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EDIBLE SPECIES OF THE FUNGAL GENUS *HEBELOMA* AND TWO NEOTROPICAL PINES

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Abstract

Mexico has one of the largest diversities of pines and ectomycorrhizal fungi known world-wide. Therefore, describing native ectomycorrizal species from the country associated with pines is important because of their biotechnological potential in the forestry and food sectors. Worldwide, *Hebeloma* has generally been considered a genus of poisonous ectomycorrhizal fungi. However, interestingly, in central Mexico there is a complex of under-studied *Hebeloma* species which are used as food in large quantities and have a great economic and social importance. Three edible species of *Hebeloma* widely marketed in the country were identified: *Hebeloma alpinum*, *H. mesophaeum* and *H. leucosarx* with scanning electron microscopy on the basis of different ornamentation patterns in the spores of these species. Synthesis was carried out by inoculating two Neotropical pines with sporomes of the three described *Hebeloma* species. To achieve this, inoculated pines were kept in greenhouse conditions during one year. A characteristic morphotype for each fungal species was observed and it is described here. The first known description of the morphotype of *Hebeloma alpinum* with pines is presented. This seminal work gives a tool to identify the morphotypes produced by the main edible ectomycorrhizal species of *Hebeloma* marketed in Mexico, with biotechnological potential to inoculate pines used in reforestation programmes in Neotropical areas.

Key words: *Pinus,* Edible ectomycorrhizal mushrooms, Forest biotechnology, Forest sustainable development.

Introduction

On a global scale, Mexico has the greatest richness of pine species, with 71 taxa of which 55% are endemic (Sánchez-González, 2008).There is also a great diversity of pine-associated ectomycorrhizal fungi that are economically important due to their use as food and medicine, and in biotechnological forestry applications. Forty percent of ectomycorrhizal fungal genera known in the world have been recorded in Mexico (Pérez-Moreno *et al*., 2015). One of these is the genus *Hebeloma*, which is widely distributed with around 250-600 species worldwide (Marmeisse *et al*., 1999). According to various reports this genus has been recorded from Europe, Australia, Asia, Africa, North America and South America (Cairney & Chambers, 1999; Aremu *et al*., 2009; Ohenoja & Ohenoja, 2010).

Worldwide, the genus *Hebeloma* has been considered toxic. The species which contain toxic metabolites include: *H. crustuliniforme*, *H. sinapizans* (De Bernardi *et al*., 1983), *H. spoliatum* (Fujimoto *et al*., 1992), *H. senescens* (Bocchi *et al*., 1992; Garlaschelli *et al*., 1995), *H. vinosophyllum* (Fujimoto *et al*., 1986; Fujimoto *et al*., 1991), *H. longicaudum* (Wichlacz*et al*., 1999) and *H. versipelle* (Liu, 2002). Some of the toxins identified include cytotoxic triterpenes, lanostane-type triterpene esters, neurotoxic cucurbitane-type glycosides and 6,7-seco-caryophyllenes and related sesquiterpenoids (Fujimoto *et al*., 1986, 1991, 1992; Garlaschelli *et al*., 1995; Wichlacz *et al*., 1999). Interestingly, in central Mexico large amounts of sporomes of the ectomycorrhizal genus *Hebeloma* are marketed and used as food (Montoya *et al*., 2008; Pérez-Moreno *et al*., 2008), and they are sold in a complex of species; this is the first study to identify which species are involved. It is worth mentioning that its edibility has only been reported in Mexico (Pérez-Moreno *et al*., 2008; this work) and in Nigeria, Africa (Aremu *et al*., 2009). However, the identification of these species and their ectomycorrhizal characterization has received little attention despite their potential for biotechnological use.

This research had the following objectives: i) Identify and describe edible species of *Hebeloma* that are widely marketed in central Mexico; and ii) Carry out synthesis of ectomycorrhizae of *Pinus patula* Schl. et Cham. and *P. pseudostrobus* Lindl. var. *pseudostrobus* by inoculation with the most common edible species of *Hebeloma* in order to describe the corresponding morphotypes.

Materials and Methods

Collection and identification of fungal species studied: The species studied were collected in the market of Ozumba, State of Mexico, which is located in the central part of Mexico, at 19° 02' latitude and 98° 48'14" longitude N/W, at an average altitude of 2340 m (Anon., 2010). The suppliers of that market collect

species of the genus *Hebeloma* in surrounding pine forests. Specifically, species of the genus *Hebeloma* were acquired and characterized according to macro and microscopic characteristics specified by Largent (1973) and Largent *et al*. (1977). The mean measurements presented correspond to the analysis of 25 sporomes for each case. The specimens were chosen from a collection of around 500 sporomes collected during the rainy season. Photographs of the spores of the *Hebeloma* species were taken using a JEOL JSM-5800LV scanning electron microscope.

Synthesis of ectomycorrhiza: Once the fungal material was collected and classified by species, the inoculum was prepared. The stipe was cut from the sporomes and only the pileus was used. The pilea were dehydrated with steam in a JERSA model L tray dryer, at a temperature of 30°C ± 2°C because at a temperature above 35°C, spores located in the pileus gills lose their viability (Brundrett *et al*., 1996).Once dehydrated, the pilea of each fungal species were milled using a Thomas hammer mill with 1 mm sieve. Finally, the spore-based inoculum obtained was stored in 1.5 mL vials at 5°C until inoculation of pines.

Prior to inoculation, seeds of *Pinus patula* and *P. pseudostrobus* were soaked in water for 24 h, then disinfected with hydrogen peroxide (H_2O_2) at 30%, while maintaining the seeds in stirring for 20 minutes, and finally rinsed with distilled water. *Pinus patula* is a native species of Mexico widely introduced to Africa, Asia and south of America. In Mexico its wood is used for construction, elaboration of boxes and poles, for protection of watershed areas; and also is widely used in reforestation and restoration of degraded areas (Patiño & Yoshio, 1991). *Pinus pseudostrobus* is also a native species of Mexico, Guatemala, Honduras and El Salvador (Cambrón-Sandoval *et al*., 2013). It has great economic importance because its wood is strong and therefore is widely used for live fences; also is used in the production of turpentine and it has some medicinal properties (Avendaño & Acosta, 2000; Iloff & Mirov, 1953; Estrada-Castillón *et al*., 2012).

The substrate used consisted of a mixture of sand, bark and soil at a 2:2:1 ratio, which was sterilized with steam for 24 h. The 140 mL containers used for sowing the pine were washed and disinfected prior to being filled with the substrate. The pines were sown in the containers filled with substrate and watered every other day. Germination of both pines occurred at about 3 weeks after seeding. In order to prevent damping off, Captan fungicide (N-trichloromethylthio-4-cyclohexene-1,2-dicarboximide) was applied immediately after germination at a ratio of 2 g L^{-1} every other day along with irrigation until the stem was lignified. Inoculation of the ectomycorrhizal fungus to the pines was conducted in two stages, the first a week after germination and the second 90 days later. The concentration of each inoculation was 10^6 to 10^8 spores per mL per pine. The concentration of these spores was determined with a hemacytometer. The experiment was run for 52 weeks in the greenhouse, at which time the destructive analysis for the morphological characterization of the mycorrhizae was performed.

Description of morphotypes: The trees were extracted from the containers and their root systems soaked for 24 h, then the shoots were cut off and the roots carefully rinsed. Roots in good condition were selected to analyze the diagnostic structures of each ectomycorrhiza (length, diameter, base-apex ratio, ramification type, tip shape, mantle texture, apex color and anatomy in the outer mantle layer) called morphotypes based on the information system for characterizing and identifying ectomycorrhizae (Agerer, 1990; Agerer & Rambold, 2015). The term morphotype refers to the structure formed by both the fungal and the plant tissues in the modified roots of the host plants, which present a characteristic size, colour, texture and branching patterns for each host-fungus combinations (Brundrett, 2008). These basic characteristics have been widely used to characterize a particular combination of ectomycorrhizal fungi and their associated hosts (Agerer & Rambold, 2004).

Photographs of the morphotypes were taken using an Olympus SZ61 model SZ2-LGB stereoscope, and of the mantle using an Olympus BX51 model U-LH100H microscope.

Results and Discussion

Diagnostic description of sporomes: Within the complex of *Hebeloma* studied, three species were identified: *H. mesophaeum* (Pers.) Quél., *H. leucosarx* P. D. Orton and *H. alpinum* (J. Favre) Bruchet.

***Hebeloma alpinum*:** It has a convex, dark brown pileus with a diameter of 1.2-5.2 cm. Its stipe is cylindrical, cream colored, 1.2-5.7 cm long and 0.4-1.8 cm wide (Fig. 1f). The gills are free and dark brown. Spore shape is elliptical with lacunose ornamentation (Fig. 1e). Like *H. leucosarx* and *H. mesophaeum*, the spores are brown with an average length and width of 8.8 µm y 4.7 µm, respectively (E=1.87).

***Hebeloma leucosarx*:** The pileus was flat with a diameter of 1.5-9.8 cm. It presented a very light brown color, giving it a whitish appearance (Fig. 1b). In some specimens there was a reddish brown colour in the center, especially in those that were at a mature stage. As for the stipe, it was almost entirely whitish and cylindrical in shape. The length of the measured stipes ranged from 2.8-8.7 cm with a width of 0.4-2.1 cm. The type of attachment of the gills to the stipe apex observed was free; i.e., the gills did not touch the stipe, and they were dark brown. The spores were elliptical with an average length and width of 8 µm and 4.8 µm respectively (E=1.66), and they were brown with dotted to light reticulate ornamentation according to what was observed in the scanning photographs (Fig. 1a).

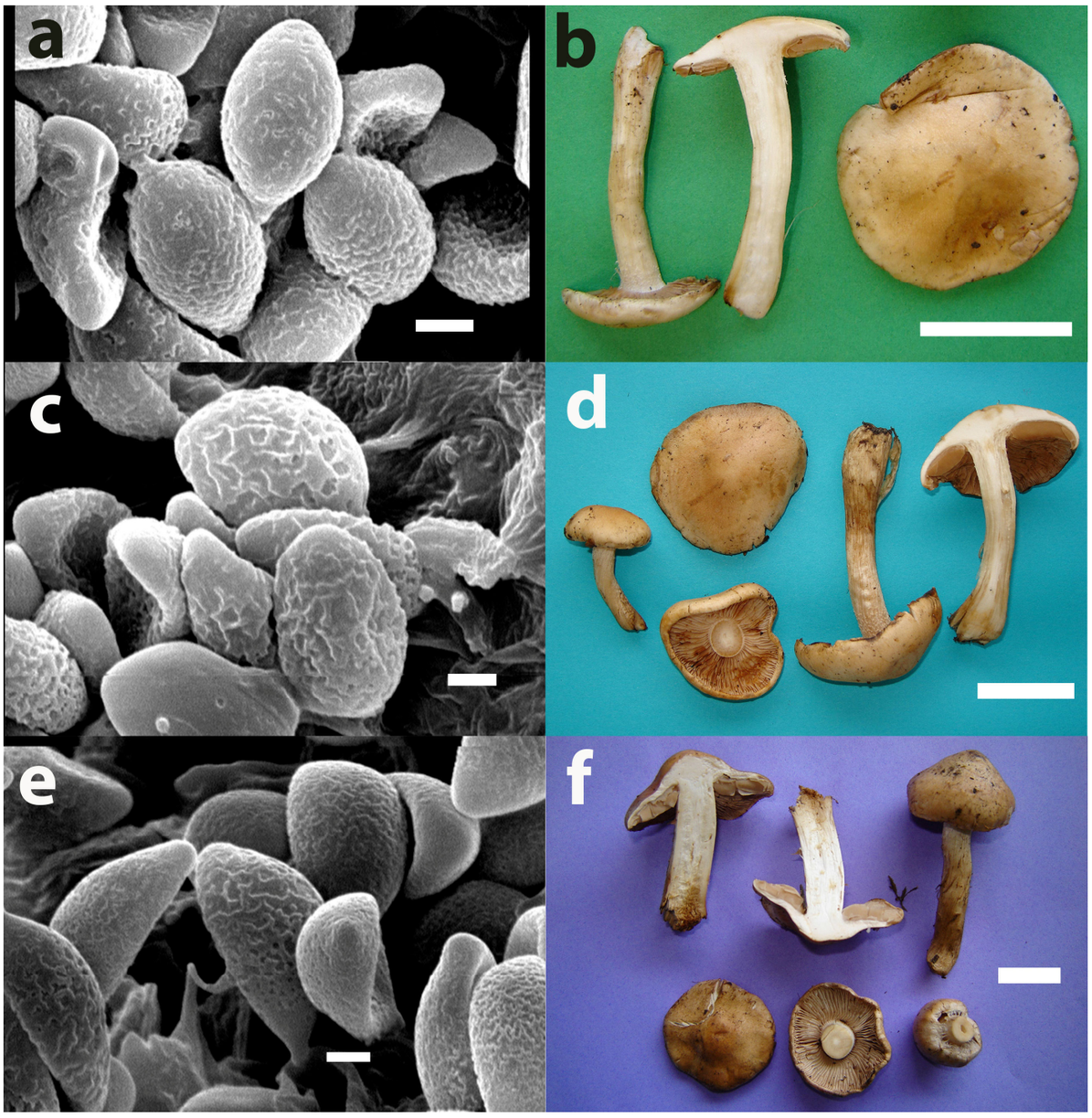

Fig. 1. Spores of *H. leucosarx* (a), *H. mesophaeum* (c) and *H. alpinum* (e) magnified al 6000x in SEM; Sporomes of *H. leucosarx* (b), *H. mesophaeum* (d) and *H. alpinum* (f).White bars= 2µm (a, c and e), 4 cm (b), 3cm (d) and 1 cm (f).

Most of the observations made in this study for *H. leucosarx* coincide with those of Vesterholt (2000), who also observed a convex pileus that expands over time, that is whitish to cream colored, and has a pink center that fades to pale cream as the distance from it increases. The color of the gills was also brown. As for the observations of Vesterholt compared to this study, there are minor differences in the measurements for the pileus (diameter: 19-60 mm), stipe (19-80 x 5-11 (5-18 mm)) and spores (10.4-12.8 x 5.8-6.9 µm).The description of the type specimen of *Hebeloma leucosarx* according to Orton, (1960) is consistent with the description of the specimen described in the present work particularly in the shape of the pileus, stipe color and the characteristic smell of fungus.

Hebeloma mesophaeum: It presented a convex pileus with a diameter of 1.7-7.1 cm, with a cream brown coloration tending to reddish in its center (Fig. 1d). The stipe had a cylindrical shape of the same color as the pileus with a length of 2-6.2 cm and a width of 0.3-1.9 cm. The gills had a free-type attachment and were dark brown. As for the spores, they were elliptical with an average length and width of 8.6 µm and 4.6 µm, respectively (E=1.87), brown and with reticulate ornamentation (Fig. 1c).These observations are consistent with those made by Arora (1979) with regard to pileus diameter and color, stipe shape, and gill type and color, but differ in that Arora (1979) reported a longer stipe length (80 mm), a smooth spore ornamentation and smaller spore width (5-6 µm).

Table 1. Comparison of diagnostic characteristics of sporomes of *H. mesophaeum*, *H. leucosarx* and *H. alpinum*, described in this study.

Structure	Characteristics	*H. mesophaeum*	*H. leucosarx*	*H. alpinum*
Pileus	Shape	Convex	Flat	Convex
	Colour	Cream brown	Whitish	Dark brown
	Diameter (cm)	1.7-7.1	1.5-9.8	1.2-5.2
Stipe	Shape	Cylindrical	Cylindrical	Cylindrical
	Color	Cream	Whitish	Cream
	Width (cm)	0.3-1.9	0.4-2.1	0.4-1.8
	Length (cm)	2-6.2	2.8-8.7	1.2-5.7
Gills	Attachment	Free	Free	Free
	Color	Dark brown	Dark brown	Dark brown
Spore	Shape	Elliptical	Elliptical	Elliptical
	Color	Brown	Brown	Brown
	Ornamentation	Reticulate	Dotted to light reticulate	Lacunose or with plate-like structure
	Length (µm)	8.6	8	8.8
	Width (µm)	4.6	4.8	4.7
Smell		Radishlike	Radishlike	Radishlike

n= 25 sporomes (see materials and methods)

The main difference between *H. mesophaeum*, *H. leucosarx* and *H. alpinum* at first sight was the size of the sporomes and the darker color of the pileus in the case of *H. alpinum* (Table 1). It was observed a slightly dotted ornamentation for *H. mesophaeum*, which differs from the smooth ornamentation reported by Arora (1979). For this reason scanning electron microscopy was conducted and clear differences were observed in the ornamentation of the three species using this technique. The ornamentation of the spores is an important feature in the species of *Hebeloma* according to Vesterholt (2005). Rücker (1987), described the ultrastructure of 55 species of *Hebeloma*, and classified the different spore ornamentations in three types termed: A, B and C. The type A was defined as spores with branched ridges, the type B spores with simple and uniform ridges and finally the type C spores, with plate-like structures. According to this classification the type of ornamentation observed in our work corresponds in the case of *Hebeloma leucosarx* to the type B and *Hebeloma alpinum* and *H. mesophaeum* to the type C. To our knowledge this is the first time that the ultrastructure of *Hebeloma alpinum* and *H. leucosarx* are illustrated using scanning electron microscopy. Specimens of these species are deposited in the collection of the Colegio de Postgraduados, Texcoco, Mexico.

Description of morphotypes: Species of the genus *Hebeloma* have a great economic, biotechnological and ecological importance in Mexico. Recently Garibay-Orijel *et al*., (2013), showed that it was one of the best represented genera in the forest spore banks of the Mexican Neovolcanic axis, which crossed the country from the Atlantic to the Pacific Ocean. As a result, biotechnologically it is very important to identify and describe the morphotypes of the different species in the genus *Hebeloma* synthesized in pine species useful for the restoration of degraded areas.

***Hebeloma leucosarx*:** The main distinctive feature of this morphotype compared with *H. mesophaeum* and *H. alpinum* is that it presents a type of ramification that is mostly absent and sparsely dichotomous, with an approximate length of 1-9 mm and a diameter of 0.3 mm. The unramified tips were moniliform and cylindrical, with light brown coloration and darker at the tip. It is important to note that mycorrhizae in their juvenile stage presented a white tip. In their mature or adult stage, the base (10 mm) was larger than the apex (2mm). In terms of the surface mantle, it was smooth with a plectenchymatous anatomy in its outer layer. Some of the observations coincide with those made by Garibay-Orijel *et al*., (2013), who described the morphotype of *Hebeloma leucosarx* P. D. Orton associated with *P. montezumae*. The similarities are in monopodial growth and emanating thin hyphae, but differ in the perception of the morphotype color since he describes them as a dense grayish brown. Also, this study did not find that the morphotype branched dichotomously. Although this morphotype has recently been described by Garibay-Orijel *et al*. (2013), to our knowledge this is one of the first studies to report its spread and synthesis in a nursery, and also to record this fungus from Neotropical areas.

One of the most important evidences that demonstrate the ectomycorrhizal status of a mushroom is their synthesis with plant host (Rinaldi *et al*., 2008). Some of the species of the genus *Hebeloma* that have been previously synthesized include**:** *H. crustuliniforme*, *H. cylindrosporum,* and *H. saccharioliens* (Fox, 1986; Obase *et al*., 2009; Debaud *et al*., 1981; Brunner, 1991; Wong & Fortin, 1989). In this work the synthesis of *H. alpinum*, *H. mesophauem*, and *H. leucosarx* was achieved with *Pinus patula* and *P. pseudostrobus*. Additionally a detailed description of the synthesized mycorrhizas is presented for the first time.

Table 2. Distinctive characteristics of short roots with mycorrhiza of *H. mesophaeum* in this paper compared with previous descriptions.

Characteristics	*H. mesophaeum* with *P. patula* and *P. pseudostrobus*	*H. mesophaeum* with *P. ponderosa*	*H. mesophaeum* with *Pinus* sp.	*H. mesophaeum* (Pers.)
Type of ramification	Dichotomous or absent in the same proportion	Dichotomous or absent	Dichotomous	Infrequent ramification
Shape of unramified tips	Straight with cylindrical ends	Straight or curved	Cylindrical	Longitudinal and thin
Rhizomorphs	Absent	Absent	Absent	n.r
Mantle texture	Cottony	Felted	n.r	n.r
Apex color (in juvenile state)	Brown	Ochre yellow with translucent robe with occasional white patches	Brown to white	From silvery to white
Colour of the apex (in state mature)	White	n.r	n.r	Rusty
Anatomy of the outer mantle	Plectenchymatous	Plectenchymatous	Plectenchymatous	n.r
Source	Present work	Barroetaveña *et al*., (2012)	Agerer & Rambold (2015)	Ingleby *et al*., (1990)

n.r. = Not reported

Hebeloma mesophaeum: The type of ramification observed was mostly dichotomous, with unramified tips 1-4 mm long and 0.2 mm in diameter. The unramified ends were straight and cylindrical at the tip, brown and elsewhere white, without rhizomorphs. The base-apex ratio of the mycorrhiza varied, with the base being larger (1-2 mm) than the apex (0.5-1.4 mm). The mantle was densely cottony and the anatomy of its outer layer plectenchymatous. Macromorphologically, abundant emanating white hyphae were observed. This species is one of the most studied within the genus *Hebeloma*. It is interesting to note that different studies have reported differences in the color of the mycorrhiza, from a yellowish ocher, to white brown and even silver. Length reported measurements of the morphotype varied from 1-10 mm (Table 2).

Hebeloma alpinum: This species showed an absent or dichotomous and sparsely tetrapodial type of ramification, and the unramified tips were straight with cylindrical ends, 1-5 mm long and 0.2 mm in diameter, without rhizomorphs. In their juvenile stage, they were brown, with the base being darker than the tip. In its mature stage, the mycorrhiza turned dark brown and was invaded by white hyphae. Like the other mycorrhizae described in their adult stage, the base (3mm) was longer than the apex (1mm). The type of mantle on the surface was smooth and the anatomy of the outer layer was plectenchymatous. It also showed the presence of abundant emanating hyphae. Macromorphologically, they appeared white. To our knowledge this is the first description of the morphotype of *H. alpinum* with pines. Debaud *et al*., (1981) obtained synthesized ectomycorrhizae in *Dryas octopetala*.

In the case of *H. leucosarx*, the species had the longest short roots followed by *H. mesophaeum* and *H. alpinum*. In addition, in most cases it presented absent-type ramification and to a lesser extent dichotomous-type, while the opposite was true for *H. alpinum*. Regarding *H. mesophaeum*, it was easy to identify because of its cottony mantle texture and its intermediate size relative to *H. alpinum* and *H. leucosarx*. Although differences were observed among fungi, they were not observed the two pines studied (*Pinus patula* and *Pinus pseudostrobus*). Although there is a great diversity of species within the genus *Hebeloma*, most research has mainly focused on two species: *H. crustuliniforme* and *H. cylindrosporum*. One reason is that *H. cylindrosporum* is easily handled in the laboratory and in the case of *Hebeloma crustuliniforme* because it has a wide variety of hosts, in addition to its potential as forest inoculum (Cairney & Chambers, 1999). However most studies of these species relate to their physiology, biochemistry, genetics and molecular biology (Kedi *et al*., 2013). In general all of the species discussed in the present contribution have received little attention world-wide (Fig. 2).

Conclusions

The inoculum based on the dried sporomes of *H. leucosarx*, *H. mesophaeum* and *H. alpinum* was successful in forming ectomycorrhizae with *Pinus patula* and *Pinus pseudostrobus*.

Morphological differences were observed in the morphology of sporomes and morphotypes of the three species of fungi studied (*H. leucosarx*, *H. mesophaeum* and *H. alpinum*) in length, diameter, ratio (base-apex), ramification type, tip shape, texture and color. However, the morphology of ectomycorrhizae analyzed were the same for both pines.

To our knowledge this study presents the first description of the ectomycorrhizal morphotype of *Hebeloma alpinum* and the spore ornamentation of *H. alpinum* and *H. leucosarx* is illustrated for the first time with scanning electron microscopy.

It is important to characterize the fungal species and their associated ectomycorrhizal morphotype, studied due to their great local use in forestry and their potential economic and social importance.

Despite the great economic, biotechnological and environmental importance of the genus *Hebeloma* in Mexico, few studies have been conducted in the country of this fungal diversity in Neotropical areas. Therefore, this is a seminal study to identify the most abundant species within the complex of species of *Hebeloma* marketed in Mexico.

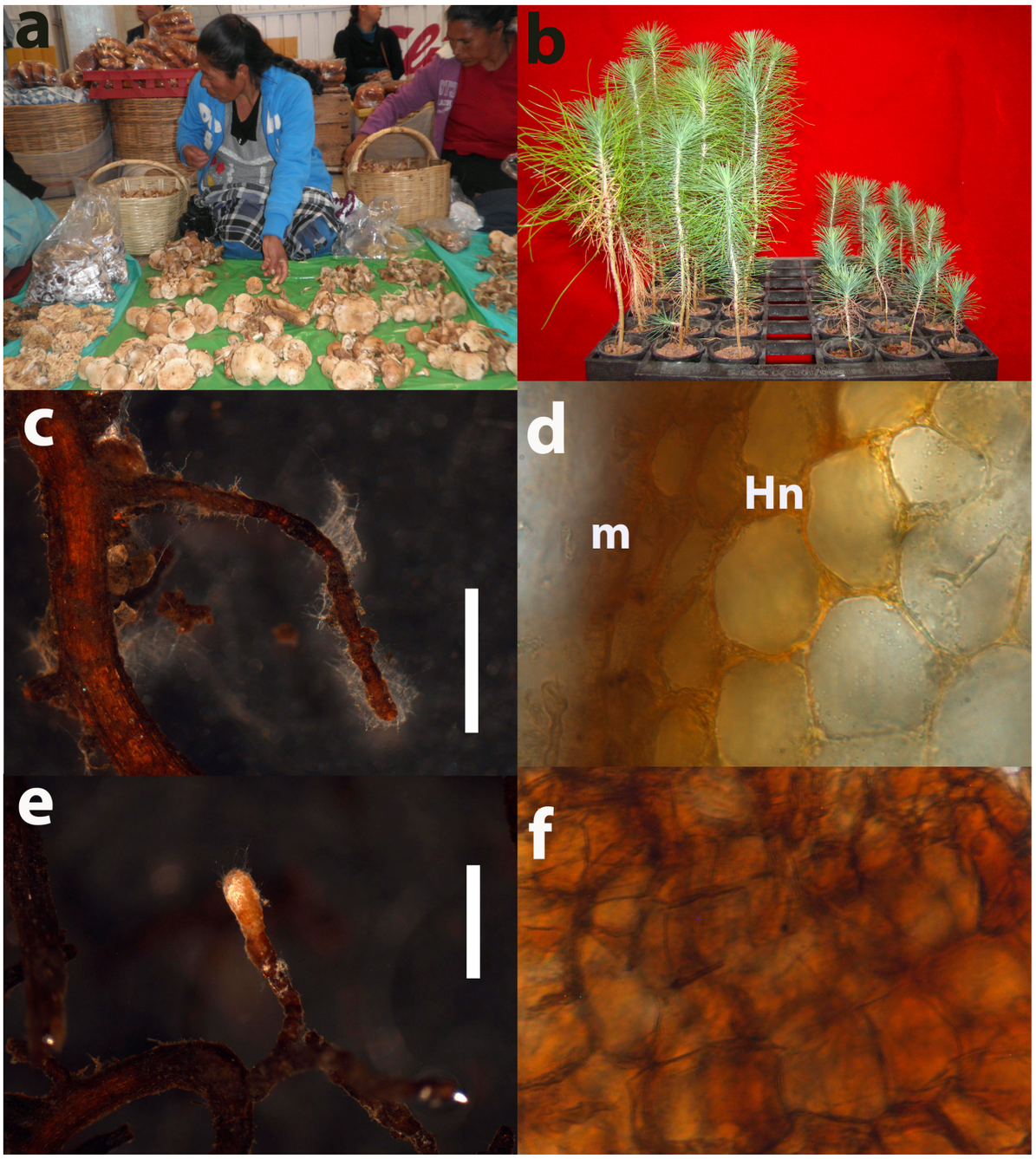

Fig. 2. a. Sale of *Hebeloma* spp. in the Ozumba market, b. Comparison of *Pinus patula* inoculated with *H. mesophaeum* (left) and uninoculated (right), c. Morphotype of *H. leucosarx*, d. Mantle (m) and Hartig net (Hn) of *H. leucosarx* and e. morphotype of *H. alpinum* and f. mantle pattern of *H. alpinum* in *Pinus patula*. Black bars= 0.5 cm (c) and 0.3 cm (e).

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