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Biocontrol potential of bacteria and fungi isolated from *Zea mays* L. (Maize) grains against fumonisins produced by *Fusarium verticillioides*

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ABSTRACT

The phytopathogenic fungus *Fusarium verticillioides* produces fumonisins, mycotoxins that contaminate grains of *Zea mays* L. (maize) and pose a risk to human and animal health. The aim of this study was to evaluate the efficiency of bacteria and filamentous fungi in controlling toxigenic *F. verticillioides* in maize. Microorganisms associated with maize grains were isolated and identified, and their ability to inhibit pathogen growth and reduce total fumonisin production was assessed. The microorganism showing the highest efficiency was subsequently identified by molecular methods. Fungi belonging to the genera *Fusarium* (85.71%), *Aspergillus* (57.14%), *Penicillium* (21.43%), and *Trichoderma* (9.52%) were recovered in maize grain samples, based on the total analyzed. 71.1% of the *F. verticillioides* isolates were toxigenic, producing fumonisin concentrations ranging from 30 to 4,010 ppb, with strain H08 showing particularly high levels. In antagonism assays, 90.7% of the bacterial isolates and 42.7% of the filamentous fungi exhibited inhibitory activity against the pathogen. *Trichoderma atroviride* strain 3 showed the highest efficiency, reducing mycelial growth by 84.94% and fumonisin concentration by 99.77%, demonstrating strong potential to reduce maize contamination by toxigenic *F. verticillioides*.

1. INTRODUCTION

Cereals such as *Zea mays* L. (maize) constitute a fundamental food source for humans and other living organisms [1]. However, infection by fungi such as *Aspergillus, Fusarium*, and *Penicillium* during agricultural cultivation, harvest, storage, and transportation causes significant economic losses in crop yield and the agro-food industry [2–4]. Fungi are considered the main biotic factor responsible for poor grain quality and yield reduction in maize, as they affect plant growth and produce mycotoxins with immunosuppressive, teratogenic, and mutagenic effects [1], and are also associated with the development of cancer in humans [5].

In tropical and subtropical regions, several toxigenic species of *Aspergillus* have been reported, including *Aspergillus flavus* and *Aspergillus ochraceus*, producers of aflatoxins and ochratoxins [6]. In addition, fungi belonging to the genus *Fusarium* are typical producers of fumonisins. Among the most relevant species are *Fusarium verticillioides* and *Fusarium proliferatum*, which cause seedling blight and rot of stems, ears, and grains [7]. Fumonisins produced by *Fusarium* are classified into four groups (FA, FB, FC, FP), among which group B stands out, including fumonisins B_1 (FB_1), B_2 (FB_2), and B_3 (FB_3). FB_1 has been described as the most toxic to humans and animals [7,8] and is classified as a group 2B possible human carcinogen [5], as it has been associated with esophageal, liver, and kidney cancers, in addition to causing neurotoxic effects through oxidative stress and cellular dysfunction [8,9].

Fusarium verticillioides is one of the filamentous fungi most frequently reported in cereals worldwide [10,11]. A study conducted in Peru detected *Fusarium* spp. at the molecular level in 95% of maize samples, with predominance of *F. verticillioides*, quantifying fumonisin levels up to 6,725 ppb in dry matter. This value significantly exceeded the maximum level of 1,000 ppb established by European regulations for maize intended for direct human consumption [12]. Therefore, preventing fungal infection in cereals and the accumulation of mycotoxins is extremely challenging. Nevertheless, toxin levels in food can be reduced through physical and chemical techniques that inhibit fungal growth; however, these approaches do not guarantee complete elimination and may generate by-products or residues that compromise food quality and safety [13]. Alternatively, biological control methods can be applied using generally recognized as safe microorganisms, which are considered safe and effective [14,15].

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These microorganisms interact with pathogens through antagonistic mechanisms such as competition, antibiosis, and parasitism [16].

In nature, several microorganisms capable of inhibiting the growth of *Fusarium* spp. and their produced toxins have been reported. These include bacteria from the genera *Lactococcus* [17], *Burkholderia, Achromobacter, Serratia* [18], *Streptomyces, Pseudomonas* [19], and *Bacillus, Lactobacillus* [20], as well as filamentous fungi from the genera *Aspergillus* [18], *Trichoderma* [21,22], and *Penicillium* [23]. Previous studies have reported biological agents for the control of toxigenic fungi such as *Fusarium* in cereals; however, they still show limited efficiency in reducing and/or suppressing these agents in crops [24,25]. Some applications yield inconsistent efficacy, which varies depending on the antagonist strain, the time elapsed post-application, or cases where fungal growth is inhibited without a corresponding reduction in the produced toxins. This limitation has been demonstrated in the control of *Fusarium graminearum* or *F. verticillioides* using *Sarocladium zeae* [26], *Streptomyces* sp. [25], and *Pseudomonas* sp. [27]. Furthermore, this lack of experimental reproducibility, combined with the complex regulatory registration processes for biopesticides, contributes to their limited availability on the commercial market [24]. Therefore, there is still a need to search for and characterize new potential microorganisms capable of biologically controlling these toxigenic agents in food under different environmental conditions. In this context, the present study aimed to determine the efficiency of bacteria and filamentous fungi recovered from *Z. mays* L. (maize) for the control of fumonisin-producing *F. verticillioides*.

2. MATERIALS AND METHODS

2.1. Isolation and Identification of Bacteria and Filamentous Fungi Associated with Maize Grains

A total of 42 maize samples (1 kg per sample) were purchased from 42 different retail markets in Lambayeque, Peru, to isolate bacteria and filamentous fungi with potential antagonistic activity, as well as *F. verticillioides*.

For the isolation of microorganisms with antagonistic potential, 10 g of maize from each sample were ground using a Nutrimixer-901 (Blackline, Peru) for 1 minute and then diluted (1:1,000) in 0.1% peptone water. Aliquots from the final dilution were plated onto potato dextrose agar (PDA) (Merck, Germany) supplemented with 25 µg/ml fluconazole (anti-fungal) to isolate bacteria and chloramphenicol (anti-bacterial) (Fisher, United Kingdom) to isolate filamentous fungi [28], and incubated at 30°C for 2 and 7 days, respectively. Representative colonies of each microbial morphotype were selected and subjected to Gram staining (bacteria) or methylene blue staining (fungi) for presumptive identification. Pure bacterial cultures were preserved on tryptic soy agar and stored at 4°C [29].

For filamentous fungi, monospore cultures were obtained. Briefly, a fragment of mycelium was transferred to a tube containing distilled water with 0.1% Tween 80 and plated onto 2% water agar. Plates were incubated for 12 hours, after which three germinated conidia were selected using a stereomicroscope and transferred onto PDA. Cultures were incubated at 30°C for 5 days and stored at 4°C [30,31]. Fungal genera were identified from these monospore cultures grown on PDA. *Fusarium* species were identified using the keys of Ramírez-Camejo *et al.* [10] and Leslie and Summerell [32]; *Aspergillus* using the keys of Cruz [33] and Piontelli [34]; *Penicillium* using Frisvad and Samson [35]; and *Trichoderma* using Pitt and Hocking [36].

2.2. Identification and Selection of Fumonisin-Producing *F. verticillioides* in Maize

Morphological identification of *F. verticillioides* grown on PDA was performed by observing reproductive structures, including the shape and chain arrangement of microconidia, the presence of scarce macroconidia, and the absence of chlamydospores [10].

Detection and quantification of fumonisins were performed using a one-step lateral flow immunochromatographic method based on a competitive immunoassay (Symmetric Fumonisin Green, Prognosis Biotech, Larissa, Greece) for the determination of toxins in cereals, grains, and feed. The method had a limit of detection (LOD) of 0.1 ppm, a limit of quantification (LOQ) of 0.15 ppm, and a quantification range of 0.15–4 ppm.

For this purpose, 10 g of maize grains were placed in Erlenmeyer flasks, disinfected with 1% sodium hypochlorite (v/v), rinsed with distilled water, and sterilized by autoclaving (121°C) for 30 minutes [37]. Subsequently, a 10-mm PDA plug from a 7-day culture of *F. verticillioides* was inoculated into each flask. After 25 days of incubation, maize grains colonized by the fungus were sterilized by autoclaving (121°C) for 30 minutes, ground, mixed with the contents of a major aqueous extraction packet, and processed according to the manufacturer's instructions.

The concentration of total fumonisins was measured using the Raptor cartridge (Lateral Flow Reader, Model 3PR MINI) with the S-Flow v.2.0.3.111 software (Prognosis Biotech, Larissa, Greece). Samples with values exceeding the quantification range were diluted (1:5 and 1:10) using the major extraction kit, and the obtained values were multiplied by the dilution factor to determine fumonisin concentration (ppm) [38].

2.3. Comparison of the Efficiency of Antagonistic Microorganisms Against Toxigenic *F. verticillioides*

The antagonistic potential of the microorganisms against the selected toxigenic *F. verticillioides* strain was evaluated using the dual culture method on PDA [39,40], with assays performed in triplicate.

For filamentous fungi, a 6-mm PDA plug from a 7-day culture was placed on one-third of the PDA plate. After 24 hours of incubation, a 6-mm PDA plug containing a 7-day culture of *F. verticillioides* was placed at the opposite edge of the plate. For bacteria, colonies were streaked on one-third of the PDA plates, and after 24 hours of incubation, a 6-mm PDA plug containing a 7-day culture of *F. verticillioides* was placed at the opposite edge of the plate. Control plates consisted of PDA inoculated only with *F. verticillioides*. Plates were incubated at 30°C for 10 days. The two perpendicular radii of fungal colonies (R1, R2) were measured to calculate colony area and inhibition area [30,41].

The three filamentous fungi and three bacteria showing the largest inhibition areas were selected for subsequent assays. Selected bacterial isolates were identified phenotypically according to Bergey's Manual of Determinative Bacteriology [42], using biochemical and physiological tests described by Amoah *et al.* [43].

The efficiency of antagonistic microorganisms in controlling toxigenic *F. verticillioides* was evaluated based on inhibition of fungal colony growth and reduction of total fumonisin production. Two experiments (1 and 2) were performed under a completely randomized design using the three selected bacterial and three filamentous fungal isolates. Seven treatments were evaluated: T1

(toxigenic fungus control), T2–T4 (toxigenic fungus + antagonistic bacteria 1, 2, and 3), and T5–T7 (toxigenic fungus + antagonistic filamentous fungi 1, 2, and 3).

2.3.1. Reduction of fungal colony area

In experiment 1, antagonism was assessed using the dual culture method on PDA as described in section "Comparison of the Efficiency of Antagonistic Microorganisms Against Toxigenic *F. verticillioides*" [39,40]. After 10 days of incubation at 30°C, the colony area of the toxigenic fungus and the inhibition area were calculated. Microscopic observations (OLYMPUS C534, 400×) of hyphae and reproductive structures were also performed for both the experimental and control treatments.

2.3.2. Reduction of total fumonisins

In experiment 2, total fumonisin production was determined by co-culturing bacteria or filamentous fungi with toxigenic *F. verticillioides*. Ten grams of previously selected maize grains (without holes or spots) were placed in Erlenmeyer flasks and disinfected and sterilized as described in section "Identification and Selection of Fumonisin-Producing *F. verticillioides* in Maize".

The inoculum (20%) consisted of a suspension of cells in 0.1% peptone water obtained from bacteria and fungi grown on PDA for 24 and 96 hours, respectively. Bacterial inoculum concentration was standardized to 10^8 CFU/ml [44], while filamentous fungal suspensions were prepared in peptone water supplemented with Tween 80 (0.0001%) and adjusted to 1–5 × 10^7 conidia/ml using a Neubauer chamber [45]. After 24 hours of incubation at 30°C, a 10-mm PDA plug containing a 7-day culture of toxigenic *F. verticillioides* was added to each flask and incubated at 30°C for 4 days with 70% relative humidity (BOECO SH-110).

Following incubation, maize grains were sterilized in an autoclave for 20 minutes, ground, and 5 g were weighed and mixed with 25 ml extraction solvent (70% methanol at a 1:5 ratio). Total fumonisins were determined using the ELISA method proposed by the manufacturer (Symmetric Total Fumonisin, Prognosis Biotech). Absorbance was measured at 450 nm using an ELISA reader [41] and analyzed with Prognosis Data-Reader v.7.6 software (Prognosis Biotech, Larissa, Greece). Manufacturer-provided standards of 0, 0.2, 0.8, 2.4, and 5 mg/kg were used. The method had a LOD of 0.15 mg/kg, a LOQ of 0.20 mg/kg, and a quantification range of 0.2–5 mg/kg.

Samples exceeding the quantification range were diluted (1:5 and 1:10) with 70% methanol, and results were multiplied by the dilution factor to determine fumonisin concentrations (µg/kg) [38,45]. The percentage reduction of total fumonisins was calculated relative to the toxigenic fungus control without antagonistic microorganisms.

2.4. Identification of Potential Microbes Biocontrolling Toxigenic *F. verticillioides*

The microorganism showing the highest efficiency in controlling toxigenic *F. verticillioides* was subjected to molecular identification. Fungal DNA was extracted using the method described by Cenis [46]. The internal transcribed spacer (ITS) region was amplified by PCR using the universal primers ITS1F and ITS4.

PCR products were sequenced using the Sanger method at the "Marcel Gutiérrez Correa" Microbiology and Biotechnology Laboratory of the Universidad Nacional Agraria La Molina. The assembled sequence was preliminarily identified using BLASTN and BOLD [47], and subsequently aligned with related ITS sequences obtained from the NCBI database [48]. Phylogenetic analysis was performed using MEGA11 [49] with the Neighbor-Joining method and 1,000 bootstrap replicates.

2.5. Statistical Analysis

The percentages of inhibition of *F. verticillioides* colony growth and reduction of total fumonisin production in co-culture with the investigated bacteria and filamentous fungi were evaluated for normality and homoscedasticity. Parametric data were analyzed using ANOVA followed by Tukey's multiple comparison test, whereas non-parametric data were analyzed using the Kruskal–Wallis test followed by Dunn's post hoc test. Statistical analyses were performed using SPSS version 26.00 with a significance level of $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Bacteria and Filamentous Fungi Associated with Maize Grains

To evaluate the biocontrol potential of the native isolates, a sequential screening strategy was employed. Microorganisms were first isolated and presumptively identified based on morphological characteristics. Following the evaluation of their antagonistic efficiency *in vitro* and *in vivo*, only the most effective strain was selected for subsequent molecular identification.

From the total number of processed samples, 54 bacterial isolates were obtained and grouped into ten morphotypes, with morphotype 3 being the most frequent. Gram-negative bacteria (n = 33, 61.1%) predominated in maize grains (Table 1).

On the other hand, 103 filamentous fungi were isolated, which were grouped into nine morphotypes (Fig. 1, Table 2). Morphotypes 1, 2, 3, and 4 were identified as belonging to the genus *Fusarium*, which was the most frequent group (n = 57; 55.3%). Morphotypes 5, 6, and 7 were identified as *Aspergillus*, morphotype 8 as *Penicillium*, and morphotype 9 as *Trichoderma*.

All these genera have been previously reported in maize grains [10,50,51]. In addition, some species within these genera are known to produce mycotoxins in food, representing a serious health risk for consumers [50]. The genus *Fusarium* is particularly noteworthy because it is dominant among fungi associated with cereal grains, causing diseases such as seedling blight and stem and root rot, which have a significant economic impact on cereal production [10].

3.2. *Fusarium verticillioides* Producing Total Fumonisins in Maize

Filamentous fungal morphotypes 1 and 2 were identified as *F. verticillioides*. Together, these two morphotypes accounted for 78.9% (n = 45) of the total *Fusarium* isolates (n = 57) obtained (Fig. 2). These isolates were recovered from 85.7% (n = 36) of the maize samples analyzed. At the microscopic level, long chains of microconidia, monophialides, and the absence of chlamydospores were observed, which are characteristic morphological features of *F. verticillioides*.

A total of 32 recovered *F. verticillioides* (71.1%) isolates were toxigenic, producing fumonisin concentrations ranging from 30 to 4,010 ppb in maize grains cultured with the fungus for 25 days. The highest fumonisin concentration (4,010 ppb) was recorded for *F. verticillioides* strain H08.

Table 1. Morphotypes of bacterial colonies isolated from maize grains.

Morphotype	Colony					Cell			Number of isolates
	Size	Shape	Margin	Elevation	Appearance	Color	Shape	Gram staining	
M1	Small	Circular	Entire	Convex	Watery	Yellow	Coccus	(-)	14
M2	Small	Circular	Entire	Raised	Watery	Cream	Coccus	(-)	6
M3	Small	Circular	Entire	Raised	Watery	Cream	Bacillus	(+)	15
M4	Large	Circular	Entire	Raised	Shiny	Cream	Coccus	(-)	5
M5	Small	Circular	Entire	Raised	Granular	Cream	Coccus	(-)	2
M6	Small	Circular	Entire	Raised	Granular	Yellow	Coccus	(-)	4
M7	Punctiform	Circular	Entire	Raised	Watery	Cream	Coccobacillus	(+)	1
M8	Small	Circular	Entire	Raised	Watery	Cream	Coccobacillus	(-)	2
M9	Small	Circular	Entire	Convex	Watery	Red	Coccus	(+)	1
M10	Small	Circular	Entire	Convex	Watery	Cream	Coccus	(+)	4

(-) Gram negative; (+) Gram positive.

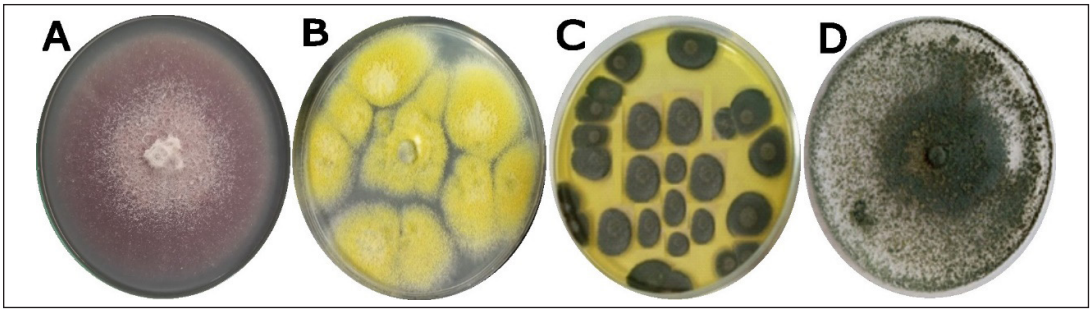

Figure 1. Plate growth of filamentous fungal colonies recovered from maize grains in this study. A) *Fusarium*, B) *Aspergillus*, C) *Penicillium*, and D) *Trichoderma*.

Table 2. Morphotypes of filamentous fungi recovered from maize grains in this study.

Morphotypes	Colony						Genus
	Shape	Obverse color	Reverse color	Elevation	Texture	Margin	
M1	Circular	Red	Red	Flat	Floccose	Entire	*Fusarium*
M2	Circular	Pink	Pink	Flat	Floccose	Entire	*Fusarium*
M3	Circular	Purple	Purple	Flat	Floccose	Entire	*Fusarium*
M4	Circular	White	White	Flat	Floccose	Entire	*Fusarium*
M5	Irregular	Yellow	Yellow	Flat	Crenate	Crenate	*Aspergillus*
M6	Irregular	Green	Green	Convex	Filamentous	Crenate	*Aspergillus*
M7	Circular	Bluish-green	Green	Convex with papillate surface	Filamentous	Crenate	*Aspergillus*
M8	Irregular	Green	Green	Convex with papillate surface	Cottony	Entire	*Penicillium*
M9	Circular	Green	Green	Flat	Filamentous	Entire	*Trichoderma*

The strain *F. verticillioides* H08 demonstrated toxigenic capacity in artificially inoculated maize grains, consistent with previous reports by Singh *et al.* [52], Rosa Junior *et al.* [53], and Muga *et al.* [54]. The concentration of total fumonisins (>4,000 ppb) produced by *F. verticillioides* H08 was higher than the 700 ppb reported by Zyl *et al.* [55] and the 2,447 ppb reported by Muga *et al.* [54]. However, even higher fumonisin concentrations have been reported in the literature, reaching up to 69,840 ppb [56] and 86,700 ppb [53]. It should be noted that not all *F. verticillioides* strains produce detectable levels of toxins. Non-toxigenic isolates have also been reported, as described by Bennett *et al.* [57] and Beccari *et al.* [56].

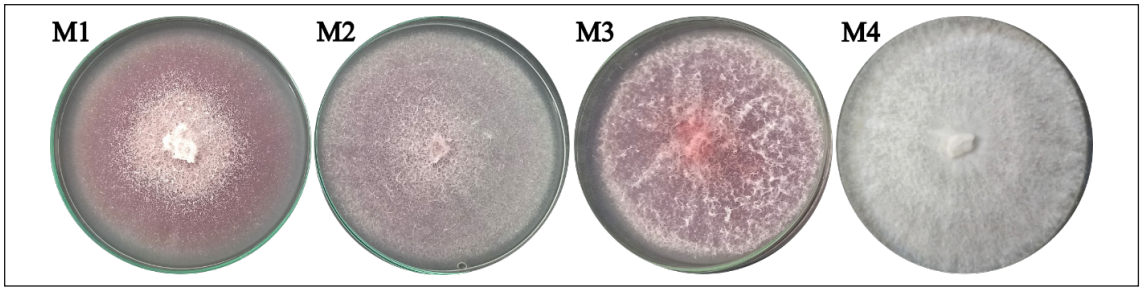

Figure 2. Morphotypes of *Fusarium* colonies grown on PDA. M1, M2, M3, and M4, respectively.

3.3. Comparative Efficiency of Antagonistic Microorganisms in the Control of *F. verticillioides*

3.3.1. Screening of antagonistic microorganisms and reduction of fungal growth

A total of 90.7% (n = 49) of the bacteria associated with maize grains exhibited antagonistic activity against the toxigenic *F. verticillioides* H08 strain, showing a reduction in fungal colony growth after 5 and 10 days of incubation. The bacterial isolates that showed the highest inhibition levels (77.85% for strain 10E, 71.97% for strain 42C, and 50.17% for strain 81D) were selected for further study and were identified as *Bacillus* sp. strain 10E, *Bacillus* sp. strain 42C, and *Pseudomonas* sp. strain 81D.

The percentage of antagonistic bacterial isolates (90.7%) obtained in this study was higher than the percentages reported in previous studies, such as 22.2% of endophytic bacteria isolated from *Euphorbia antiquorum* [58], 60% in isolates of *Lactococcus lactis subsp. lactis* [17], 61.6% in *Streptomyces* spp. [25], and 68.6% in *Bacillus amyloliquefaciens* [58], all evaluated for antagonistic activity against *Fusarium* species.

Antagonistic bacteria have also been reported to inhibit conidial germination, reduce fumonisin production [59], and promote detoxification of FB_1 [17]. These bacteria may produce chitinases [59], antibiotics, siderophores [25], lipases, and chitinases [60]. Additionally, the culture supernatant of *L. lactis subsp. lactis* has been shown to cause dysregulation of genes involved in the synthesis and virulence of FB_1, fusaric acid, and chitin produced by *F. verticillioides* [17].

The inhibition range of *F. verticillioides* colony growth by the bacterial isolates evaluated in this study (6.93%–77.85%) was higher than the 5.04%–40.20% reported by Jahuddin *et al.* [61] and the 59%–62% reported by Castro *et al.* [62]. However, this range was lower than the 45%–85% reported by Figueroa *et al.* [63] and the 56.6%–76.9% reported by Nguyen *et al.* [64].

On the other hand, 42.7% (n = 44) of the filamentous fungi (n = 103) associated with maize grains exhibited antagonistic activity against the toxigenic *F. verticillioides* H08 strain. The growth of the fungal colony was inhibited by 10%–30% by species of *Aspergillus* and *Fusarium*, 31%–51% by other species of *Fusarium* and *Aspergillus* (Fig. 3). The antagonistic fungi showing the largest inhibition areas against the toxigenic fungus were *Trichoderma* sp. strain 3 (89%), *Trichoderma* sp. strain 17 (88%), and *Trichoderma* sp. strain 14 (86%), which were therefore selected for further analyses.

The antagonistic activity of filamentous fungi against *F. verticillioides* was evidenced by the reduction in colony growth, consistent with previous reports by Mirsam *et al.* [65] and Ferrigo *et al*. [66]. Alterations in the morphology of the pathogen's hyphae were also observed, which may be associated with enzymatic lysis of the cell wall mediated by β-(1,3)-glucanases, chitinases, and cellulases [67].

The mechanisms by which filamentous fungi exhibit antagonism include competition for space and nutrients [66], mycoparasitism associated with enzymatic activity [68], modulation of genes related to induced systemic resistance and systemic acquired resistance [66], biotransformation of mycotoxins [69], as well as the production of bioactive compounds [70] and volatile compounds [71]. The inhibition area of *F. verticillioides* H08 colony growth reached 89%. This value was higher than the 57.4% inhibition previously reported for *F. verticillioides* by *Trichoderma harzianum* [66].

The specific reduction of the fungal colony area was subsequently evaluated using only the selected microorganisms: *Bacillus* sp. strain 10E, *Bacillus* sp. strain 42C, *Pseudomonas* sp. strain 81D, *Trichoderma* sp. strain 3, *Trichoderma* sp. strain 17, and *Trichoderma* sp. strain 14. The inhibition of colony growth of the toxigenic fungus ranged from 43.39% to 48.26% when treated with bacteria and from 80.96% to 84.94% when treated with filamentous fungi. Regarding colony morphology, changes in color, texture, and colony margin were observed in the toxigenic fungus, particularly due to the effect of the filamentous fungus *Trichoderma* spp., whereas no such changes were observed with antagonistic bacteria. The inhibition of *F. verticillioides* colony growth by the microorganisms selected in the first assay ranged from 50.17% to 77.85% for the bacterial isolates and 86%–89% for the fungal isolates. However, in the second assay, conducted under similar conditions, the inhibition ranges decreased to 43.39%–48.26% and 80.96%–84.94%, respectively. This more pronounced reduction in bacterial antagonistic activity may be attributed to the effects of in vitro subculturing on the antagonists, consistent with the findings of Clough et al. [72] and Valeros et al. [73]. In an initial assay, five strains of the phytopathogen Ralstonia solanacearum were inhibited by the cell-free supernatants of fluorescent *Pseudomonas* spp.; however, in subsequent assays, the inhibitory effect declined. This reduction was attributed to the development of increased tolerance in the phytopathogen, resulting from reduced susceptibility to the lipopeptide antimicrobials secreted by the antagonists [72]. Likewise, after eight in vitro subcultures, the entomopathogenic fungus *Metarhizium rileyi* exhibited changes in colony morphology, reduced germination and sporulation rates, and decreased virulence against Spodoptera frugiperda larvae [73].

Macroscopic alterations in the colony of the toxigenic *F. verticillioides* H08 caused by antagonism from *Trichoderma* spp. strains 3, 17, and 14 included a shift of the mycelium to a white coloration and the development of an irregular colony margin, in contrast to the red coloration and regular margin observed in the control. Microscopically, plates showing antagonistic activity exhibited deformed, collapsed, and coiled hyphae, with few conidia and the absence of conidial chains, unlike the control plates.

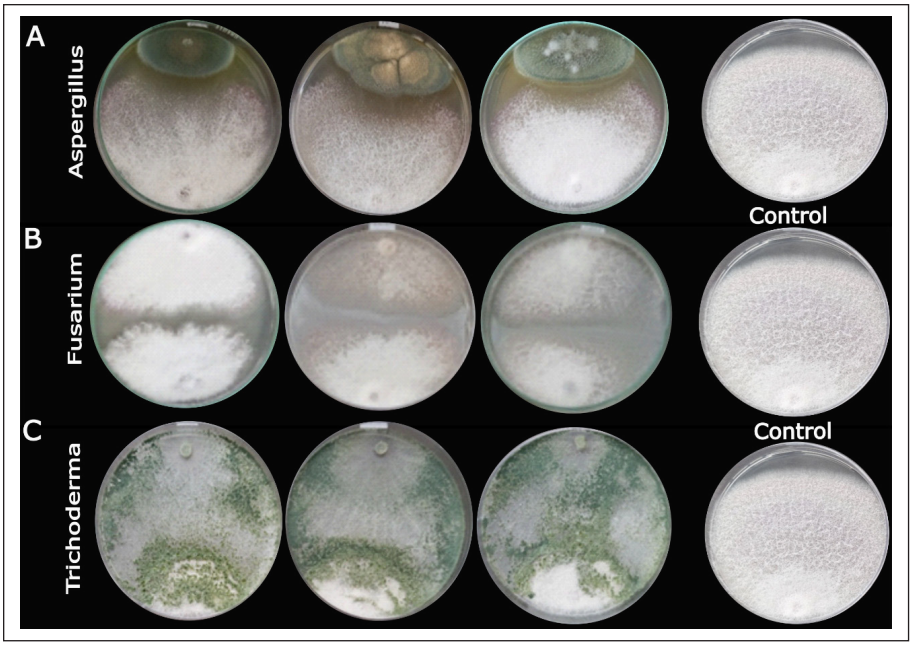

Figure 3. Macroscopic observation of dual cultures of fungi from the genera *Aspergillus* (A), *Fusarium* (B), and *Trichoderma* (C) exhibiting antagonistic activity against the toxigenic strain *F. verticillioides* H08.

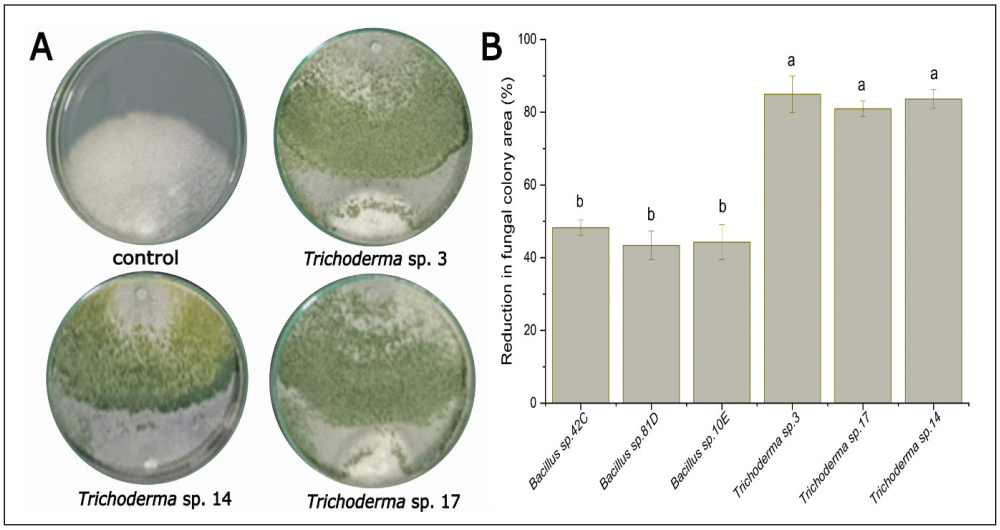

Figure 4. A) Macroscopic observation of the dual culture of antagonistic *Trichoderma* spp. strains against the toxigenic *F. verticillioides* H08. B) Reduction in the colony area of toxigenic *F. verticillioides* H08 caused by antagonistic bacteria and filamentous fungi. Different letters indicate significant differences among treatments ($p < 0.05$).

The Kruskal–Wallis test indicated significant differences in the percentage of inhibition of *F. verticillioides* H08 colony growth among the seven treatments. The Dunn's test showed that the highest inhibition value was obtained with *Trichoderma* sp. strain 3, with no significant differences compared to *Trichoderma* sp. strains 14 and 17, but with significant differences compared to the three bacterial strains evaluated in this study (Fig. 4).

3.3.2. Reduction of total fumonisins

After 4 days of co-culture, the mycelial growth of toxigenic *F. verticillioides* H08 was limited in the presence of antagonistic bacteria and filamentous fungi. Maize grains from the treatments showed a green coloration due to the growth and sporulation of the antagonistic fungi, whereas in the control treatment, the maize grains were colonized by the characteristic white filamentous mycelium of *F. verticillioides*.

The concentration of total fumonisins produced by the toxigenic fungus ranged from 713.33-821.67 ppb in co-cultures with antagonistic bacteria, while it ranged from 9.68 to 576.67 ppb in co-cultures with antagonistic filamentous fungi. The highest percentage reduction of the toxin (99.77%) was observed with *Trichoderma* sp. strain 3.

ANOVA revealed significant differences in the percentage reduction of fumonisin produced by toxigenic *F. verticillioides* H08 among the

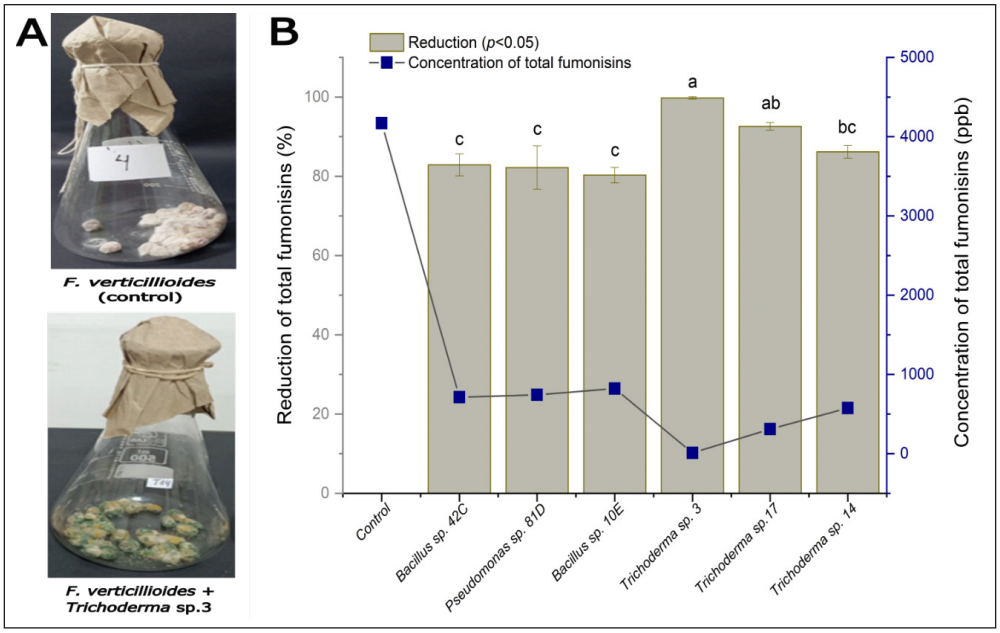

Figure 5. A) Observation of the mycelial growth of toxigenic *F. verticillioides* H08 in co-culture with *Trichoderma* sp. strain 3 on maize grains. B) Reduction and concentration of total fumonisins produced by toxigenic *F. verticillioides* H08 in the presence of antagonistic bacteria and filamentous fungi. Different letters indicate significant differences among treatments ($p < 0.05$).

seven treatments. Tukey's test indicated that the greatest reduction in fumonisin concentration was achieved with *Trichoderma* sp. strain 3, showing no significant differences compared with *Trichoderma* sp. strain 17, but significant differences compared with the other treatments (Fig. 5).

Trichoderma sp. strain 3 showed the highest efficiency in controlling *F. verticillioides*, reducing colony growth by 84.94% and the concentration of total fumonisins produced by 99.77%. The antagonistic bacteria and fungi evaluated in this study reduced both colony growth and total fumonisin production by *F. verticillioides*, as also demonstrated by Bennett *et al*. [57] and Strub *et al*. [25]. Similarly, Zanon *et al.* [20] showed that *Bacillus velezensis* reduced the growth of *F. graminearum* by 45.3% and decreased the production of the mycotoxin deoxynivalenol by 81.8%, whereas *Lactobacillus plantarum* reduced the growth of *F. graminearum* by 32% but did not reduce toxin production.

Fungi of the genus *Trichoderma* showed the highest percentage of inhibition of *F. verticillioides* colony growth. This result is consistent with several previous studies demonstrating the antagonistic capacity of different species within this genus, including *T. harzianum, Trichoderma asperellum, Trichoderma erinaceum* [74], and *Trichoderma viride* [67]. In addition, adhesion, coiling, penetration, and lysis of *F. verticillioides* hyphae by *T. harzianum* have been reported [67].

The antagonistic activity of *Trichoderma* spp. varies depending on the species, as reported by Jambhulkar *et al.* [74], who observed inhibition of *F. verticillioides* growth of up to 80% with *Trichoderma brevicompactum* and *Trichoderma atroviride*, 85% with *T. harzianum* and *Trichoderma ghanense*, and 75% with *T. asperellum*. Variability has even been observed among strains of the same species, as described for *T. harzianum*, which reached inhibition percentages of 80% with strain 4, 85% with strain 6, and 87% with strain 29.

Trichoderma spp. have also demonstrated plant growth-promoting properties and the ability to control root rot under field conditions [74]. Likewise, *T. harzianum* reduced the incidence of maize ear rot by 30.7%–37.1% and disease severity by 32.6%–43.5%, in addition to markedly decreasing contamination with deoxynivalenol and eliminating fumonisin in maize grains [66].

3.4. Molecular Identification of the Microorganism with the Highest Efficiency in the Control of Toxigenic *F. verticillioides*

The filamentous fungus *Trichoderma* sp. strain 3 demonstrated the highest efficiency in controlling the toxigenic strain of *F. verticillioides*. Therefore, a molecular analysis was performed to identify this strain at the species level. Comparison of its ITS sequence (GenBank accession number: PX974310.1) using BLASTN searches and the BOLD database, followed by phylogenetic inference with other filamentous fungi, indicated that *Trichoderma* sp. strain 3 corresponds to an isolate of *T. atroviride,* although it shows close phylogenetic affinity with *T. koningii* (Fig. 6).

Although the initial identification of the strain was performed by sequencing the ITS region, several studies have demonstrated that this region has limited discriminatory power for closely related species within the genus *Trichoderma*, particularly between *T. koningii* and *T. viride* [75,76]. In such cases, analyses based solely on ITS sequences may yield high similarity percentages with multiple species, making a conclusive taxonomic assignment difficult. For this reason, a more robust identification approach involves multilocus analyses incorporating markers with greater resolving power, such as the translation elongation factor 1-α gene, considered one of the most informative markers for *Trichoderma*, as well as RPB2 and calmodulin [77,78].

In the present study, the highest efficiency in controlling *F. verticillioides*, measured as inhibition of colony growth and reduction of fumonisin production, was achieved with *T. atroviride* strain 3. These findings

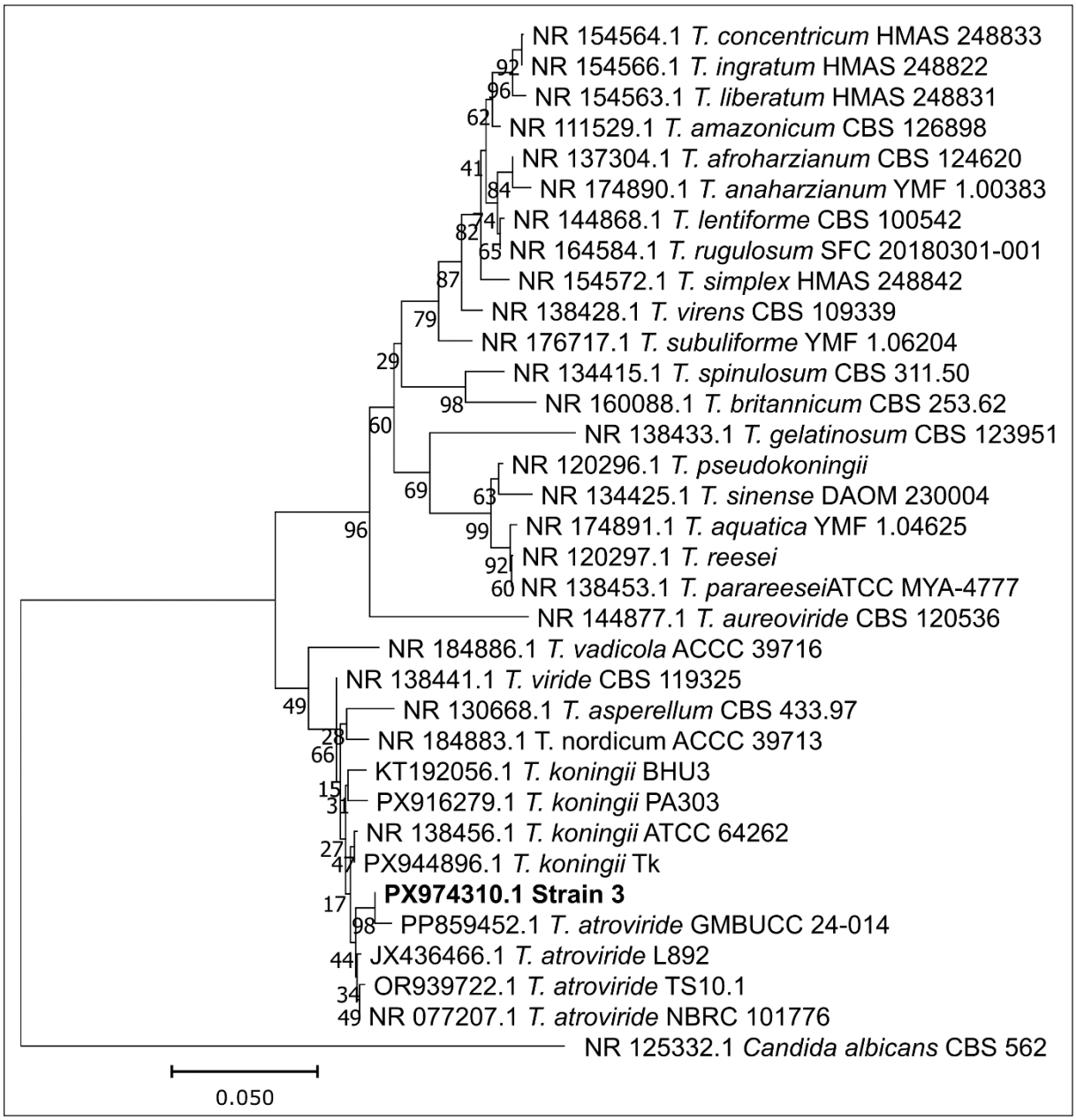

Figure 6. Phylogenetic tree inferred from the ITS region of multiple filamentous fungal species, including *Trichoderma* sp. strain 3, with bootstrap values shown at branch nodes and *Candida albicans* used as the outgroup.

are consistent with the results reported by Jambhulkar *et al.* [74] and Tian *et al*. [69]. The mechanisms by which *Trichoderma* spp. act as biological control agents against phytopathogens include the production of lytic enzymes (chitinases, glucanases, and proteases), the synthesis of bioactive secondary metabolites, and the ability to promote both systemic acquired resistance and induced systemic resistance in plants [68,79]. An important advantage of native fungi as biocontrol agents is their better adaptation to the environmental conditions in which they will be applied [71]. In this context, the *T. atroviride* strain recovered in this study shows potential for use as a biocontrol agent against toxigenic *F. verticillioides*.

Although several studies have reported the biological control of *F. verticillioides* [17,21], the present study differs from previously published work in three main aspects. First, it employs native bacteria and fungi isolated directly from local maize in Lambayeque, ensuring high ecological adaptability to the specific environmental conditions of the region. Second, rather than relying solely on *in vitro* radial growth assays, this study emphasizes a comparative *in vivo* approach using maize grains, simultaneously evaluating native bacteria and filamentous fungi under the same conditions. Third, while some previously reported antagonists inhibit fungal growth without significantly reducing toxin levels [27], or exhibit a reduction in toxin production that is reversible over time [25], our selected *T. atroviride* strain 3 demonstrated a highly efficient dual-action synergistic effect, reducing total fumonisin production (99.77%) alongside strong mycelial inhibition (84.94%). This dual efficacy highlights its superior practical potential for postharvest management compared to agents that target only the physical growth of the fungus.

4. CONCLUSION

The high proportion of bacteria and filamentous fungi isolated from maize samples showing antagonistic activity against the

toxigenic *F. verticillioides* H08 strain highlights the potential of native microorganisms as biocontrol agents. Among the isolates evaluated, *T. atroviride* strain 3 stood out for its high efficacy, achieving a significant reduction in both mycelial growth (84.94%) and total fumonisin production (99.77%). These results suggest that this strain not only limits the development of the pathogen but also interferes with its toxigenic capacity, representing a key advantage for integrated management strategies. In this context, the use of *T. atroviride* strain 3 as a biocontrol agent could represent a sustainable alternative for reducing total fumonisin contamination in maize, thereby contributing to food safety and reducing reliance on synthetic fungicides.

5. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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7. CONFLICT OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

8. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

9. DATA AVAILABILITY

All the data are available from the authors and shall be provided upon request.

10. PUBLISHER'S NOTE

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11. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declare that they have not used artificial intelligence (AI) tools for writing and editing of the manuscript, and no images were manipulated using AI.

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