



Isolation, Identification, and Preserval of Filamentous Fungi Found at Soils of Viotá, Cundinamarca

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Abstract

Objectives: This study focuses on the isolation, identification, and conservation of strains of filamentous fungi recovered from soils of Viotá, Cundinamarca; and the subsequent conformation of a strain collection. **Methods:** An adaptation of the sampling method proposed by Arias and Piñeros was carried out. Identification was carried out by macroscopic, microscopic, and DNA sequencing. Conservation techniques were evaluated based on the index of effectiveness of the behavior of viability, purity, and morphological stability. An ANOVA TUKEY test was performed using both an alpha value of $\alpha = 0.05$ with 95% confidence to assess significant differences. **Results:** Of the 125 samples analyzed, 165 isolates were recovered, and nine species were identified: *Trichoderma spirale* 15.53%; *Trichoderma ovalisporum* 13.66%; *Hypocrea nigricans* 13.04%; *Penicillium implicatum* 13.04%; *Penicillium citrinum* 11.80%; *Fusarium incarnatum* 11.18%; *Fusarium verticiloides* 9.94%; *Cunninghamella elegans* 6.83%, and *Aspergillus cf. versicolor* 4.97% that had the lowest number of isolates. *Aspergillus*, *Penicillium*, *Trichoderma*, *Fusarium*, and *Cunninghamella* are among the most common genera isolated soils. The most efficient conservation method was cryopreservation, with an efficiency rate of 100%. However, although the cultivation technique had an efficiency percentage of 70%, it is considered that it can be used in collections with limited financial support or as a backup preservation method. Ángel, in 2006 supports the use of serial cultivation for periods of time such as those handled in this study (15 days). **Application/Improvements:** The results obtained will contribute to the knowledge of the diversity of this group and its biotic potentials; for the generation of strategies that promote the development of this region.

Keywords: Conservation, Isolation, Identification, Filamentous Fungi, Soils.

1. Introduction

The Municipality of Viotá is located southwest of the Department of Cundinamarca, being part of the province of Tequendama to which it belongs in the regional context, along with nine other municipalities. Its good water supply due to the influx of rivers such as Calandaima and Lindo; and streams such as Modelia, La Neptuna, La Aguardienta; in addition to the quality of its soils, make the municipality a region with agricultural potential, with coffee being the main cultivated product, which places it as the main producer of the department. However, Viotá shows a current critical environmental situation in terms of water quality, basic sanitation services, environmental effects on the components of water, soil, air, and landscape [1], perhaps as a consequence of the armed conflict and the lack of management by the State. As a reference to the above, there are studies carried out by entities such as the Corporación Autónoma Regional de Cundinamarca (CAR) and Biology Research Group of La Universidad el Bosque (GRIB), related to the management of water resources in the Bogotá river basin [2] and on the evaluation of water quality in the Bogotá river sub-basin in the municipality of Viotá, Cundinamarca [3–4]; research showing relevant information related to the physical aspects, ecosystems, environmental sanitation and socioeconomic aspects relevant to understanding the complexity and needs of the area; It urges the continuation of studies that contribute to the knowledge of diversity and allow the identification of biotic potentials that can promote the development of the region.

The main objective of this work is to study the diversity of filamentous fungi present, in soils of the village of La Bella, Viotá-Cundinamarca, to determine their conservation techniques for the conformation of a collection that allows studies of their biotic potentials; for the generation of strategies that promote the development of this region.

Field studies and analyses were carried out for five sampling events. An adaptation was made to the method proposed by Arias and Piñeros of the diagonal route sampling system [5], to take five samples per point, for a quantity of 25 samples per sampling event. Strains were identified by macroscopic and microscopic observation, and amplification tests were performed in the ITS 1 and ITS 4 regions for subsequent sequencing of the DNA fragment to confirm microscopic identification. Finally, tests were carried out to evaluate two methods of preservation, cryopreservation, and serial periodic cultivation, considering the behavior of viability, purity, and morphological stability of the strains, for the conformation of the collection of strains of La Universidad El Bosque, in order to preserve the genera of filamentous fungi.

2. Materials and Methods

2.1. Study Area

The soil used to isolate filamentous fungi was collected in a mixed cultivation area of coffee, banana, avocado, and citrus, located in the village of La Bella, in the municipality of Viotá, in the lower basin of the Bogotá river, with coordinates N 4 ° 25,228'W 74 ° 28,146', in an area of 1 hectare.

2.2. Method of Sampling

Five samples were taken between the months of October 2016 and May 2017 in which the diagonal sampling pattern [5] was applied, consisting of a zig-zag displacement in 5 m² quadrants and obtaining 22.5 cm³ of corner soil and the center of the quadrant with a hole, as shown in Figure 1. The samples were transported in hermetic bags in a refrigerated container to the INBIBO Biology Research laboratory at Universidad El Bosque for analysis.

2.3. Laboratory Phase

In the laboratory, the samples were cleaned by extracting the waste material and then sieving (2 mm pore) to determine pH, moisture percentage, and isolation in selective agars.

The pH of the samples was measured potentiometrically by mixing 20 g of soil in distilled water at a ratio of 1:1.25.

To determine the percentage of moisture, a gravimetric method was used, which consists of taking 10 g of sample to dry in an oven at 110 °C for 24 hours. The moisture percentage was obtained by dividing the difference of the wet soil mass minus the dry mass over the dry mass, multiplied by 100 [5].

2.3.1. Isolation of Strains

A modification was made to the strain isolation method referenced by Arias and Piñeros [5]. A solution of 100 ml of pre-enrichment of 0.005 mg/ml of biochemical chloramphenicol, Tween 80 at 5% and 1% of peptone and 10 g of the soil sampled was used. Subsequently, they were kept in constant agitation at 150 rpm for 48 hours. They were then subjected to static incubation at 28 °C for 7 days. Subsequently, triplicate isolation was performed in PDA, incubated at 28 °C for 7 days.

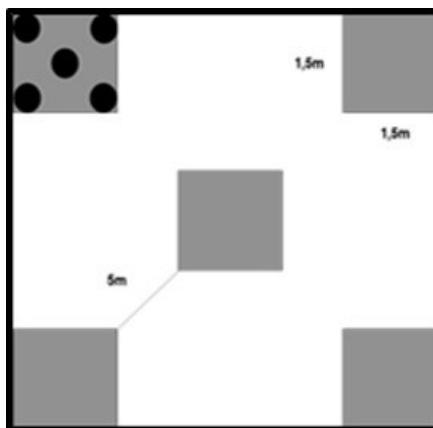


FIGURE 1. Sampling method. The displacement followed and the way in which the points for sampling are selected are shown.

2.3.2. Identification of Isolates

The identification was carried out by macroscopic observation of aspects such as color and texture on both the reverse and the front of the petri dish. For microscopic identification, lactophenol blue staining was used using the adhesive tape printing technique; taxonomic keys. In addition, DNA and PCR extraction tests were carried out to amplify the ITS regions of the isolated species for subsequent sequencing to corroborate what was found.

2.3.3. Conservation Methods

For the conservation of the strains, inoculums from the samples supplemented in the chloramphenicol solution were used. They were kept in constant agitation at 150 rpm during a period of 5 days to achieve an inoculum concentration of 3×10^8 spores/ml. This concentration was confirmed by the Neubauer chamber count.

From these inoculums, tests were performed to evaluate the two conservation methods; inclined tube cultivation with PDA and Sabouraud agar (SAB), and cryopreservation. In order to verify the results obtained, the effectiveness of each conservation method was determined by calculating the percentage of successful trials in the tests carried out, multiplying it by 100, and thus choosing the appropriate method to form the filamentous fungi collection.

2.3.3.1. Serial Cultivation Method

For the serial cultivation, the cultivation was made in PDA and SAB simultaneously, from inoculates of each species with a concentration of 3×10^8 spores/ml, in quadruplicate in an inclined tube to avoid the drying of the cultivation medium. These were stored at room temperature for 7 days without completely sealing the tubes as aerobic organisms. Once the 7 days are fulfilled, the samples are transferred to refrigeration at 4 °C to delay the aging of the strains and to postpone the period between the reseedling to 15 days, adapting the method used by Angel in the evaluation of conservation techniques for filamentous fungi and yeasts [6] for a total of 6 repetitions.

2.3.3.2. Cryopreservation Method

For cryopreservation, the microorganisms were preserved in cryogenic vials in liquid nitrogen, from cultivation that was at the beginning of the stationary phase of growth of the strain in the inoculum, using 10% glycerol v/v as cryoprotector to avoid cell damage and decrease the freezing point allowing a quick freezing [7–8]. This has as premises to use cells that were in the stationary phase of growth; to maintain a short time during the freezing and thawing of the vials to its last one to a temperature of 37 °C. After one month, the criterion was evaluated by recovering three of the five cryogenic vials of each species in PDA and SAB, in triplicate, incubated at 28 °C for five days [6].

2.3.4. Evaluation Criteria

The parameters established were viability: formation of typical colonies in the cultivation medium [9]; purity: only typical colonies of evaluated isolation are recovered [6];

and morphological stability: the strains maintain the macroscopic and microscopic characteristics [6] corresponding to those defined during their isolation and identification.

2.4. Conformation of the Cepary

Finally, in order to deposit the strains, an admission format and technical sheets were designed with the pertinent information of the isolated and identified species, together with a protocol for the entry of material and the maintenance of the strains. An Arduino DTH111 (Arduino) sensor was also programmed and installed to control the temperature and relative humidity conditions of the strain collection.

2.5. Statistical Analyses

For statistical analyses, once the data on the number of isolates vs. sampling times are established, as the assumptions of homogeneity of variances and normality are fulfilled, an ANOVA test is performed in conjunction with a TUKEY test using in both an alpha value of $\alpha = 0.05$ with 95% confidence to evaluate significant differences. In the conservation process, the percentage of effectiveness of each conservation method was evaluated for each species and for all of them, understanding the effectiveness as the capacity of each method to present the expected results of maintaining viability, morphological stability, and purity.

3. Results and Discussions

3.1. Frequency of Insulation

Of the 125 samples analyzed, 165 isolates were recovered, and nine species were identified, see Figure 2: *Trichoderma spirale* 15.53%; *Trichoderma ovalisporum* 13.66%; *Hypocrea nigricans* 13.04%; *Penicillium implicatum* 13.04%; *Penicillium citrinum* 11.80%; *Fusarium incarnatum* 10.30%; *Fusarium verticilloides* 7.27%; *Cunninghamella elegans* 4.85%; *Aspergillus cf. versicolor* 4.85%.

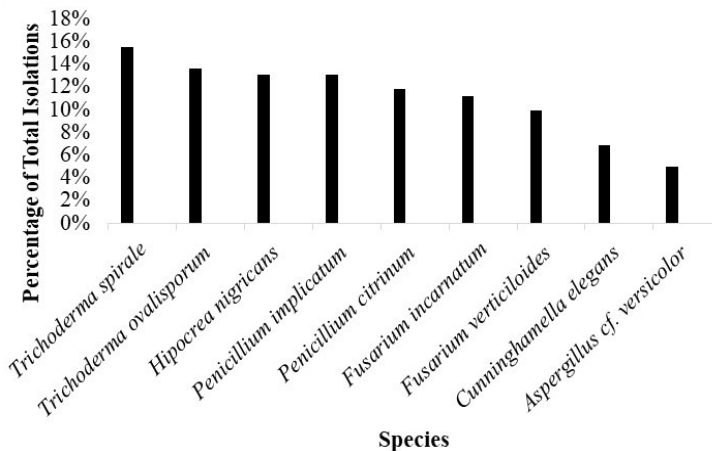


FIGURE 2. Number of isolations (N:A) of each species per sampling season.

incarnatum 11.18%; *Fusarium verticillioides* 9.94%; *Cunninghamella elegans* 6.83% and *Aspergillus cf. versicolor* 4.97% that had the lowest number of isolates. *Aspergillus*, *Penicillium*, *Trichoderma*, *Fusarium*, and *Cunninghamella* are among the most common genera isolated on the ground, corroborating the referenced by [10].

3.2. Characteristics of Filamentous Fungal Species

The isolated strains were identified considering their microscopic and macroscopic morphological characteristics. Each of these characteristics is contrasted with other characteristics reported by different authors in the tables:

- ***Aspergillus cf. versicolor*:**

Description, Figure 3a: the colonies of slow growth, small, that are initially of whitish color and from the fifth day to acquire a very abundant yellow pigmentation and an incrustants aspect, although when the sowing in dilution its growth can occupy all the means. The conidia are small and abundant up to 2 microns and the conidiophores are long and very hyaline, as seen in the table the description of the species made by the authors is similar in comparison to the description of Arias and Piñeros in Czapeck agar [5] (Table 1).

- ***Cunninghamella elegans* (Lendn. 1905):**

Description, Figure 3b: the colonies show a rapid growth in PDA, in the first 48 h the colonies occupy three quarters of the box with a diameter of 7 cm, after 72 h they can occupy the entire box. Its color is initially white, and the passage of time acquires the

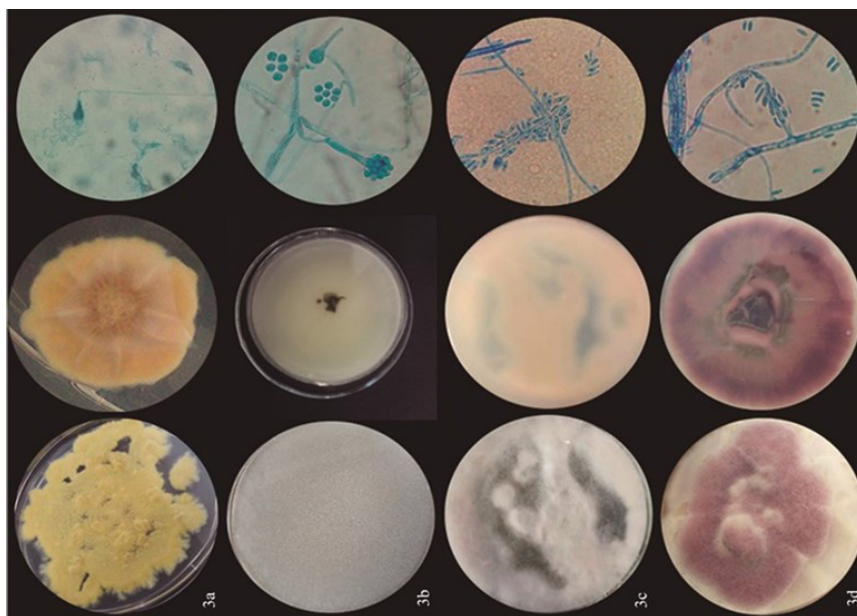


FIGURE 3. Characteristics of filamentous fungal species. From left to right on PDA: front, back, microscopic image enlarged to 100×. a: *Aspergillus cf. versicolor*, b: *Cunninghamella elegans*, c: *Fusarium incarnatum*, d: *Fusarium verticillioides*.

TABLE 1. *Aspergillus cf. versicolor*

Character	Isolated <i>Aspergillus cf. versicolor</i>	<i>Aspergillus cf. versicolor</i> in Czapeck ^a
Growth and development in agar.	Small whitish colonies, initially flocky, in days form crustal yellowish layers, highly pigmented.	Velvety white colonies with different shades between yellow and red. They reach up to 3 cm in diameter in 15 days.
Conidiophores.	Very long hyaline.	Hyaline or slightly pigmented.
Conidia.	Small, globular, hyaline with smooth walls. 2 µm.	Slightly rough balloons up to 3 µm in diameter.

^aDescription of Arias and Piñeros in Czapeck agar.

color gray storm. Mycelium without septa. The sporangiophores have a variable size; they are usually abundant, with a width of between 2.7 and 3 µm in the basal part and up to 7 µm in the apical part. The sporangioles are globose or pyriform, slightly abundant grayish or measured between 2 and 4 µm. As can be seen in Table 2 for the authors' species descriptions compared to the Lendn strain description in PDA [11].

• ***Fusarium incarnatum*:**

Description, Figure 3c: Slow growing colonies, 1 cm at 7 days in PDA, initially white, flocky, cream color on the inverse, with salmon pigmentation as the vine ages. With hyaline hyphae, long partitions and thin conidiophores, with polyphialideslides, although also with simple phialides, the macroconidia have 3 septa, are straight and elongated, and can measure up to 9 µm, the description of the species, made in this text agrees with the description of Carrillo in PDA [12] in Table 3.

• ***Fusarium verticillioides*:**

Description, Figure 3d: Colonies of fast growth, cottony, of 3 cm of diameter to the first 48 h, of 4.5 cm to the first 72 h, with abundant aerial mycelium. Coloration initially white, then violet. Pigment violet agar. Cream back. Conidiophores densely branched, septa rarely evident. Macroconidia are abundant, almost always between 7 and 10 µm, predominance of globose chlamydiospores. As can be seen in Table 4 for the description of the species made by the authors, which agrees with the description of Arias and Piñeros in PDA [5].

TABLE 2. *Cunninghamella elegans* (Lendn. 1905)

Character	Isolated <i>Cunninghamella elegans</i>	<i>Cunninghamella elegans</i> Lendn (ATCC [®] 36112) ^a
Growth and development in agar.	A velvety colony of massive growth occupies the entire plaque after 72 hours, initially whitish in color and gradually turns gray.	Initially whitish fast-growing colonies are gradually transformed to grey with dark conidia spots.
Sporangiophore.	Straight, 5–6 µm wide, variable length, reaming.	Right, verticillate or solitary.
Esporangiolas.	Globose, 5–9 µm.	Globose, 7–11 µm.

^aDescription of Lendn. 1905

TABLE 3. *Fusarium incarnatum*

Character	Isolated <i>Fusarium incarnatum</i>	<i>Fusarium incarnatum</i> in PDA ^a
Growth and development in agar.	At 28 ° C they begin their growth as white flocculant colonies after 5 days.	Flocculant colonies with aerial mycelium of dense salmon color to brown coffee with brown bottom.
Hiphae. Conidiophores.	Septated and hyaline. Simple and branched and polyphialide.	Septated and hyaline. Single or branched and polyphialide.
Macroconidia.	Straight, 6–9 × 2–3 μm.	Straight, 3 to 5 septa. Measures 7.5–35 × 2.5–4 μm.

^aDescription of Carillo in PDA.

TABLE 4. *Fusarium verticillioides*

Character	Isolated <i>Fusarium verticillioides</i>	<i>Fusarium verticillioides</i> in PDA ^a
Growth and development in agar.	Initially white colonies of woolly aspect, when growing it turns of violet tones, with cottony aspect. In 7 days, it occupies the whole surface of the box (7 cm in diameter). The obverse side is dyed dark violet in PDA agar or exhibits reddish tones.	PDA agar colonies reach a diameter of 3–5.5 cm in 7 days at 25 °C, velvety yellowish-white with a pinkish shade. Reverse yellow or purple.
Conidiophores. Microconidia.	Densely branched. Straight to the ellipsoid, they sometimes form long chains and often proliferate fialidos.	With or without septa. 0 to 2 septa, abundant, variable in shape and size, oval-ellipsoidal to cylindrical, 5–12 × 2.2–3.5 μ.

^aDescription of Arias and Piñeros in PDA agar.

• *Hipocrea nigricans* (Yoshim, 1972):

Description, Figure 4c: After the first 96 h it already occupies the middle completely in PDA. Its color is bright white with a dusty blue-green center. After one week, it has a green disc on the outermost part, with rigid and whitish pustules. With a cream-colored back, the yellow agar is slightly pigmented. It has cylindrical phialides with globular conidia of smooth walls. The description of the species by the authors corresponds to the description of Kamala and his collaborators [13] in Table 5.

• *Penicillium citrinum* (Thom, 1910):

Description, Figure 4a: In the PDA, colonies grow at a rate of 0.3 to 0.5 cm per day. Velvety blue-green and white border. It presents yellow pigmentation in the agar and exudation. The conidiophores are hyaline and generally biverticilated with up to three metules in each branch. Phyalides are bottle-shaped, shorter than metula with an average size of 2 μm in length. The conidia form chains of 8 or more, have a green color, are globose and have

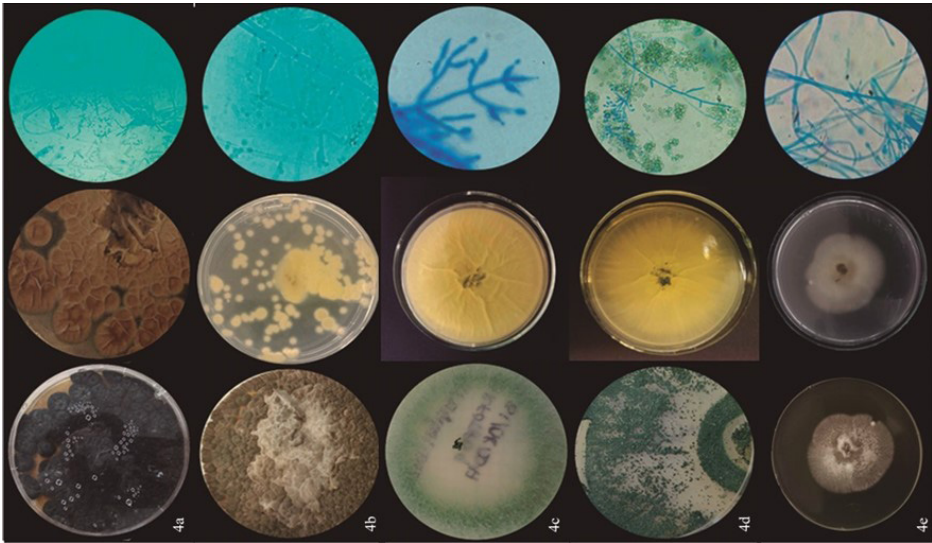


FIGURE 4. Characteristics of filamentous fungal species. From left to right on PDA: front, back, microscopic image enlarged to 100×. a: *Penicillium citrinum*, b: *Penicillium implicatum*, c: *Hipocrea nigricans*, d: *Trichoderma ovalisporum*, e: *Trichoderma spirale*.

TABLE 5. *Hipocrea nigricans*

Character	Isolated <i>Hipocrea nigricans</i>	<i>Hipocrea nigricans</i> ^a
Growth and development in agar.	The radius of the colony in the first 72 hours can be up to 3 cm, at 7 days it occupies the entire medium, presents pustules and yellow pigmentation.	The radius of the colony 45–50 mm formed pustules in culture, conidiophores added forming poorly developed pustules. Yellow pigment.
Phyalide.	Cylindrical, 7.6– 8 µm long and 2– 2.3 µm wide.	Straight, 7.6 µm long, 2.0 µm wide at the base, 2.8 µm wide in the widest form.
Conidia.	Globose 2 µm.	3.2 × 2.8 µm, globose to subglobose.

^aDescription of Kamala et al.

smooth walls. As can be seen in Table 6, the description of this species made by the authors of this text agrees with the description made by Arias and Piñeros in Czapeck agar [5].

• ***Penicillium implicatum* (Biourge, 1923):**

Description, Figure 4b: Small colonies of slow growth in PDA, velvety, initially whitish later with greenish center and white and thick irregular edges, produce pigmentation in the agar from the first 24 h. The conidia are short and tend to be biverticillated, although it is common to observe monoverticillates, with long metulas, one per branch, to which up to 8 cylindrical phialides can be attached, the conidia are globose with thick walls and roughness, as observed in Table 7 for the description of the species by the authors in accordance with the description of Arias and Piñeros in Czapeck agar [5].

TABLE 6. *Penicillium citrinum*

Character	Isolated <i>Penicillium citrinum</i>	<i>Penicillium citrinum</i> in Czapeck ^a
Growth and development in agar.	In Czapek agar, colonies grow slowly after 7 days and the diameter becomes 0.5 cm. Green color with wide white and irregular border.	Czapek agar colonies grow slowly and reach a diameter of 1.5 cm in 7 days. Light green to bluish green, yellow back. Colony round, no exudation.
Conidiophores.	2 µm wide and up to 60 µm long, smooth walls and hyalines.	2 µm wide and up to 60 µm long, smooth walls and hyalines.
Metulae.	Up to 3 Metulaes 2–2.5 µm wide.	3 to 5 divergent metulaes.
Phyalide.	Bottle shaped smaller than metulae.	Cylindrical between 8–10 µm long and 2–3 µm wide.
Conidia.	Globose and 24 ub globosa up to 2 µm diameter.	Globose and 24 ub globosa, hyaline or green 2.5–3 µm.

^aDescription of Arias and Piñeros in Czapeck agar.

TABLE 7. *Penicillium implicatum*

Character	Isolated <i>Penicillium implicatum</i>	<i>Penicillium implicatum</i> in Czapeck ^a
Growth and development in agar.	In slow-growing, reduced-growing czapeck agar colonies less than 3 cm in diameter, accumulation of spores in the centre, slightly greenish in colour, inverse cream in colour.	The colonies grow very little and slowly reach 1.5 to 2 cm in diameter in 7 days. The thin white margin produces abundant spores of light and dark green color, powdery texture, exudation of limited transparent color. Back cream color with coffee center.
Conidiophores.	Biverticised short conidiophores.	Short conidiophores, which are divided into two branches, biverticilled or triverticilled.
Metulae.	Only one long metula of 1.5–2 µm.	–
Phyalide.	Up to 8 Phyalide.	Phyalide united from 8 to 12 by verticilium.
Conidia.	Globose or semi-globose conidia of approximately 2 µm thick-walled, with roughnesses.	Globular conidia with smooth and thick walls or with slight roughness.

^aDescription of Arias and Piñeros in Czapeck agar.

• *Trichoderma ovalisporum*:

Description, Figure 4d: Rapidly growing colonies, initially white and flocky, but which quickly (48 h) acquire a greenish powder coating, presenting rigid pustules. The conidiophores are long, with phyalides paired in the shape of a flask, produce abundant green globular conidia with thin walls that tend to accumulate. As can be seen in

TABLE 8. *Trichoderma ovalisporum*

Character	Isolated <i>Trichoderma ovalisporum</i>	<i>Trichoderma ovalisporum</i> in PDA ^a
Growth and development in agar.	Fast growing, dark green cologne with whitish or greenish pustules and mycelium stuck to the medium, powdery. Fill the Petri dish after 7 days.	Fast growing, dark green cologne with whitish or greenish pustules and mycelium stuck to the medium, powdery. Fill the Petri dish after 7 days.
Conidia.	Globular to oval, green.	Green, ovoid to broadly ellipsoidal or subglobose.
Phialide.	Elongated, semi-stated, shaped flask.	Paired or arise in spirals, usually at 90 °, in the shape of a flask.
Conidiophores.	Semiglobose 2.3 × 2 µm wide, green.	Conspicuous, arises at or about 90° to the main axis, in a range between 1.7–2 and 4–6.2 µm wide.

^aDescription of Kamala et al. in PDA agar.

Table 8, the description of the authors' species matches the description of Kamala and his collaborators in PDA agar [13].

• *Trichoderma spirale*:

Description, Figure 4e: Cotton colonies, slow growth, initially whitish, from the ninth day shows a change from color to light green. Agar pigmented yellow, white on the back with the inside cream to dark brown. As seen in the description in Table 9 made for this species

TABLE 9. *Trichoderma spirale*

Character	Isolated <i>Trichoderma spirale</i>	<i>Trichoderma spirale</i> in PDA ^a
Growth and development in agar.	White cotton-colored colonies that dye the agar yellow on the back. It grows rapidly in both media, remains cottony and covers the agar, until the eighth or ninth day, when it begins to pigment light green.	The colony forms concentric rings more or less distinguishable. Pustules are typically formed, powdery to subglobose, greenish-grey (0.5–1.5 mm), compact, yellowish-green colony. The hyphae form a cottony layer, cold white mycelium, with pustules formed around the periphery of the colony.
Conidia.	Green smooth walled.	5–4.5 × 2.5–30 µm. Green, oblong to very ellipsoidal (2.5–3.0 µm), smooth.
Conidiophores.	Cylindrical and elongated.	They form a sterile strand shorter than the base from which they emerge, broad fertile branches.
Phialide.	Elongated ellipsoidal.	8.8–9.2 × 1.0–4.2 µm long and wide, arise directly from either of the branches, or arise in the form of spirals at the end of the branches.

^aDescription of Kamala et al. in PDA agar.

by the authors of the text is similar with the description of Kamala and collaborators in PDA [13].

3.3. Fungus Behavior in Relation to pH

Additionally, to understand the behavior of these species, the pH and average moisture percentage were recorded in Table 10, followed by the number of isolates for each sampling period shown in Figure 5.

Initially, for the months of October 2016 and March 2017, an average of humidity was evidenced in relation to the other sampling periods. In April 2017, neutral pH values were recorded.

Similarly, in the number of isolates for each species per sampling period (Figure 5), you can see how many isolates were recorded on the dates of the sampling events, generally for the species of the genus *Trichoderma* recorded between four and five isolates, for the genus *Fusarium* between two and five isolates, for *Penicillium* between two and six,

TABLE 10. Fungus behavior in relation to pH and % humidity

Sampling periods	Average% humidity	Average pH
1: 28/10/2016	33.77	6.65
2: 05/12/2016	42.44	6.31
3: 24/03/2017	34.12	6.64
4: 28/04/2017	45.85	7.16
5: 26/05/2017	40.42	6.29

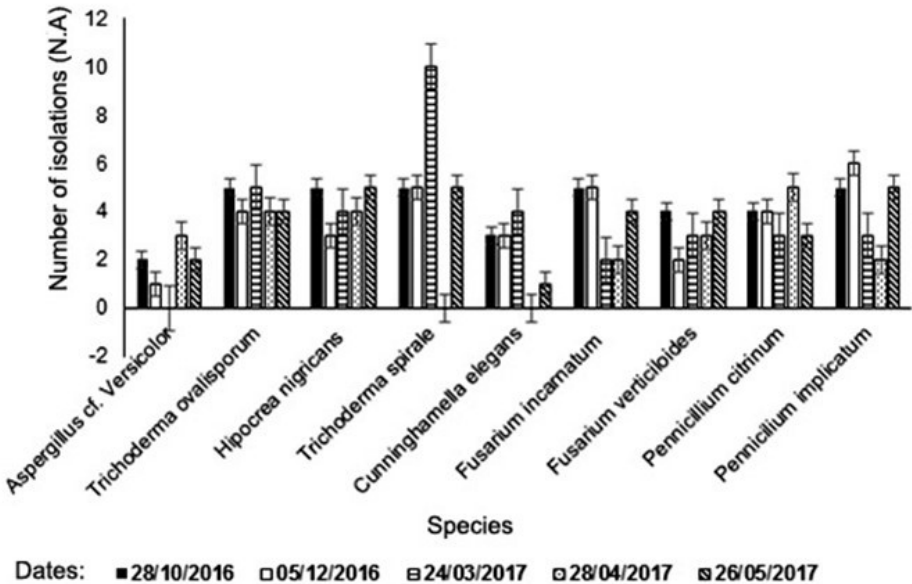


FIGURE 5. Number of isolations of each species per sampling season. The graph shows the number of isolations (N: A) that were registered for each sampling season.

Cunninghamella and *Aspergillus* between one and four isolates on the five dates the soil samples were collected.

On the table of average values for pH and humidity percentage (Table 10) for the March sampling event in which the pH is 6.31, slightly acidic, and the average percentage of humidity is 34.12% up to 10 isolates were recorded, while for the next time when the pH is slightly neutral and the humidity is higher, 45.85% did not register any. For *Cunninghamella elegans*, the highest number of isolates was on March and none for April. Otherwise, for *Aspergillus cf. versicolor* for the month of March there were no isolations, for the next date, which corresponds to the month of April, it had the highest number of isolations, corresponding to three. The number of isolations registered in relation to the sampling times statistically does not present significant differences with a significance of $P = 0.391$. *Aspergillus*, *Fusarium*, and *Trichoderma* present a high tolerance so they can grow in a variety of conditions and can be easily isolated in different environments [4], in the case of *Cunninghamella* they can grow and sporulate quickly and easily in environments that are rich in carbohydrates and in soils [14].

3.4. Serial Cultivation

The nine isolated and identified species were preserved in La Universidad El Bosque herbarium collection. Seven of the nine species were conserved under the serial cultivation method in an inclined tube and all species in cryopreservation, the results show the relevance in the use of each method according to the requirements for the maintenance of each species.

Fusarium incarnatum and *Aspergillus cf. versicolor* could only be recovered by purifying the vine; in the case of *Fusarium incarnatum*, it was only possible to preserve it in petri dishes with PDA and by cryopreservation. In the case of *Aspergillus cf. versicolor*, after recovering the vine, it was possible to continue preserving it through serial cultivation in both PDA and Sabouraud agar, in addition to cryopreservation.

Of these nine species analyzed, only seven had an efficiency percentage higher than 70.83% under this method, viability higher than 79%, purity higher than 83% and for the tubes without contamination showed a morphological stability higher than 77%, both in PDA and in Sabouraud agar (see Figure 6a). The contamination generated in some of the tubes for *Cunninghamella elegans* and *Penicillium implicatum* were yeasts of the genus *Rhodotorula*.

Fusarium incarnatum showed contamination after 15 days, in both agars, in all tubes with another fungus of the genus *Penicillium*, 15 days later it was no longer viable. In contrast, *Aspergillus cf. versicolor* lost purity in the second trial and viability in the third trial, with the effectiveness of this method in these two species being only 41.67% and 16.67%, respectively, in PDA (Figure 6b).

It can be seen that, in relation to purity, there is only a difference of 0.46% between the agars (Figure 6a), being slightly greater when using the PDA. Morphological stability is greater when the growth in PDA to preserve it by subcultivation in periodic series, with a difference of 1.39% using Sabouraud agar. On the contrary, for viability, a difference of 0.46% was greater in Sabouraud agar than in PDA.

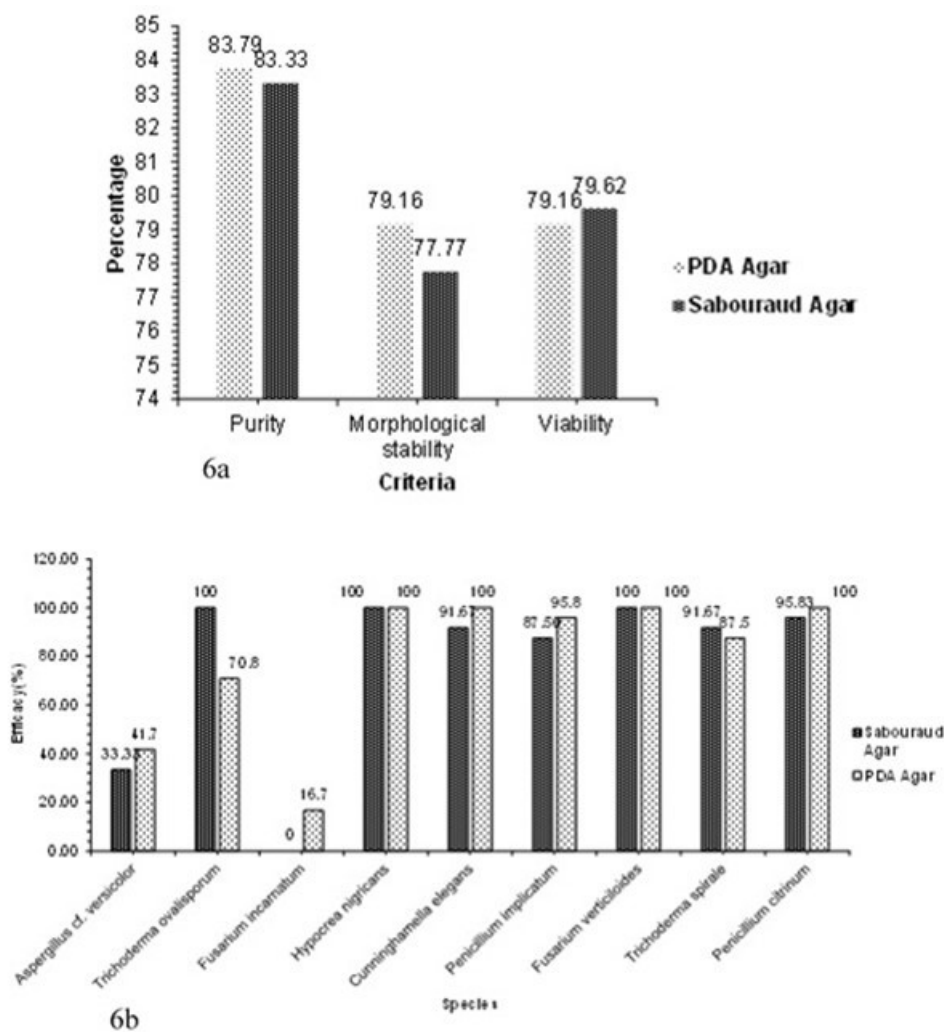


FIGURE 6. Serial cultivation. a: Percentage of each evaluation criterion for serial cultivation. Comparing cultivation media. b: Comparison of the effectiveness of the serial cultivation method. For each species, comparing the cultivation media.

For the first trial, *Cunninghamella elegans*, showed incomplete growth in the PDA, the remaining species grew completely in most of the tubes. As for Sabouraud agar, *Fusarium incarnatum* was viable only in two tubes, but was not pure, and *Trichoderma spirale* grew in three out of four tubes, the rest of the strains were viable and pure in most of the tubes.

For the second trial, the only morphotype in PDA not showing viability was *Fusarium incarnatum*, all other morphotypes showed growth, by *Aspergillus cf. versicolor* a purity of 50% was obtained. Similarly, in Sabouraud agar, *Fusarium incarnatum* showed no viability, exclusively *Cunninghamella elegans* and *Trichoderma spirale*, showed no growth in a single tube and *Aspergillus cf. Versicolor* showed contamination in all tubes.

In contrast, during the third trial, *Trichoderma ovalisporum* was viable, pure, and morphologically stable in 25%, unlike the rest of the strains in PDA, excluding *Aspergillus cf. versicolor* and *Fusarium incarnatum*, which were no longer viable in PDA in the first case and for both agars in the second. *Penicillium implicatum* was viable in 75%, *Cunninghamella elegans*, showed contamination in one of the tubes, *Aspergillus cf. versicolor* grew in two tubes but was contaminated, and the other strains were optimally met with the three criteria mentioned in the Sabouraud agar.

As for the fourth trial, in both agars the optimum purity, viability, and morphological stability were observed for all strains, excluding *Aspergillus cf. versicolor* which was viable at 25%, but without purity or morphological stability and *Fusarium incarnatum*, without viability, purity, or morphological stability.

In the fifth trial, viability, purity, and morphological stability were optimal in both agars for the strains, even after recovery and purification, *Aspergillus cf. versicolor* presented 50% purity in Sabouraud and PDA, unlike *Fusarium incarnatum*.

The last trial maintained the viability, purity, and morphological stability of all strains in both agars, with the exception of *Penicillium implicatum* in Sabouraud agar.

3.4.1. Viability Percentage

The viability of *Aspergillus cf. versicolor*, *Penicillium implicatum*, and *Fusarium incarnatum* was compromised by using this method on Sabouraud agar compared with other species (Figure 7a).

3.4.2. Percentage of Purity

The purity of *Trichoderma ovalisporum* in PDA, *Penicillium implicatum* and *Cunninghamella elegans* in Sabouraud agar was good but not optimal, since at some point in the procedure they presented contamination of the medium with yeasts of the genus *Saccharomyces* and *Rhodotorula*, fungi of the genus *Penicillium* and actinobacteria of the genus *Streptomyces*, so the percentage of purity and efficacy of these species was affected (Figure 7b).

3.4.3. Percentage of Morphological Stability

The percentage of morphological stability for the species remained above 80% in both agars, however, there were certain particularities such as *Trichoderma ovalisporum*, which their morphological stability in PDA did not exceed 71% (Figure 7c). In addition, as mentioned above, *Aspergillus cf. versicolor* and *Fusarium incarnatum* in any of the agars did not exceed 50%.

3.5. Cryopreservation

This conservation method was carried out with 100% efficacy for all strains, starting in September and performing an analysis of purity, viability, and morphological stability, one month later.

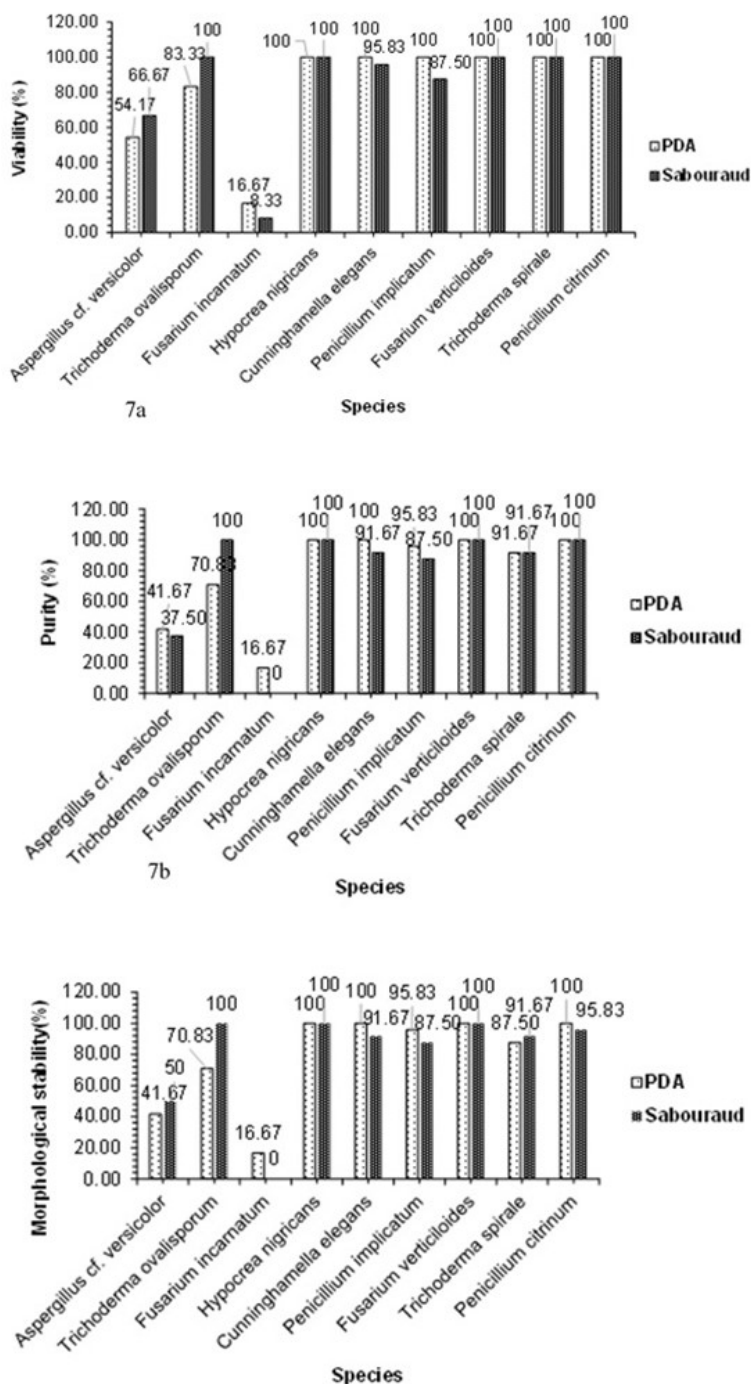


FIGURE 7. Serial cultivation. a: Viability percentage for periodic serial subculture method. Corresponding to each species, in PDA agar culture medium compared to Sabouraud; b: Percentage of purity for the serial cultivation method. For each species comparing the cultivation media; c: Percentage of morphological stability for the serial cultivation method. For each species comparing the cultivation media.

3.5.1. Percentages of Criteria Assessed

All strains showed optimal viability, morphological stability, and purity.

3.6. Choosing the Best Method of Preservation

The conservation of filamentous fungi varies according to the type and degree of sporulation [15]; it is likely that the conservation method per serial cultivation is not compatible with the strains of *Aspergillus cf. versicolor* and *Fusarium incarnatum* due to these factors.

According to García and Uruburú, Nakasone and collaborators, and González, despite being the traditional method for the preservation of microorganisms, serial cultivation is not a very practical method for storing large quantities of mushroom cultivation, as it takes a long time, and has disadvantages such as: be prone to contamination, genetic and physiological changes (degeneration, aging) that increase during the long-term serial cultivation, losing the characteristics of the organism [8,16–17], which was evidenced test to test in some of the species analyzed in this work, Gonzalez also highlights the danger of crop loss [17].

However, the serial cultivation method is still used in collections with limited financial support or in most other collections as a backup preservation method [18]. Angel [6], in 2006 supports the use of serial cultivation for periods of time such as those handled in this study (15 days), also clarifies the disadvantages of frequently performing this process and the loss of the characteristics of the organism by keeping them at room temperature [6]. That is why the technique was applied by growing and keeping the strains at room temperature for 7 days, then cooling them to complete 15 days at 4 °C, in order to delay aging, which also mentions that Angel was carried out in his work. Since the lapse between two peals is prolonged [6].

On cryopreservation, Leslie and Summerell, for the genus *Fusarium*, have reported that the use of 15% sterile glycerol and maintenance of freezing at –70 °C, the isolates have remained viable for more than 10 years [19]. According to Gonzalez, cryopreservation in liquid nitrogen is one of the methods available in all national collections of strains and many industrial and university collections that have adequate facilities, however, this method demands a constant supply of liquid nitrogen [19] and despite the effectiveness obtained by applying this method to the species in this study, no preservation method can be applied universally to all fungi and intraspecific variability makes it impossible to apply standard protocols at the species level. Therefore, as a strategy in the collection of La Universidad El Bosque, it will be the use of more than one conservation technique to reduce the chances of deterioration or death. It is necessary to emphasize that the evaluation was carried out in a relatively short period of time, unlike the studies indicated, which evaluate it in longer periods (annual, half-yearly, etc.).

4. Conclusions

Of the species identified in soils of the municipality of Viotá, we find filamentous fungi of important economic interest, such as *Trichoderma ovalisporum* and *Hipocrea nigricans*.

According to the statistical analysis, it is possible to appreciate that the sampling dates did not have a significant incidence on the variation of the isolates obtained from the soil samples.

In order to guarantee the cepary, temperature and humidity must be measured constantly. For cryopreservation with liquid nitrogen, the tank should be filled once every three months, which ensures that the average temperature inside is kept below -70°C , in addition, it is necessary to keep the strains in culture media in hermetic boxes, covered individually with parafilm at an average temperature of 4° to 8°C .

The most efficient conservation method was cryopreservation, with an efficacy percentage of 100%, maintaining optimal viability, morphological stability, and purity, although the subculture in periodic series had an efficacy higher than 90%, especially for *Trichoderma ovalisporum*, in agar Sabouraud, *Cunninghamella elegans*, *Penicillium citrinum* and *Penicillium implicatum* in PDA, finally, the efficacy of *Hipocrea nigricans* was the same in both agars. The best culture medium that maintained morphological stability and purity for longer was PDA, Sabouraud agar was better to maintain viability; however, the difference between the two agars was 1.39% effective.

These results were used to establish the protocol for the conservation of filamentous fungal strains of La Universidad El Bosqueperium.

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