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Viability of *Hirsutella* spp. strains preserved in mineral oil

Viabilidad de cepas de *Hirsutella* spp. conservadas en aceite mineral

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RESUMEN

Antecedentes: a las colecciones de cultivos les corresponde buscar métodos alternativos para conservar especies fúngicas sensibles a técnicas específicas debido a sus características como tamaño vacuolar y de los conidios, la susceptibilidad a desecación o a bajas temperaturas, producción de esporas y crecimiento lento.

Objetivo: recuperar 18 aislados de *Hirsutella citriformis* y dos de *Hirsutella thompsonii* conservados en aceite mineral (AM), para valorar su viabilidad y sugerir este método como alternativa para su preservación, en laboratorios que no disponen de los recursos para la conservación a -70 y -196 °C.

Métodos: los aislados se preservaron en AM y se recuperaron en Agar Dextrosa Sabouraud (ADS) con 1 % de extracto de levadura (ADSY), después de seis años de conservación, además se valoró su viabilidad, morfología y pureza.

Resultados y conclusiones: 19 cepas de *Hirsutella* spp. se mostraron viables (95 %), excepto CHE-CNRCB 333. La cepa CHE-CNRCB 345 mostró diferencias en su crecimiento, respecto a las características iniciales observadas para *H. citriformis*. En SDAY *H. citriformis* produjo los sinemas característicos de la especie. El AM puede utilizarse como una alternativa de conservación para *Hirsutella* spp. permitiendo prolongar su preservación, comparado con el agua estéril y la liofilización donde se reduce su viabilidad.

Palabras clave: conservación *ex situ*, hongos entomopatógenos, Hypocreales, Ophiocordycipitaceae

ABSTRACT

Background: culture collections are responsible for the search for alternative methods to preserve fungal species that are sensitive to specific techniques due to their characteristics such as the vacuolar size and conidia, susceptibility to desiccation or low temperatures, spore production and slow growth.

Objective: to recover 18 isolates of *Hirsutella citriformis* and two of *Hirsutella thompsonii* preserved in mineral oil (MO), to assess their viability and suggest this method as alternative for its conservation, in laboratories that do not have the resources for preserve at -70 and -196 °C.

Methods: isolates were preserved in MO and recovered in Sabouraud Dextrose Agar (SDA) with 1 % yeast extract (SDAY) after six years of preservation, and their viability, morphology and purity were assessed.

Results and conclusions: 19 strains of *Hirsutella* spp. were viable (95 %), except for CHE-CNRCB 333. The CHE-CNRCB 345 strain showed differences in its growth, with respect to the initial characteristics observed for *H. citriformis*. In SDAY *H. citriformis* produced the characteristic synnemata of the species. MO can be used as a preservation alternative for *Hirsutella* spp. allowing their preservation, compared to sterile water and lyophilization where their viability is reduced.

Keywords: entomopathogenic fungi, *ex situ* conservation, Hypocreales, Ophiocordycipitaceae

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INTRODUCTION

All research or applied studies that involve the use of living microorganisms require a constant supply of them in optimal conditions (Humber 2012). The study, management, and utilization of microscopic fungi require the use of proper conservation techniques (Mier *et al.* 2005). In some cases, the process of identifying taxonomic fungal species may take a long time and can affect the viability of strains. Therefore, it is crucial to preserve them for later identification (Kannojia *et al.* 2020, Smith *et al.* 2001). Furthermore, certain isolates have unique characteristics that are of practical value for medical or biotechnological applications, such as producing enzymes, proteins, bioactive compounds, antibiotics, or biopolymers (Prakash *et al.* 2013, Sievers 2015, Kannojia *et al.* 2020). Methods that employ low temperatures, such as lyophilization, ultracold-freezing at -70 °C, and cryopreservation at -196 °C, generally provide optimal conservation for most species of fungi. However, there are exceptions where the choice of a method poses certain limitations, either due to the microorganism's sensitivity to the method or its slow growth (Mier *et al.* 2005, Humber 2012). Fortunately, alternative conservation methods are available. One of these methods is preservation with mineral oil (MO) or paraffinic oil, which is a simple and accessible technique for conserving both sporulating and non-sporulating fungi, regardless of laboratory size or infrastructure (Kannojia *et al.* 2020). This economical and effective procedure was first used by Lumière and Chevrotier (1914), and later by Buell and Weston (1947), and is still in use today. The mechanism of this preservation method involves the prevention of dehydration, leading to a reduction in metabolic activity and growth (Smith and Onios 1994, Singh *et al.* 2018, Kannojia *et al.* 2020). The primary advantage of this technique is its ability to maintain the viability of certain fungal species for extended periods. Additionally, this method enables the preservation of species that are intolerant to other preservation methods, including low-temperature techniques, while also preventing mites contamination, without the requirement of specialized equipment (Smith and Onios 1994). However, the drawbacks of this method include the possibility of contamination by fungi that generate airborne spores (such as *Aspergillus* and/or *Penicillium*), slow growth of recovered strains, and alterations in pathogenicity and/or virulence after decades of storage (Smith and Onios 1994, Mier *et al.* 2005).

The Colección de Hongos Entomopatógenos of the Servicio Nacional de Sanidad, Inocuidad y Calidad Agroalimentaria (SENASICA), in conjunction with the Dirección General de Sanidad Vegetal (DGSV) of the Government of Mexico, implements biological control programs to manage various pests using entomopathogenic fungi (EPF). Among the prominent genera is *Hirsutella*, a group of asexually reproducing fungi in the Ophiocordycipitaceae family, originally described by mycologist Narcisse Théophile Patouillard in 1892. The genus includes over 70 species, some of which are pathogens of insects, mites, and nematodes, making them suitable as microbial agents for biological control (Sung *et al.* 2007). One particularly useful species is *Hirsutella citriformis* Speare, which is naturally abundant and plays an important role in the control and/or reduction of pests in citrus crops (Raymundo-Jiménez *et al.* 2019). Field observations have shown a 23 % infection rate by the fungus in adult Asian citrus psyllids, *Diaphorina citri* Kuwayama, perched on leaves. However, its laboratory conservation presents some challenges due to its slow growth and limited conidial production (Raymundo-Jiménez *et al.* 2019), as well as its susceptibility to preservation methods such as freeze-drying or limited viability in sterile distilled water (less than 6 months) (Ayala-Zermeño *et al.* 2017). This study aims to evaluate MO as a method of prolonged conservation for 20 isolates of *Hirsutella* spp.

MATERIALS AND METHODS

Fungi

The multi–spore isolates of *H. citriformis* and *Hirsutella thompsonii* Fisher (Hypocreales: Ophiocordycipitaceae) used in this study are part of the Colección de Hongos Entomopatógenos (CHE) at the Departamento de Control Biológico (DCB), an institutional partner of the World Data Centre for Microorganisms (WDCM), with registration number 1034/CHE-CNRCB (Table 1). The isolates used in this study CHE-CNRCB 328, 329, 330, 331, 332, 333, 335, 337, 338, 339, 341, 342, 345, and 347 of *H. citriformis* were previously characterized (Berlanga-Padilla *et al.* 2018), in ano-

ther research, Pérez-González *et al.* (2015a, 2015b) characterized the isolate 392 (IB-Hir-2), the rest of the strains used 384, 386, and 388 have been studied and morphologically characterized in the CHE (unpublished results by Berlanga-Padilla A. 2022, Com. Pers.). In this study, two strains of *H. thompsonii*, 327 and 377, were also evaluated for comparison with strains of *H. citriformis*. Although these strains belong to the same genus, they have different growth and sporulation characteristics, which makes them resistant to drying and freezing conditions and tolerate preservation in techniques such as freeze-drying (lyophilization) and silica gel, which are not conducive to *H. citriformis.*

MO conservation method

Glass vials, 18 × 65 mm and of 12 mL capacity, were filled with 3 ml of SDA culture medium, supplemented with 1 % yeast extract (SDAY) [g/L: 40 dextrose, 10 peptone, 10 yeast extract, 15 agar], and sealed with Bakelite caps. The vials were sterilized at 121 °C for 15 min and then cooled down by tilting them at an angle of around 45°. Each vial was quadruplicatedly inoculated with mycelial segments and/or synnema from each of the strains and incubated at 25 °C until synnema formation was observed, which took approximately 34 days. MO (density at 25 °C, 0.848 g/mL, and viscosity 83.5 SUS) was sterilized at 121 °C for 15 min, followed by a second sterilization at 180 °C for one hour after 24 to 48 h to eliminate the retained moisture (Smith *et al.* 2001). Under aseptic conditions, the cultures were covered with 8 ml of MO, leaving a one-centimeter gap above the culture. Finally, the vials were sealed with Parafilm® paper, labeled, and stored upright at a room temperature of 25 ± 2 °C (Montesinos-Matías *et al.* 2015).

Initial quality control tests and recovery of isolates stored for more than six years

The quality control tests involved recovering the fungus one week after preservation. To achieve this, all the oil was removed from one vial of each of the 20 isolates, and the excess was allowed to drain off. Using a sterile mycological loop, conidia and/or mycelium with synnemata were detached and placed in Petri dishes with ADSY culture medium. Observations were made under a stereoscopic microscope to verify the mycelial growth of each isolate after recovery. Since the fungus still retains a layer of oil, it can take up to two weeks to grow under these conditions. Therefore, it is recommended to take sections of the colonial growth and transfer them an oil-free medium.

For isolates stored for more than 6 years at 25 ± 2 °C, they were recovered in a similar way as the quality control in SDAY medium (Table 2). From this growth, 5 mm sections of mycelium were placed in SDAY. The media were sterilized for 15 min at 121 °C. In all cases, they were seeded in triplicate and grown for 34 days at 25 ± 2 °C (Table 2). The characteristics were evaluated qualitatively, observing that the colonial growth and synnema production corresponded to the initial characteristics observed and inherent to the corresponding species.

Table 1. Characteristics of isolates of *Hirsutella* spp.

Acronyms	Host	Host culture	Locality
Hirsutella thompsonii			
327	*Aceria guerreronis* [Aracnidae: Acari: Eriophyidae]	Coconut tree	Cuba 23°0'47.282'' N 80°49'58.349'' W
377	Citrus rust mite [*Phylocoptruta oleivora*]	Orange	El Galage, Ixtlahuacan, Colima, México. 19° 0' 4.284'' N 103° 44' 10.967'' W

Acronyms	Host	Host culture	Locality
Hirsutella citriformis			
328	*Diaphorina citri* [Hemiptera: Psyllidae]	*Citrus aurantifolia*	El Roble, Cofradía de Morelos, Tecomán, Colima, MX. 18°51'16.3" N 103°50'58.9" W
329	*Diaphorina citri* [Hemiptera: Psyllidae]	Mexican lemon	El Roble, Cofradía de Morelos, Tecomán, Colima, MX. 18°51'16.3" N 103°50'58.9" W
330	*Diaphorina citri* [Hemiptera: Psyllidae]	*Citrus aurantifolia*	Tecuanillo, Tecomán, Colima, MX. 18°49'00.1" N 103°52'58.8" W
331	*Diaphorina citri* [Hemiptera: Psyllidae]	*Citrus aurantifolia*	Tecuanillo, Tecomán, Colima, MX. 18°49'00.1" N 103°52'58.8" W
332	*Diaphorina citri* [Hemiptera: Psyllidae]	*Citrus aurantifolia*	Cofradía de Morelos, Tecomán, Colima, MX. 18°52'09.8" N 103°50'16.8" W
333	*Diaphorina citri* [Hemiptera: Psyllidae]	Mexican lemon	El Roble, Cofradía de Morelos, Colima, MX. 18° 52' 4.26" N 103° 50' 12.048" W
335	*Diaphorina citri* [Hemiptera: Psyllidae]	*Citrus aurantifolia*	Cofradía de Morelos, Tecomán, Colima, MX. 18°52'09.8" N 103°50'16.8" W
337	*Diaphorina citri* [Hemiptera: Psyllidae]	*Citrus aurantifolia*	El Pandelo, Tecomán, Colima, MX. 18°53'21.0" N 103°53'10.7" W
338	*Diaphorina citri* [Hemiptera: Psyllidae]	*Citrus latifolia*	Tecuanillo, Tecomán, Colima, MX. 18°49'00.1" N 103°52'58.8" W
339	*Diaphorina citri* [Hemiptera: Psyllidae]	*Citrus latifolia*	Tecomán, Colima, MX. 18°55'37.9" N 103°53'01.7" W
341	*Diaphorina citri* [Hemiptera: Psyllidae]	*Citrus latifolia*	Tecomán, Colima, MX. 18°55'37.9" N 103°53'01.7" W
342	*Diaphorina citri* [Hemiptera: Psyllidae]	*Citrus aurantifolia*	Tecomán, Colima, MX. 18°55'37.9" N 103°53'01.7" W
345	*Diaphorina citri* [Hemiptera: Psyllidae]	*Murraya paniculata*	Tecomán, Colima, MX. 18°55'37.9" N 103°53'01.7" W
347	*Diaphorina citri* [Hemiptera: Psyllidae]	*Citrus aurantifolia*	Cofradía de Morelos, Tecomán, Colima, MX. 18°52'09.8" N 103°50'16.8" W
384	*Diaphorina citri* [Hemiptera: Psyllidae]	Mexican lemon	San José del Progreso, Tututepec, Oaxaca, MX. 16° 13' 33.577" N 96° 13' 27.445" W
386	*Diaphorina citri* [Hemiptera: Psyllidae]	Mexican lemon	San José del Progreso, Tututepec, Oaxaca, MX. 16° 13' 33.577" N 96° 13' 27.445" W
388	*Diaphorina citri* [Hemiptera: Psyllidae]	Mexican lemon	La Cañada, Tututepec Juquila, Oaxaca, MX. 16° 0' 0.047" N 96° 59' 59.949" W
392 (IB-Hir-2)	*Diaphorina citri* [Hemiptera: Psyllidae]	Persian lemon	Tecomán, Colima, MX. 18°55'37.9" N 103°53'01.7" W

All fungal isolates were obtained from the "Colección de Hongos Entomopatógenos" of the "Centro Nacional de Referencia de Control Biológico" (CHE-CNRCB; Mexico).

Table 2. Species, acronym, years of conservation, recovered vials and presence of contaminants of the *Hirsutella* sp. isolates recovered from MO

Species/Acronym CHE-CNRCB	Years of conservation	Vials with live fungus	Presence of contaminants
Hirsutella thompsonii			
327	6.33	2 of 3	-
327	6.33	3 of 3	-
Hirsutella citriformis			
328	6.33	3 of 3	-
329	6.16	2 of 3	1 vial
330	6.33	2 of 3	-
331	6.16	1 of 3	2 vial
332	6.16	1 of 3	-
333	NV**	0 of 3	-
335	6.33	2 of 3	1 vial
337	6.16	2 of 3	-
338	6.16	2 of 3	-
339	6.66	2 of 3	-
341	6.83	3 of 3	-
342	6.66	3 of 3	-
345	6.83	3 of 3	-
347	6.66	3 of 3	-
384	6.66	1 of 3	-
386	6.83	2 of 3	-
388	6.83	1 of 3	-
392 (IB-Hir-2*)	6.66	2 of 3	-

*IB-Hir-2: acronym from the Biotechnology Institute at the Biological Sciences Faculty, Autonomous University of Nuevo León, Mexico (IB-UANL)

**NV: not viable

RESULTS

Figure 1 (a-e) show the colonial growth of some isolates CHE- CNRCB 335, 338, 339, 388 and 392 of *H. citriformis* grown in SDAY before to their preservation in MO. After six years of preservation in MO, viable growth was observed, and 19 isolates were recovered, except for isolate CHE-CNRCB 333 of *H. citriformis* (Tables 1, 2). In all fungi recovered using SDAY, initial growth was observed from the fifth to the sixth day. Isolates 327 (Figure 2a) and 377 of *H. thompsonii* (Figure 2b) were recovered in SDAY; in this culture medium, they exhibited irregular, raised, rough colonies, wavy margins, gray color, and no exudates were observed. The isolates of *H. citriformis* grown in SDAY displayed circular, flattened colonies with varying presence of striations, and gray to white color, along with different amounts of synnemata and exudates. These features were consistent with those observed in isolates 328, 329, 331, 332, 335, 339, 341, 384, 386, and 388 (Figure 2). However, isolate 392 (Figure 2s) exhibited rough type colonies. Isolates 342, 345, and 347 (Figure 2m, n, o) showed irregular, raised colonies with striations, gray-to-white color, and no synnemata or exudates. Isolate 345 (Figure 2n) demonstrated similar characteristics with rough-type colonies, which were distinct from other growths. In the same culture medium, isolates 330, 337, and 338 (Figure 2e, i, j) displayed sinuous, rough colonies without exudates, but with a unique characteristic that made them similar to the original cultures: the growth of mucilaginous-looking colonies containing yeast-like bodies on the periphery of the colony. Isolate 339 also showed this type of growth although only in a small area of the colony (Figure 2s). Optical microscope observations at 40× magnification showed fungal hyphae and yeast-like bodies (Figure 3a, b).

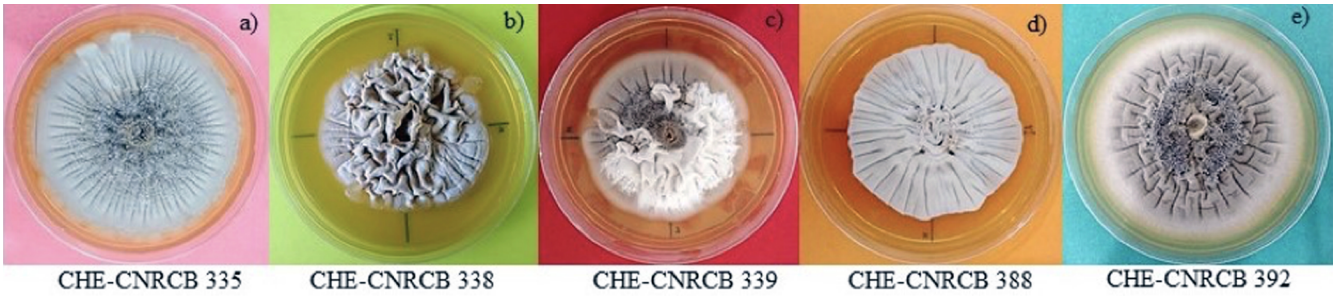

Figure 1. Colonial growth of some isolates of *Hirsutella citriformis* grown in SDAY culture medium, at 34 days, 25 ± 2 °C, prior to their preservation in MO.

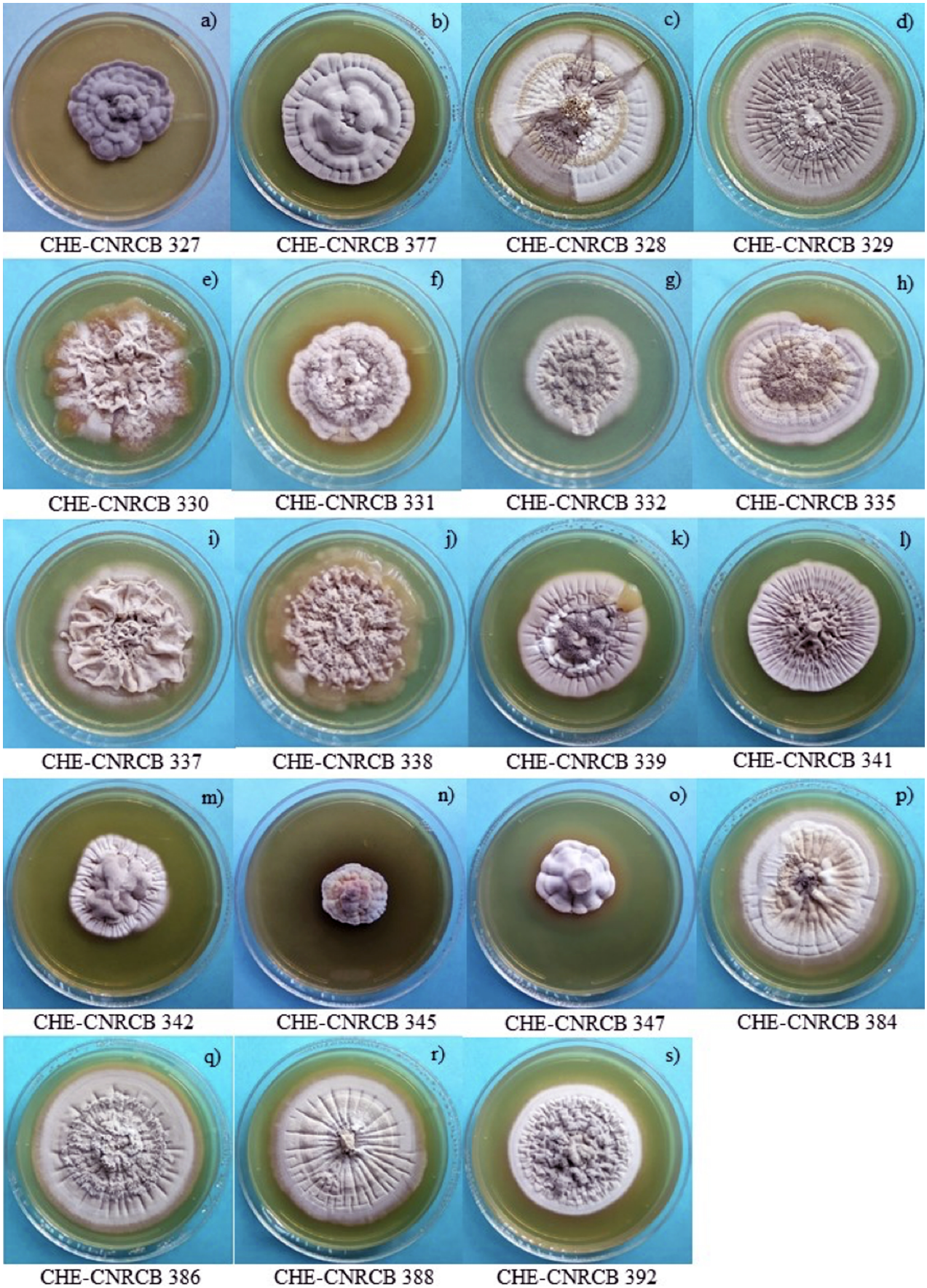

Figure 2. Strains of *Hirsutella* spp. recovered from MO and grown on SDAY culture medium, at 34 days, 25 ± 2 °C.

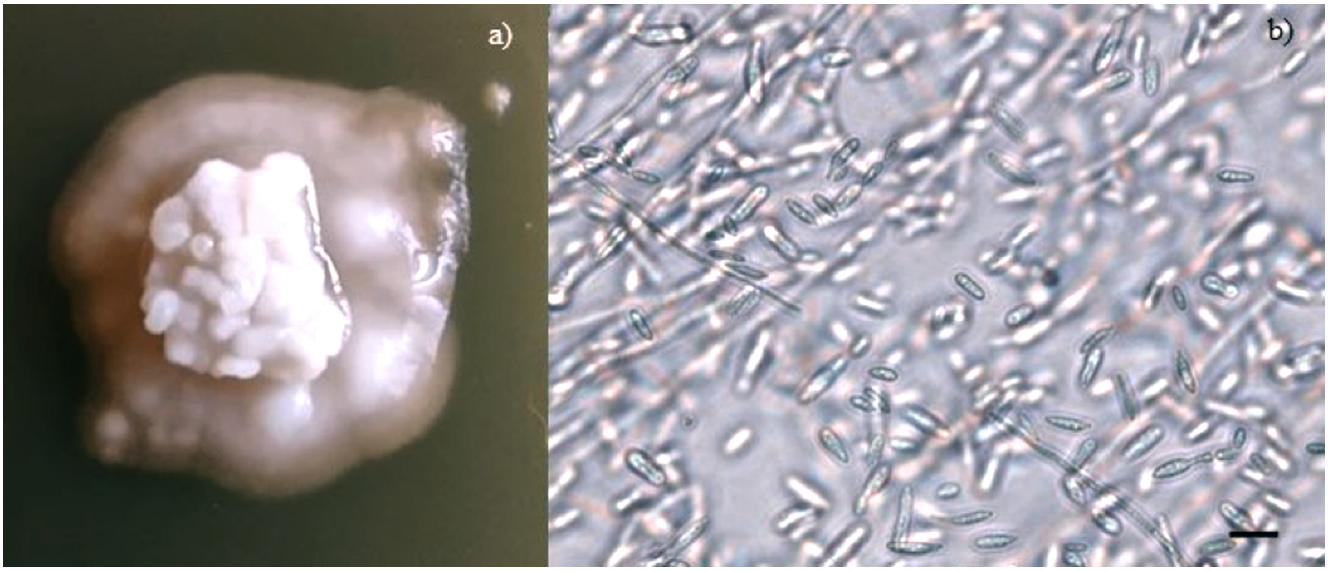

Figure 3. Colonial growth of the *Hirsutella citriformis* isolate CHE-CNRCB 332. a) mucilaginous-looking growth is observed in the initial stages of colonial development, and also on the periphery of mature culture. b) the microscopy (40×) allows to appreciate that the mucilage is made up of hyphae and yeast-like bodies (scale bar = 10 µm).

DISCUSSION

The results of the study demonstrate that 17 isolates of *H. citriformis* and two of *H. thompsonii* (95 %) can be preserved in MO for more than six years at ambient temperature (25 ± 2 °C). According to Schönborn (1989), a minimum percentage of cultures preserved using this technique lose their viability after 5-6 years. In the present study, a similar result was observed with the CHE-CNRCB 333 isolate of *H. citriformis*, which was found to be non-viable.

Undoubtedly, procedures based on low temperatures (-70 °C, and -196 °C) are the best methods for the preservation of microorganisms. In a previous work by Ayala-Zermeño *et al.* (2017) the fungi *H. citriformis* and *H. thompsonii* were recovered viable in ultracold-freezing at -70 °C, and cryopreservation at -196 °C after two years of conservation with a DICE similarity coefficients from AFLP analysis show a value of 100 % for both species, as reported by López-Lastra *et al.* (2002) with *H. thompsonii* that was observed viable at 12 and 18 months in ultracold-freezing at -20 and -80 °C, respectively.

This study suggests the use of MO for the conservation of *Hirsutella* spp. as an alternative in laboratories that do not have the resources to implement ultracold-freezing at -70 °C, and cryopreservation at -196 °C, in addition to the limitations presented by *H. citrifomis* in other preservation methods, such as sterile distilled water (where the species survives for less than six months) (Ayala-Zermeño *et al.* 2017) or lyophilization (which is not suitable for fungi that are susceptible to this procedure) (Mier *et al.* 2005).

Several studies have reported on the viability of different fungal species using MO preservation. For instance, Smith and Onions (1983) reported 32 years (for example *Verticillium theobromae*, *Torula* sp., *Penicillium* sp., *Aspergillus* sp., and others) and Smith and Onions (1994), Rico *et al.* (2004), and Panizo *et al.* (2005) reported 47 (*Candida parapsilosis* and *Candida tropicalis*) and 48 years (*Aspergillus niger*). In the case of Hypocreales fungi, Little and Gordon (1967) reported 12 years of viability for *Beauveria* sp., and Mier *et al.* (2005) reported 1.5 years of viability for *P. farinosus* and *V. lecanii*. In entomopathogenic Entomophthorales fungi, Humber (2012) reported 1.5- and 0.25-years viability for *Conidiobolus thromboides* and *Zoophthora radicans*, respectively.

Buell (1947) reported that long-term preservation of fungi in MO can impact their development, conidial formation, and dimorphic process, due to microaerobiosis, nutrient loss, and toxic metabolite accumulation. Barnes (1984) noted that differences in species, temperature, and culture may affect a species' ability

to survive prolonged preservation using this method. Other factors, such as the timing of metabolic activity reduction, inoculum age, layer depth, and oil quality, can also affect preservation success (Fennell 1960, Pumpyanskaya 1964). To prevent deterioration and loss of viability (a cause of intraspecific variability), Smith and Onions (1994) recommend regularly transferring fungi preserved in this manner every one to two years. Although we did not conduct intermediate recoveries during the six-year preservation period, our samples were stored at a controlled temperature of 25 ± 2 °C. Most recovered colonies showed no apparent macroscopic alterations compared to some colonies of *H. citriformis* grown in SDAY prior to preservation in MO (Figure 1 a-e), except for the CHE-CNRCB 345 isolate, which exhibited distinct morphological differences in growth on both culture media tested and differed significantly from inherent characteristics of the *H. citriformis* species. Panizo *et al.* (2005) report that 4.6 % of 241 species of filamentous fungi preserved in oil for 3 to 47 years displayed micro-morphological alterations. In contrast, Surja *et al.* (2020) found no morphological alterations or contamination after preserving *Aspergillus* sp., *Trichophyton mentagrophytes*, and *Candida albicans* fungi for six months, likely due to the short preservation period. However, the fungi did show reduced inhibition zones against antifungal agents. In studies carried out by Ayala-Zermeño *et al.* (2017) and (2023) tested the genetic stability of a strain of *H. citriformis* recovered at two and 7.2 years from the MO conservation method the DICE similarity coefficients from AFLP analysis show a values of 99.24 % and 98.07 % (standard deviation range from 0.57 to 3.51 %), respectively, which indicating a high genetic stability contrasted with the original isolate in this conservation method.

Concerning the *H. citriformis* isolates recovered in SDAY, some of them exhibited a phenotype with mucilaginous-looking colonies containing yeast-like bodies during the early stages of colony development (1-5 cm) and persisted for up to 34 days of growth (Figures 3a, b). Pérez-González *et al.* (2015b) also reported similar growth in *H. citriformis* isolates from Mexico; however, the authors noted that this phenotype is exclusively expressed under dark conditions, which is in contrasts to the present study where the isolates grew under both light and dark conditions.

Ex situ conservation is a crucial step in the utilization of microorganisms to preserve their inherent characteristics for prolonged periods. In the present study, *H. citriformis* and *H. thompsonii* fungi were conserved in oil, and most of the isolates were successfully recovered viable. However, it will be essential to evaluate the genetic integrity of these isolates over extended periods in future studies.

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