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Morphological and molecular characterization of *Trichoderma* strains isolated from the BioHumisol® biofertilizer

Karla Marina Báez-Parra[1], Edith Salazar-Villa[2], Josefina León-Félix[3], Miguel Ángel Angulo-Escalante[3], Indira Rojo-Báez[2]*

[1]Facultad de Ciencias Químico-Biológicas, [2]Facultad de Biología, Universidad Autónoma de Sinaloa, Calzada de las Américas, Blvd. Universitarios, Cd. Universitaria, C.P. 80040, Culiacán, Sinaloa, México; [3]Centro de Investigación en Alimentación y Desarrollo, A. C. Coordinación Culiacán, Carretera Culiacán-El dorado Km 5.5, Campo El Diez, C.P. 80110, Culiacán, Sinaloa, México.

***Corresponding Author:**
Indira Rojo-Báez
indira@uas.edu.mx

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ABSTRACT

Background/Objetive. Biofertilizers exist that contain microorganisms beneficial to plants and the environment. However, some commercial products provide little information about the species they use. Therefore, this study aimed to identify, culturally, morphologically, morphometrically, and molecularly, the *Trichoderma* species present in the BioHumisol® fertilizer used in agriculture in Culiacán and Navolato, Sinaloa, México.

Experimental development. Fungal colonies were isolated and purified from the BioHumisol® liquid biofertilizer. For cultural, morphological, and morphometric identification, three Petri dishes were used, each containing one fungal isolate. The following characteristics were recorded: color, mycelium shape and arrangement in the culture medium, days of sporulation, and the size and shape of conidia, phialides, and chlamydospores. The mycelial growth rate was also recorded. At the molecular level, the ITS region and the TEF-1 α gene were amplified. The sequences were registered in GenBank with the accession numbers: PP956784, PP956785 for ITS; PP968400, PP968399 for TEF-1 α. Phylogenetic analysis of the concatenated sequences was performed using the maximum likelihood and Bayesian inference method with a bootstrap of 1000 replicates.

Results. The fungal colonies presented hyaline, branched, non-whorled conidiophores; phialides either single or in groups with terminal branches worth average measurements of 4.1-6.2 × 9.7-15.3 µm; unicellular ovoid conidia with average dimensions of 3.6-5.09 × 3.27-6.41 µm and subglobose intercalary and terminal chlamydospores measuring 21.41 × 21.72 µm consistent with *Trichoderma*. Currently accurate identification requires cultural, morphological and morphometric characterization, complemented by molecular characterization. BLAST analysis of partial ITS sequences showed 99% homology, and the TEF -1 α gene showed 100% homology with *T. asperellum*. Phylogenetic analyses confirm genetic proximity to species of the Viride clade.

Conclusion. Based on the cultural, morphological, morphometric and molecular characterization, the species found in the liquid biofertilizer BioHumisol® is *Trichoderma asperellum*.

Keywords: *Trichoderma asperellum*, ITS, TEF -1 α, Biocontrol

INTRODUCTION

Agriculture in Mexico relies on fertilizers that supply nutrients to crops to increase both yield and product quality. This enables producers to achieve higher productivity and, consequently, greater economic income (SAGARPA, 2025). In Sinaloa, chemical fertilizers are used in approximately 98% of agricultural production. However, beyond the economic cost of their acquisition, the excessive use of chemicals has ecological and environmental repercussions (Debnath *et al.*, 2020).

Biofertilizers contain microorganisms capable of synthesizing chemical compounds that promote plant growth, help fix atmospheric nitrogen, and solubilize or mobilize soil nutrients; an example is the fungus *Trichoderma* (Shahwar *et al.*, 2023). The benefits of using *Trichoderma* spp. include promoting plant growth and development through growth factors, lowering soil pH, solubilizing components of plant metabolism, and reducing soil pollution through bioremediation (Rodríguez-García and Vargas-Rojas, 2022). Various mechanisms have been proposed to explain how *Trichoderma* species promote plant growth. Among these are the biosynthesis of secondary metabolites, which promotes the production of enzymes such as xylanase, cellulase, and glucanase; the solubilization of phosphorus; the synthesis of indole-3-acetic acid; and the stimulation of root and stem growth, as well as the enhancement of carbohydrate metabolism, photosynthesis, and plant defense mechanisms (Contreras-Cornejo *et al.*, 2024).

In addition, *Trichoderma* functions as a biological control agent by combating phytopathogenic bacteria, fungi, and nematodes through various mechanisms, including competition for substrate and space, antibiosis, antagonism, mycoparasitism, recognition, chemotrophic growth, lytic activity, and the promotion of induced systemic resistance in plants (Yao *et al.*, 2023; Sánchez-Rivera *et al.*, 2025). In Mexico, *T. harzianum*, *T. viride*, *T. koningii*, *T. asperellum*, *T. longibrachiatum*, *T. virens*, *T. lignorum*, and *T. aureoviridae* are reproduced and commercialized as biological control agents (Allende-Molar *et al.*, 2022; SENASICA, 2025). Certified *Trichoderma* species used for biotechnological purposes in biofertilizer formulations include *T. harzianum*, *T. viride*, *T. asperellum*, *T. virens*, *T. atroviride*, *T. gamsii*, *T. hamatum*, and *T. polysporum*. Most biofertilizers are prepared using *T. viride*, *T. virens*, and predominantly *T. harzianum* (Hernández-Melchor *et al.*, 2019; Yao *et al.*, 2023).

The company Humisol Orgánico S.A. de C.V. produces and commercializes the liquid biofertilizer BioHumisol®, which, according to its label, contains anaerobic and nitrifying bacteria; however, the *Trichoderma* species present in the commercial product are unknown. The BioHumisol® biofertilizer is used in crops such as berries, asparagus, and vegetables. Since no certified strain is available and the *Trichoderma* species present in this biofertilizer are unknown, cultural, morphometric, and molecular characterization was necessary (BioHumisol, 2025). Therefore, the aim of this study was to identify, at the cultural, morphological, morphometric, and molecular levels, the *Trichoderma* species present in the commercial liquid biofertilizer BioHumisol®, used in agriculture in Culiacán and Navolato, Sinaloa, Mexico.

EXPERIMENTAL DEVELOPMENT

Sampling, isolation, purification, and preservation. Sampling was conducted at the company Humisol Orgánico S.A. de C.V., located in Cofradía de San Pedro, Navolato, Sinaloa, Mexico, where the liquid biofertilizer BioHumisol® (registration RSCO-

381/X/21) is produced and commercialized in the municipalities of Culiacán and Navolato, Sinaloa. For fungal isolation, a stock solution of the final product was prepared by placing 50 mL of the liquid biofertilizer into a glass flask containing 450 mL of sterile distilled water and shaking it for 30 s (stock solution). Subsequently, six serial dilutions (10^{-1} to 10^{-6}) were prepared by transferring 1 mL of the previous dilution into a glass tube containing 9 mL of sterile distilled water, followed by 30 s of vortex mixing (Cis-Lab, Mexico) to homogenize the solution (Suárez-Palacios *et al.*, 2023). From this suspension, serial dilutions were made up to 1×10^{-6}. Additionally, 100 µL of the conidial suspension were spread on Petri dishes containing potato dextrose agar (PDA, Bioxon®) supplemented with lactic acid (PDA-LA). The conidial suspension was homogenized using sterile 0.8 mm glass beads. Petri dishes were inoculated in triplicate for each dilution and incubated at (±) 28 °C for four days in an incubator (Ecoshel, USA) (Savín-Molina *et al.*, 2021). Purification was performed using the hyphal-tip technique (Cuervo-Parra *et al.*, 2024). Once pure hyphal-tip cultures were obtained, 5 mm mycelial disks were taken with a cork borer and preserved in 1.5 mL tubes containing sterile distilled water (Castellani method), then stored at 4 °C until further use (Fernández *et al.*, 2013).

Cultural, morphological, and morphometric identification. For cultural, morphological, and morphometric identification, three Petri dishes with fungal growth per isolate were used. The following characteristics were recorded: color, mycelial form and arrangement on the culture medium, sporulation time, and the size and shape of conidia, phialides, and chlamydospores (Ynfante-Martínez *et al.*, 2023). The structures were observed using a digital camera (Dino-Lite) attached to a compound microscope, CxL series (Labomed®) (Sánchez-Hernández *et al.*, 2018). Measurements of conidia, phialides, and chlamydospores (n = 50) were taken using the Dino Capture 2.0 software (Zainudin *et al.*, 2023). Cultural, morphological, and morphometric characteristics were compared with the taxonomic features reported by Barnett and Hunter (1998) and Samuels and Hebbar (2015). In addition, the mycelial growth rate of the strains was determined by continuous measurement of Petri dishes containing PDA-LA culture medium incubated at (±) 28 °C under 12 h light / 12 h dark conditions for four days. The mycelial growth rate was calculated by measuring the radial growth diameter in perpendicular directions with a vernier caliper every 24 h for four days. Growth rate was expressed in mm/day (Samaniego *et al.*, 2018).

Molecular identification. Three Petri dishes with contamination-free fungal growth per isolate were used. Mycelium was scraped with a sterile spatula from Petri dishes containing seven-day-old fungal cultures grown on PDA-LA medium. Subsequently, 150 mg of mycelium were transferred into sterile 1.5 mL tubes. Fungal DNA extraction was then performed following the manufacturer's instructions for the commercial ZR Fungal/Bacterial DNA Miniprep® Kit (Zymo Research, USA) (Manfredini *et al.*, 2025). To verify DNA concentration and purity, samples were analyzed using a spectrophotometer (QIAxpert, QUIAGEN®), and their integrity was confirmed by electrophoresis on a 1% agarose gel. DNA samples were stored in an ultrafreezer at −80 °C until further use (Matas-Baca *et al.*, 2022). The Internal Transcribed Spacer (ITS) region was amplified using the primers ITS4 (5′TCCTCCGCTTATTGATATGC3′) and ITS5 (5′GGAAGTAAAAGTCGTAACAAGG3′), under the following amplification conditions: initial denaturation at 94 °C for 5 min, followed by 29 cycles of denaturation at 94 °C for

30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 45 s, with a final extension at 72 °C for 5 min (Ríos *et al.*, 2016).

In addition, the Elongation Factor 1 alpha (TEF-1α) gene of isolates ATV and ATB was amplified using the primers EF1-728 (5′CATCGAGAAGTTCGAGAAGG3′) and TEF1R (5′GCCATCCTTGGGAGATACCAGC3′), under the following amplification conditions: initial denaturation at 94 °C for 2 min, followed by 29 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min, with a final extension at 72 °C for 10 min (Lee *et al.*, 2020). Amplification was performed in a Biorad C1000 thermocycler with a final volume of 25 µL (Samuels *et al.*, 2002; García-Nuñez *et al.*, 2017). The integrity of the PCR products was verified by electrophoresis on a 1% agarose gel stained with ethidium bromide, and the gel was visualized using a UV photodocumentation system (Axygen™, USA) (Lee *et al.*, 2020).

The PCR products obtained were purified and sequenced using the Sanger method and sent to the Advanced Genomics Unit of LANGEBIO, Cinvestav, Irapuato. The resulting sequences were analyzed with the BioEdit software (García-Nuñez *et al.*, 2017) and submitted to GenBank under the following accession numbers: PP956784, PP956785, PP968400, and PP968399. Subsequently, they were compared with other sequences available in the GenBank database of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/) using the BLAST (Basic Local Alignment Search Tool) algorithm (NCBI, 2024).

Phylogenetic analysis. Partial nucleotide sequences of the ITS region and the TEF-1α gene were aligned with reference sequences obtained from GenBank, and phylogenetic relationships were inferred based on multiple sequence alignment using the MAFFT program (Vences *et al.*, 2022). The sequences were concatenated with the Mesquite software, version 3.8. The phylogenetic tree was constructed using the maximum likelihood and Bayesian inference methods. To determine confidence values for the clades within the resulting tree, a bootstrap analysis with 1000 replicates was performed using the TPM2+F+G4 substitution model in IQTREE, and the tree was visualized in FigTree version 1.4.4 (Zhao *et al.*, 2024). The sequences used in the phylogenetic analysis are shown in Table 1. A sequence of *Fusarium oxysporum* (GenBank accession numbers: OR734798 and ON316841) was used as the outgroup.

Table 1. Species and GenBank accession numbers of the sequences used in the phylogenetic analysis

Specie	Strain number	GenBank number (ITS)	GenBank number (TEF 1-α)
T. atroviride	IMI206040	OR975627.1	MK644115.1
T. composticola	CBS133497	MT187974.1	DQ841716.1
T. asperellum [z]	**ATV**	**PP956784**	**PP968400**
T. asperellum [z]	**ATB**	**PP956785**	**PP968399**
T. asperellum	CBS43397	OR770586.1	OL825045.1
T. asperellum	CBS43397	MN727373.1	MW457023.1
T. flagellatum	CBS130626	MH865822.1	FJ763158.1
T. orientale	CBS130428	PP464121.1	OL757487.1
T. bissettii	CBS137447	MW295457.1	HG931271.1
T. virens	FT333	ON357718.1	OQ702629.1
T. crassum	CBS33693	PP860359.1	KJ871144.1
T. alni	CBS120633	MK459321.1	KJ665356.1
T. amazonicum	CBS126898	MH864268.1	HM142378.1

[z] Sequences from this study.

Cultural, morphological, and morphometric identification. This study provides the cultural, morphological, morphometric, and molecular identification of two isolates with characteristics like *Trichoderma*, designated ATV and ATB, obtained from the agricultural liquid biofertilizer BioHumisol®. After four days, the colonies consisted of white, powdery-textured mycelium that turned green with age; abundant sporulation and radial growth were observed. The average daily radial growth rate of the two isolates over four days was 26, 57, 84, and 90 mm. The fungal colonies exhibited morphological characteristics such as hyaline, branched, non-verticillate conidiophores; phialides simple or in terminally branched groups; turquoise, unicellular, ovoid conidia; and subglobose intercalary and terminal chlamydospores (Figure 1). Conidia (n = 50) measured 3.6–5.09 × 3.27–6.41 µm, phialides (n = 50) measured 4.1–6.2 × 9.7–15.3 µm, and chlamydospores measured 21.41 × 21.72 µm. Based on cultural, morphological, and morphometric characteristics, both isolates were tentatively identified as *Trichoderma* according to the taxonomic keys of Barnett and Hunter (1998) and Samuels and Hebbar (2015).

The size of the conidia coincides with that reported for *T. asperellum* (Calle-Cheje *et al.*, 2023); however, in this study, the conidia were larger than those described in other morphological characterizations of *T. asperellum* (Sebumpan *et al.*, 2022; Andrade-Hoyos *et al.*, 2023). *Trichoderma* is widely used as a biofertilizer and biopesticide in Mexican agriculture for various economically important crops due to its multiple mechanisms of action. *T. asperellum* is employed in bioformulations in Mexico and exhibits morpho-cultural variability (Andrade-Hoyos *et al.*, 2023). Furthermore, Foyate (2023) reported diversity in morphological traits such as pigmentation, and the size and shape of conidia and phialides. This morphological variability may be related to the type of formulation used in commercial products. On the other hand, morpho-cultural variability makes precise identification of *Trichoderma* sp. difficult, thus requiring the use of different taxonomic keys and molecular identification (Ynfante-Martínez *et al.*, 2023).

Molecular identification. The sequences of isolates ATV and ATB were deposited in GenBank (ITS: PP956784, PP956785; TEF 1-α: PP968400, PP968399). The BLAST analysis of the partial ITS sequences (549 bp and 588 bp) showed 99% similarity with *T. asperellum* isolates (KY750369, KY750373), while the partial TEF 1-α sequences (631 bp and 624 bp) showed 100% similarity with *T. asperellum* (JQ040494, KP747448). The phylogenetic tree obtained from the concatenated sequences of this study and other *Trichoderma* species from different clades indicate that the isolates obtained from the BioHumisol® biofertilizer belong to the *Viride* clade (Figure 2).

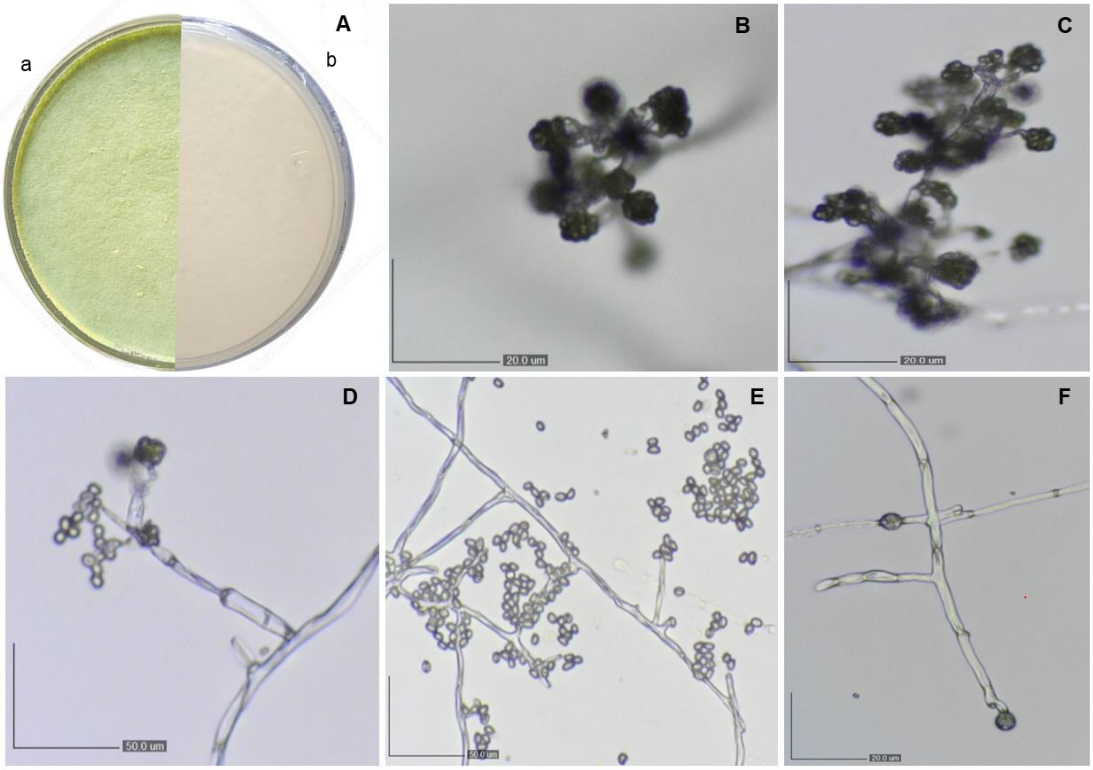

Figure 1. Morphology of *Trichoderma asperellum* isolates. **A)** Petri dish after seven days of growth on PDA-LA: a) front view, b) reverse view; **B–D)** conidiophores with phialides and clustered conidia; **E)** hyphae and conidia; **F)** chlamydospores.

Currently, accurate identification requires cultural, morphological, and morphometric characterization complemented by molecular characterization. Several studies have addressed the molecular characterization of *T. asperellum* using the ITS gene region (Ma *et al.*, 2020; Sehim *et al.*, 2023). In the present study, the percentage of homology with *T. asperellum* using the ITS region was 99%. Reports of molecular studies employing the ITS region have mentioned a 98% similarity (GenBank accession number: MN950427) for a native *Trichoderma asperellum* species isolated from soil in Guasave, Sinaloa, Mexico (Matas-Baca *et al.*, 2022). In Mexico, the molecular identification of *T. asperellum* is primarily based on the ITS region and the TEF-1α gene (Allende-Molar *et al.*, 2022). The universal marker for molecular identification in fungi is the ITS region (Maldonado-Bonilla *et al.*, 2024); however, it does not allow differentiation among closely related species and provides low resolution within the *Trichoderma* genus (Samuels *et al.*, 2002). Consequently, this genus is considered notoriously difficult to identify, and the use of more than one gene is recommended to increase specificity and provide greater robustness to molecular identification (Samuels and Hebbar, 2015).

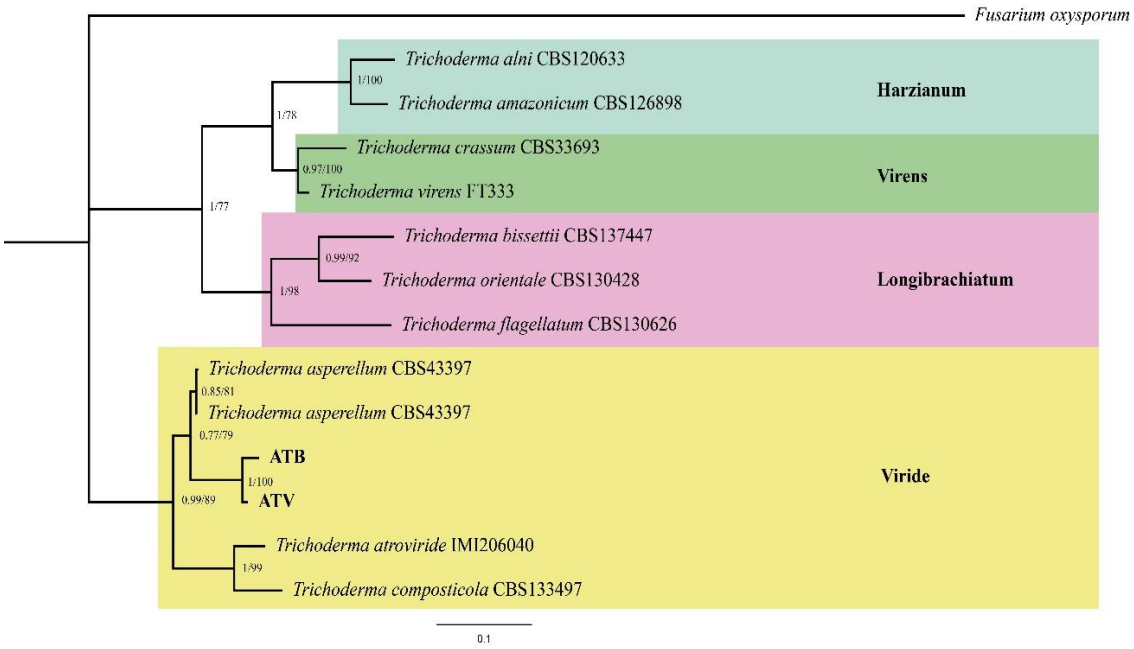

Figure 2. Phylogenetic tree based on concatenated partial ITS and TEF 1-α sequences of *Trichoderma* species. The tree was constructed using the maximum likelihood and Bayesian inference methods with 1000 replicates. Bayesian posterior probabilities (PP > 0.5) and bootstrap values (BS > 50) are shown at the nodes. The sequences from this study are shown in bold. *Fusarium oxysporum* (GenBank accession numbers: OR734798 and ON316841) was used as the outgroup.

A widely used gene for the molecular identification of *Trichoderma* is TEF-1α, as it allows for the assessment of the accuracy and robustness of genetic markers used in fungi, aiding in the identification of previously undescribed species. The TEF-1α gene has been reported to possess high phylogenetic efficacy due to its stronger association with the identification of unknown species and its alignment among *Trichoderma* species (Hewedy *et al.*, 2020). According to García-Nuñez *et al.* (2017), the identity percentage for the molecular identification of *T. asperellum* using the TEF-1α region was 99%, and they suggest that phylogenetic analysis is more accurate when both the ITS region and the TEF-1α gene are used. This analysis should be complemented with morphological characterization for reliable identification. Molecular identification of *Trichoderma* requires the use of one or more markers—ITS, TEF1, ACT, CAL, ACL1, CHI18-5, and RPB2—along with phylogenetic analysis. Based on this approach, the genus is grouped into 24 clades (Samuels and Hebbar, 2015), with the Harzianum, Viride, Virens, and Longibrachiatum clades being particularly notable (Rodríguez *et al.*, 2021).

The *Viride* clade encompasses the largest number of species (54), which are morphologically similar, including *T. asperellum*, *T. asperelloides*, and *T. viride* (Samuels and Hebbar, 2015; Allende-Molar *et al.*, 2022). The phylogenetic tree based on ITS and TEF-1α markers confirms that *T. asperellum* isolated from the Biohumisol® biofertilizer produced by Humisol Orgánico S.A. de C.V. belongs to the *Viride* clade—a species previously reported within this group (Samuels and Hebbar, 2015). The isolates obtained in this study are genetically related to other *T. asperellum* species when compared with other species grouped within the same clade (*T. atroviride* and *T. composticola*). According to Sánchez-Miranda *et al.* (2021), phylogenetic analysis allows the association of various native *Trichoderma* species, including *T. asperellum*, isolated from different crops and localities. The genetic proximity among *Trichoderma* sequences isolated from distinct

geographical regions may be due to the genetic regions used, which are highly conserved among species, resulting in high homology even in geographically distant isolates. Moreover, this genus is cosmopolitan and exhibits high ecological adaptability, which facilitates its global dispersal.

CONCLUSIONS

Cultural, morphological, and morphometric characterization (with average measurements of phialides: 4.1–6.2 × 9.7–15.3 µm; conidia: 3.6–5.09 × 3.27–6.41 µm; and chlamydospores: 21.41 × 21.72 µm), together with molecular characterization of *Trichoderma* strains isolated from the agricultural liquid biofertilizer Biohumisol® in Culiacán and Navolato, Sinaloa, Mexico, enabled the identification of *Trichoderma asperellum*. The biotechnological importance of this species supports the effectiveness of the Biohumisol® liquid biofertilizer in regional agriculture. Moreover, the integration of morphological and molecular methods ensures precise identification, which is essential for developing sustainable agricultural management strategies based on native microorganisms with high adaptive capacity.

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