* strains with broad antagonistic potential, strengthening sustainable disease management and improving the resilience of barley production systems.

Declarations

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Ethics Approval

Not applicable. This study does not contain any studies with human participants or animals performed by any of the authors.

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Author Contribution

All authors contributed to the study conception and design. Material preparation, data collection and analyses were performed by Gabriela Pupo Hagemeyer, Simone Cristine Izidoro-Morimitsu, Cacilda Marcia Duarte Rios Faria; Jackson Kawakami; Paulo Roberto da Silva and Felipe Liss Zchonski. The first draft of the manuscript was written by Adriana Knob and Gabriela Pupo Hagemeyer and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data Availability

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

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Figures

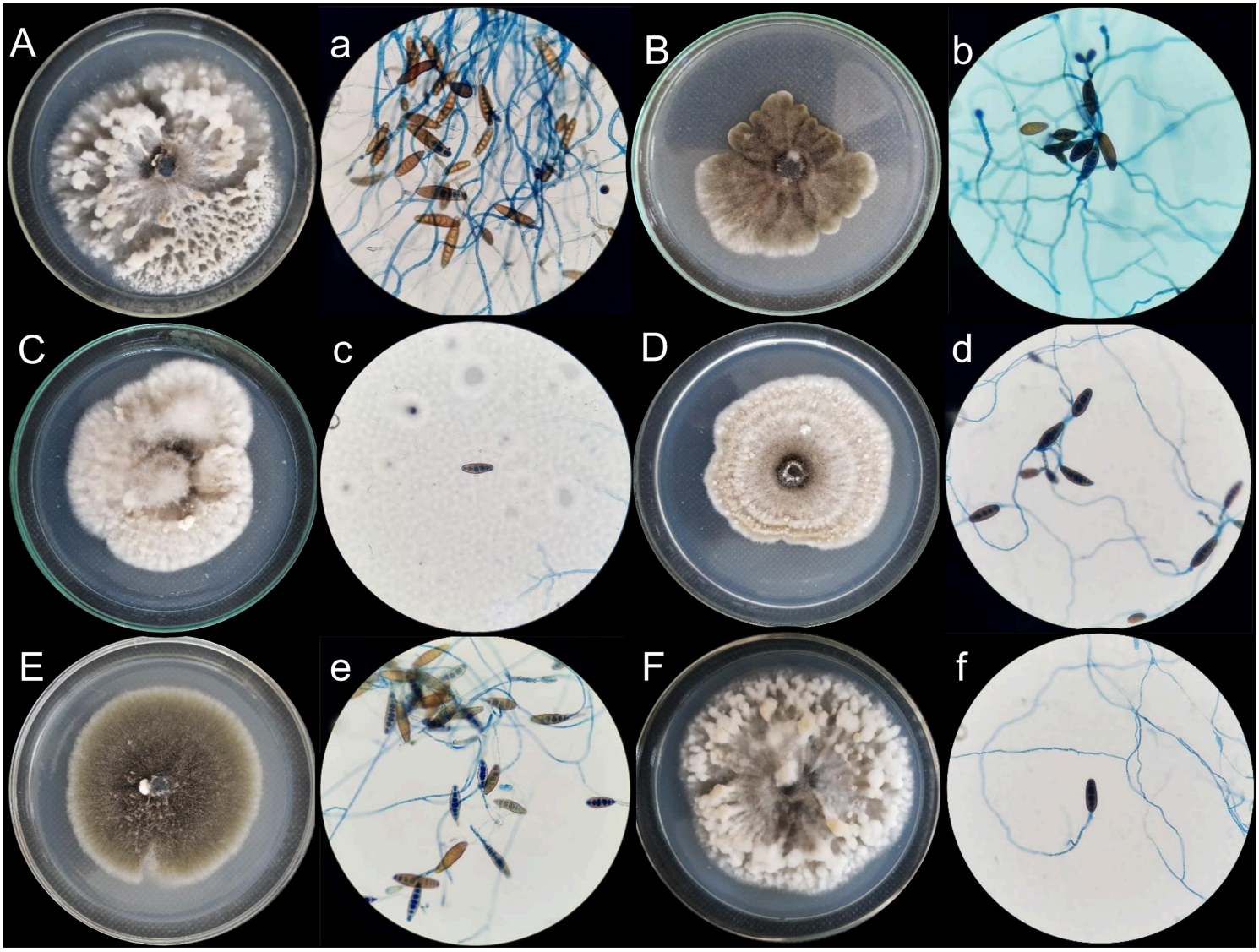

Figure 1

Macroscopic (A-F) and microscopic (a-f, 400×) morphology of six *B. sorokiniana* isolates. Microscopic structures were obtained using the slide micro-culture technique and incubated at 25 °C for 4 days. A, a: BS1; B, b: BS2; C, c: BS3; D, d: BS4; E, e: BS5; F, f: BS6

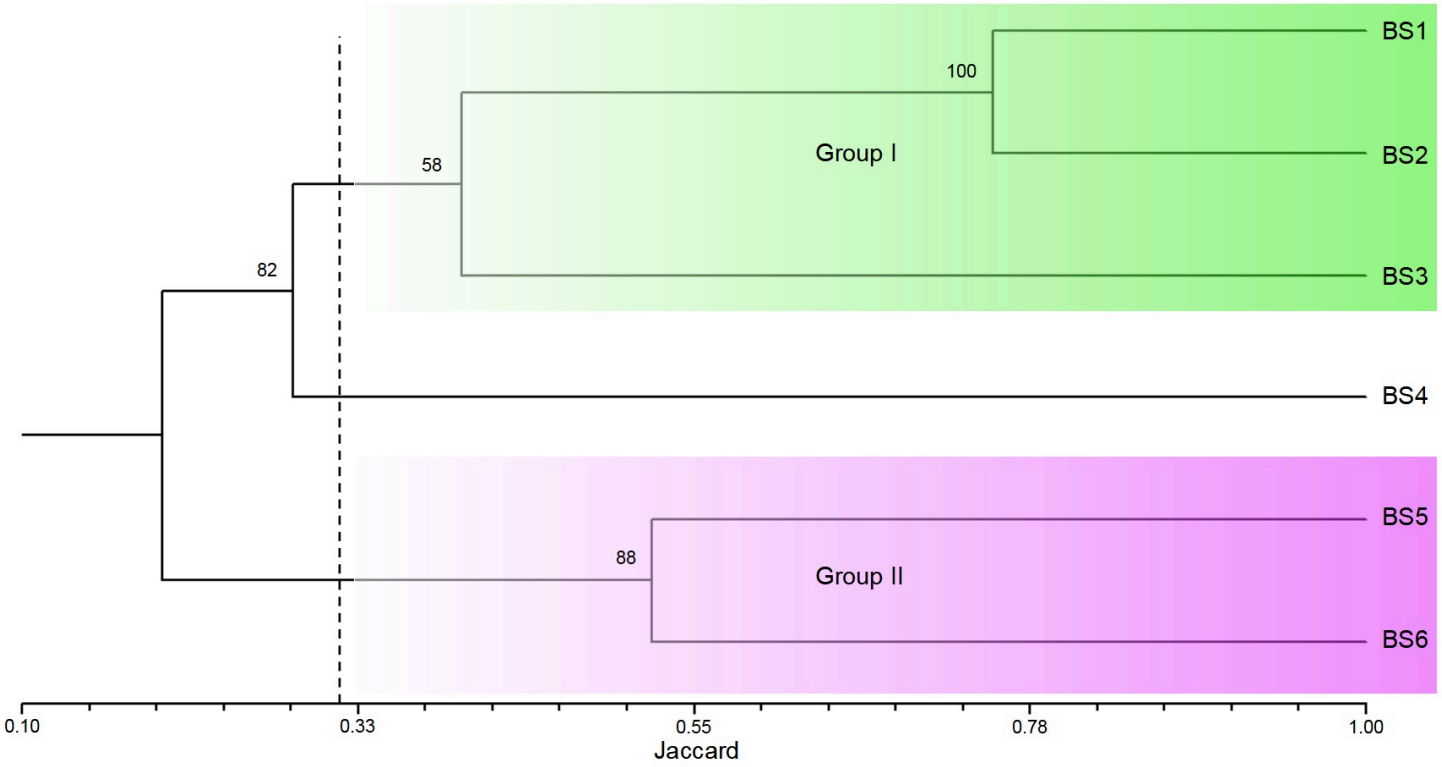

Figure 2

Dendrogram showing the genetic relationships among *B. sorokiniana* isolates based on ISSR markers. The analysis was performed using the Jaccard similarity coefficient and the UPGMA clustering method. Numbers at the nodes represent bootstrap support values based on 1,000 replicates. The dashed line indicates the similarity threshold (0.33) used to define major clusters

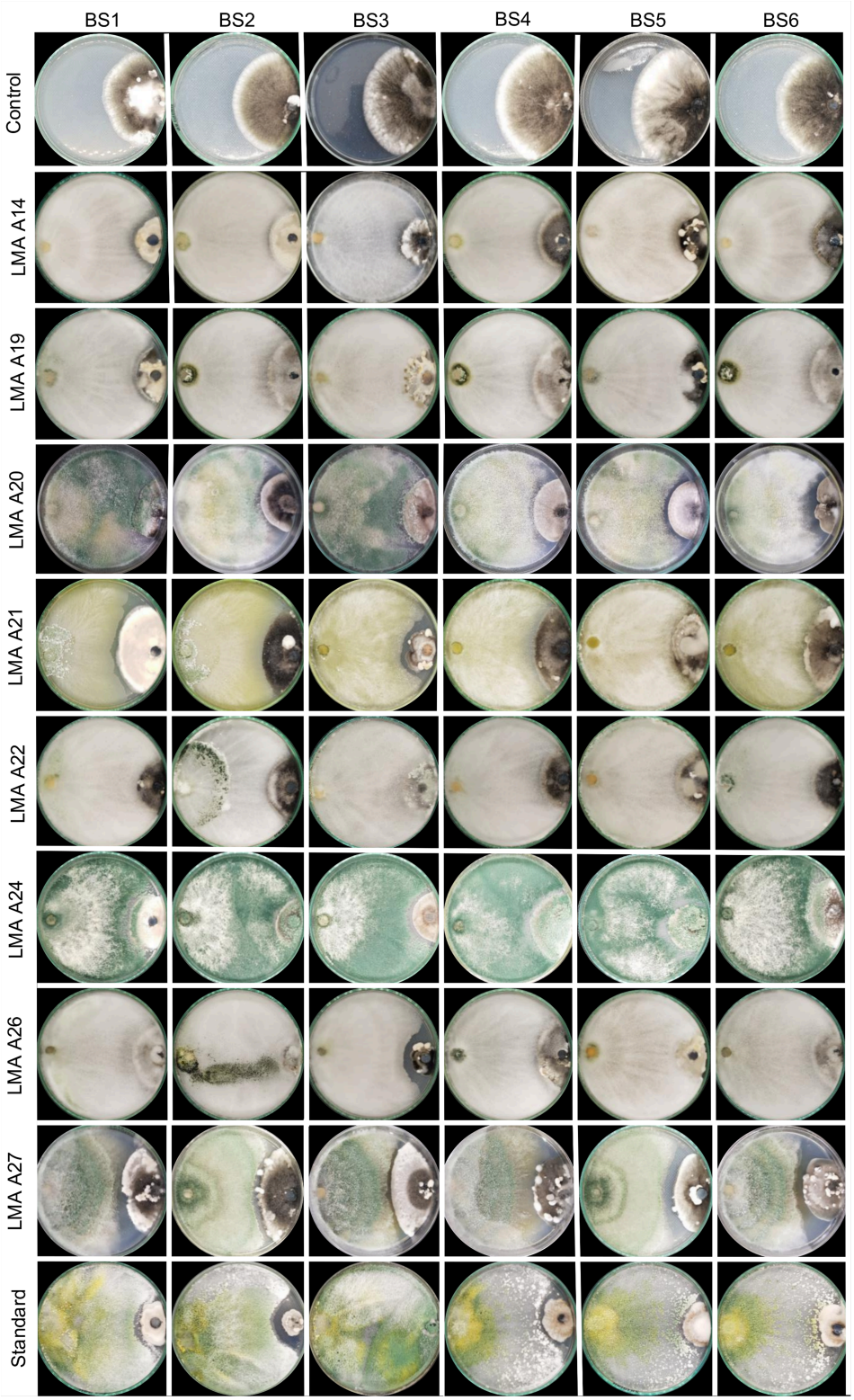

Figure 3

Dual culture assays showing the antagonistic activity of *Trichoderma* isolates against *B. sorokiniana*. Plates were incubated at 25 ± 2 °C for 7 days. *Trichoderma* isolates rapidly colonized the medium, suppressing pathogen growth through overgrowth or formation of inhibition zones

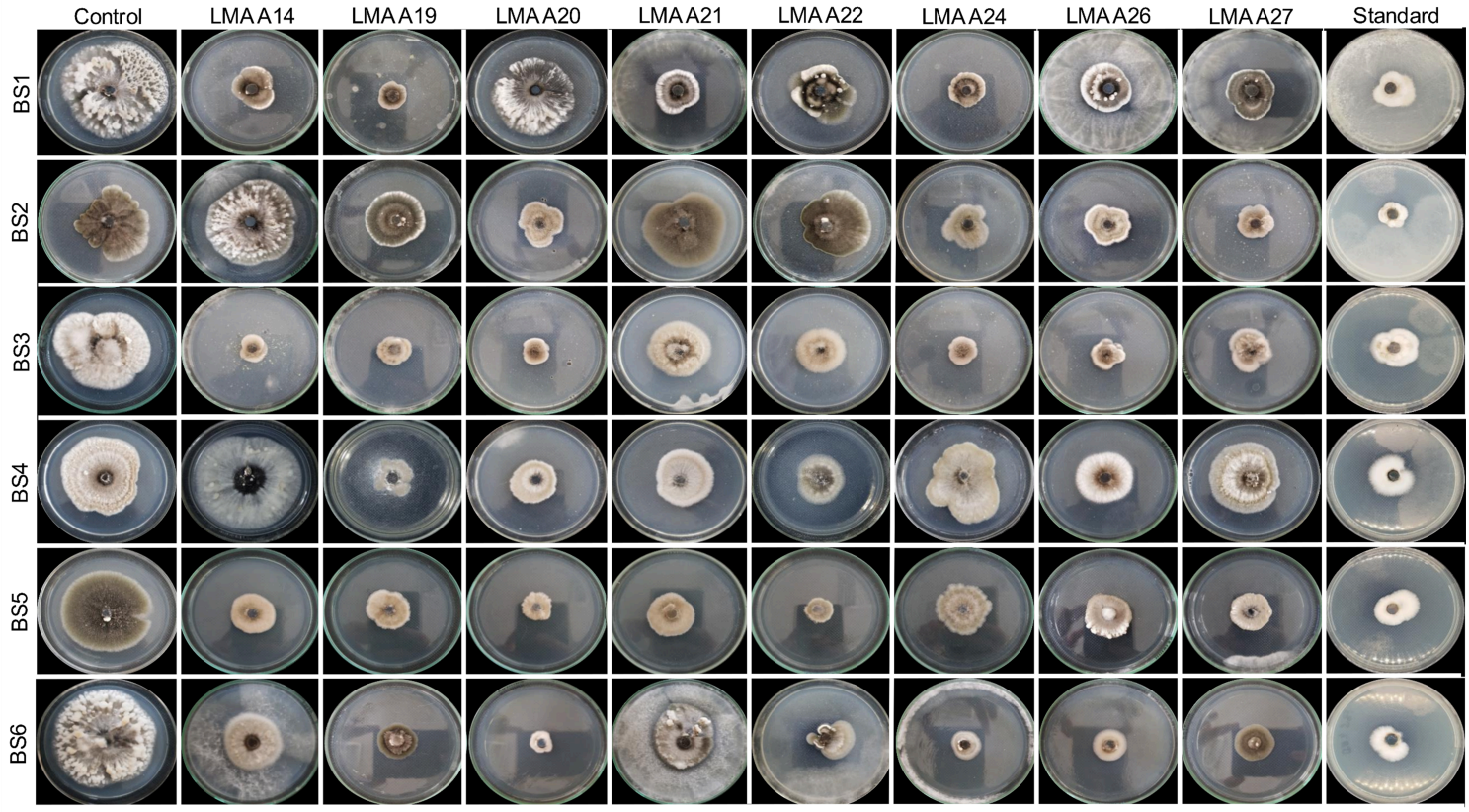

Figure 4

Inhibition of *B. sorokiniana* by volatile secondary metabolites (VOCs) produced by *Trichoderma*isolates

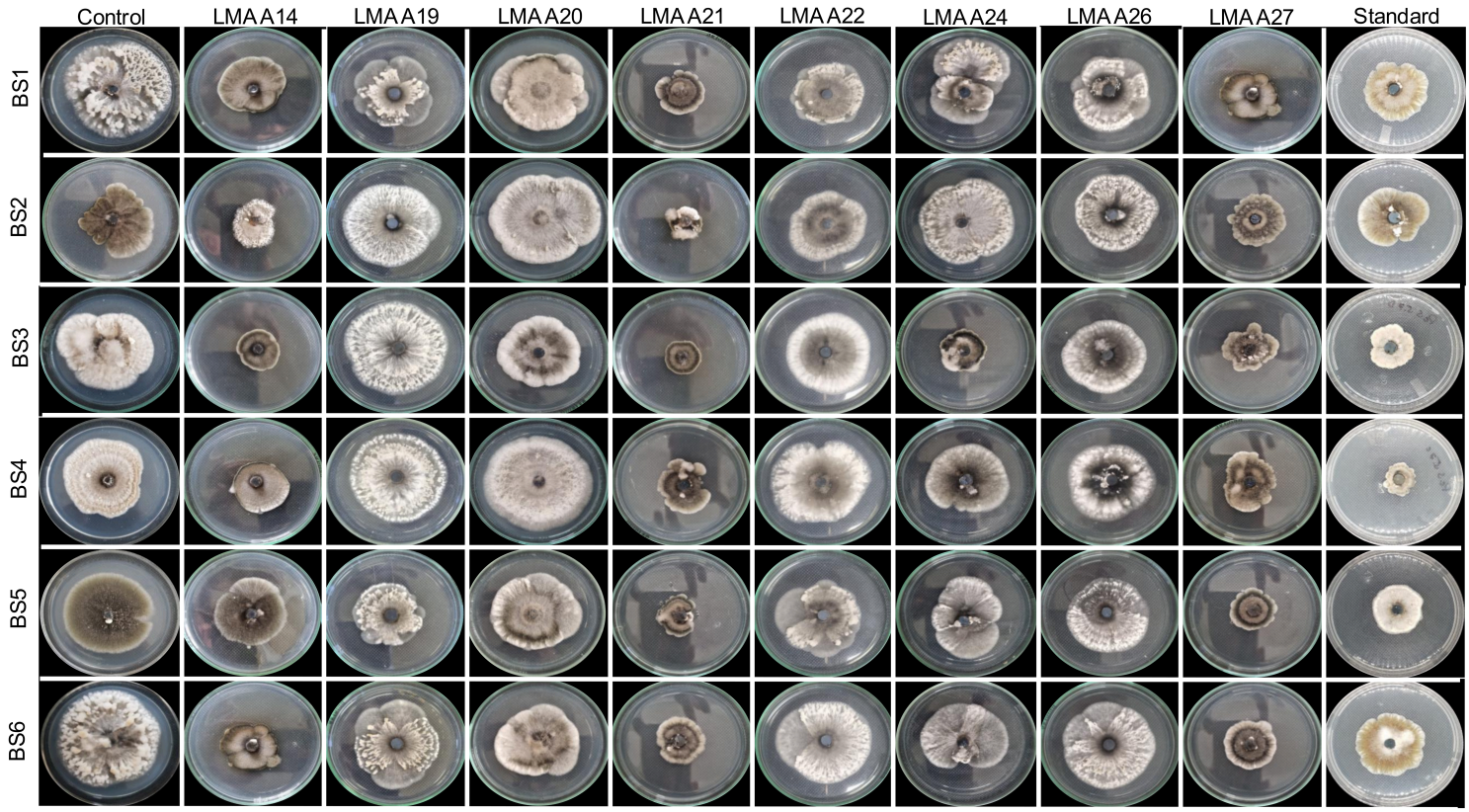

Figure 5

Inhibition of *B. sorokiniana* by non-volatile secondary metabolites (non-VOCs) produced by *Trichoderma*isolates