https://doi.org/10.32854/agrop.v14i2.1871

Mycelial disturbance stimulates the formation of sporomes of edible ectomycorrhizal fungi associated with two neotropical pines

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ABSTRACT

Objective: To determine the effect of mycelial disturbance on the formation of sporomes of two edible ectomycorrhizal fungi of great biocultural relevance in Mexico (*Laccaria laccata* and *Hebeloma leucosarx*) associated to two Neotropical pines with economic and ecological importance, *Pinus greggii* and *P. montezumae*.

Design/Methodology/Approach: Spore inoculum was produced using ground pilei of the evaluated ectomycorrhizal fungi; each pine plant was inoculated with 10^7 to 10^8 fungal spores. A completely randomized experimental design was used with four treatments and six replicates per treatment for each pine species, having a total of 48 experimental units, each one consisting in an inoculated tree. During two years the sporome production was evaluated in the treatments with and without mycelial disturbance. The duration of the experiment, since seed germination, was 5 years.

Results: The mycelial disturbance originated a higher formation of sporomes in both fungal species, regardless of the associated tree species. The highest sporome formation was recorded in plants inoculated with *H. leucosarx* compared to those inoculated with *L. laccata* in both pine species. Mycelial disturbance, originated a higher number of sporomes in *Pinus greggii* compared to *P. montezumae*.

Study Limitations/Implications: The evaluation of factors influencing sporome formation in edible ectomycorrhizal fungi requires long term experiments.

Findings/Conclusions: This study shows for the first time that mycelial disturbance increases sporome formation in Neotropical ectomycorrhizal fungi. Additionally, a differential influence of the fungal and tree species on the number of produced sporomes was found. These findings shed some light on potential cultivation methods for edible ectomycorrhizal mushrooms.

Keywords: spore inoculum, ectomycorrhizal symbiosis, wild edible fungi, mycelial disturbance.

Agroproductividad: *Vol. 14, Núm. 2, febrero. 2021. pp: 93-99.*
Recibido: *noviembre, 2020.* **Aceptado:** *enero, 2021.*

INTRODUCTION

Ectomycorrhiza is a mutualistic symbiosis of paramount structural and functional importance in forest ecosystems. The edible ectomycorrhizal mushrooms *Laccaria laccata* and *Hebeloma leucosarx* have great biocultural and economic relevance in Mexico and they are commercialized in numerous Mexican markets (Pérez-Moreno *et al*., 2008; Pérez-Moreno *et al*., 2019). The genus *Hebeloma* has been reported as a toxic fungus in other latitudes (De Bernardi *et al*., 1983; Liu *et al*., 2002). However, in central Mexico, large amounts of diverse species of that genus are consumed and commercialized (Montoya *et al*., 2008; Pérez-Moreno *et al*., 2008). Worldwide there is high demand for the production of edible ectomycorrhizal fungi which represent a potential agroindustry of high economic and environmental importance, due to their relevance in successful reforestation programs and given their nutritional quality and medicinal use. However, despite their importance, the factors that originate the sporome formation of edible ectomycorrhizal fungi have been scarcely studied. Previously, it has been reported that the light quality is a factor that influences the formation of ectomycorrhizal sporomes (Villegas-Olivera *et al*., 2017). In addition, it has also been shown that the application of electrical impulses stimulates the formation of sporomes both in saprotrophic and ectomycorrhizal fungi, including: *Pholiota nameko*, *Lentinula edodes*, *Lactarius deliciosus*, *Laccaria laccata* and *Tricholoma matsutake* (Guerin Laguette *et al*., 2000; Ohga *et al*., 2011; Takaki *et al*., 2009; Ohga *et al*., 2012). However, it is unknown whether the rupture of hyphae is a factor that stimulates the formation of edible ectomycorrhizal fungi sporomes. This study assessed the hypothesis that mycelial disturbance increased the sporome formation of two edible ectomycorrhizal fungi associated with two Neotropical pines of great economic and ecological importance, *P. greggii* and *P. montezumae*.

MATERIALS AND METHODS

Fungal material and preparation of the inoculum

The fungal material used was acquired in the Ozumba market, Estado de México, and collected in pine forests adjacent to this region. The inoculum was obtained from fresh sporomes of *H. leucosarx* and *L. laccata*, separating the pileus from the stipe. The pilei were dehydrated at 35°C (JERSA drier, Cuatitlán Izcalli, Mexico). Later these pilei were ground and sieved in a mesh with opening of 1.19 mm to homogenize the particle size. The spore concentration was evaluated, by using a Neubauer chamber (Marienfeld, Lauda-Königshofen, Germany), which was estimated to be of 10^7-10^8 spores per cm^3 of inoculum. The final inoculum was stored in vials of 1.5 cm^3 at 5 °C, until it was used.

Establishing the experiment

Seeds of *P. greggii* and *P. montezumae* were collected in a natural forest of Xochicoatlán, Hidalgo, and Cofre de Perote, Veracruz, respectively. Previous to sowing, the seeds were soaked in running water for 24 hours, and then they were disinfected with H_2O_2 at 30% for 20 minutes and rinsed 4 times with sterile distilled water, under aseptic conditions. Sowing consisted in placing three seeds at a depth of 2 cm from each pine species in black plastic forest tube containers with a volume of 140 cm^3. These containers had 150 g of substrate made from a mixture of river sand, pine bark and forest soil, in proportion 2:2:1. The substrate was sterilized previously with vapor at a pressure of 1.3 kg cm^2 and a temperature of 125 °C for 3 h, then it was left resting for two days and, on the fourth day, it was sterilized again for 2 h. Once the seeds germinated, and when the plants had their first true leaves, each plant was inoculated with 10^7 to 10^8 spores of *L. laccata* and *H. leucosarx*. The inoculum was placed at three centimeters depth; then covered with a layer of substrate until filling the container, and a layer of sterilized volcanic rock was placed on their surface. The plants were kept in a greenhouse with irrigation every third day with sterile distilled water during 3 years (Figures 1 a-c).

Mycelial disruption

To induce mycelial disruption, 3-year-old *P. greggii* (Figura 1a) and *P. montezumae* (Figura 1b) plants were selected, grown in forest tubes of 130 cm^3 volume, following the methodology previously described, with mycorrhization percentages of at least 90%. These plants were transplanted to PVC tubes (polyvinyl chloride) with capacity of 6 kg (Figure 1c). Five months after the transplant, perforations of 5×5 cm^2 were made at three heights of the tube, in the superior, middle and inferior parts. As inducer of mycelial disruption, soil was extracted with roots with the aim of provoking the split of hyphae. Finally, the perforations were covered with a mesh with opening of 0.3 cm. The same number of perforations was made on the PVC in the case of the experimental units of the control treatments and the

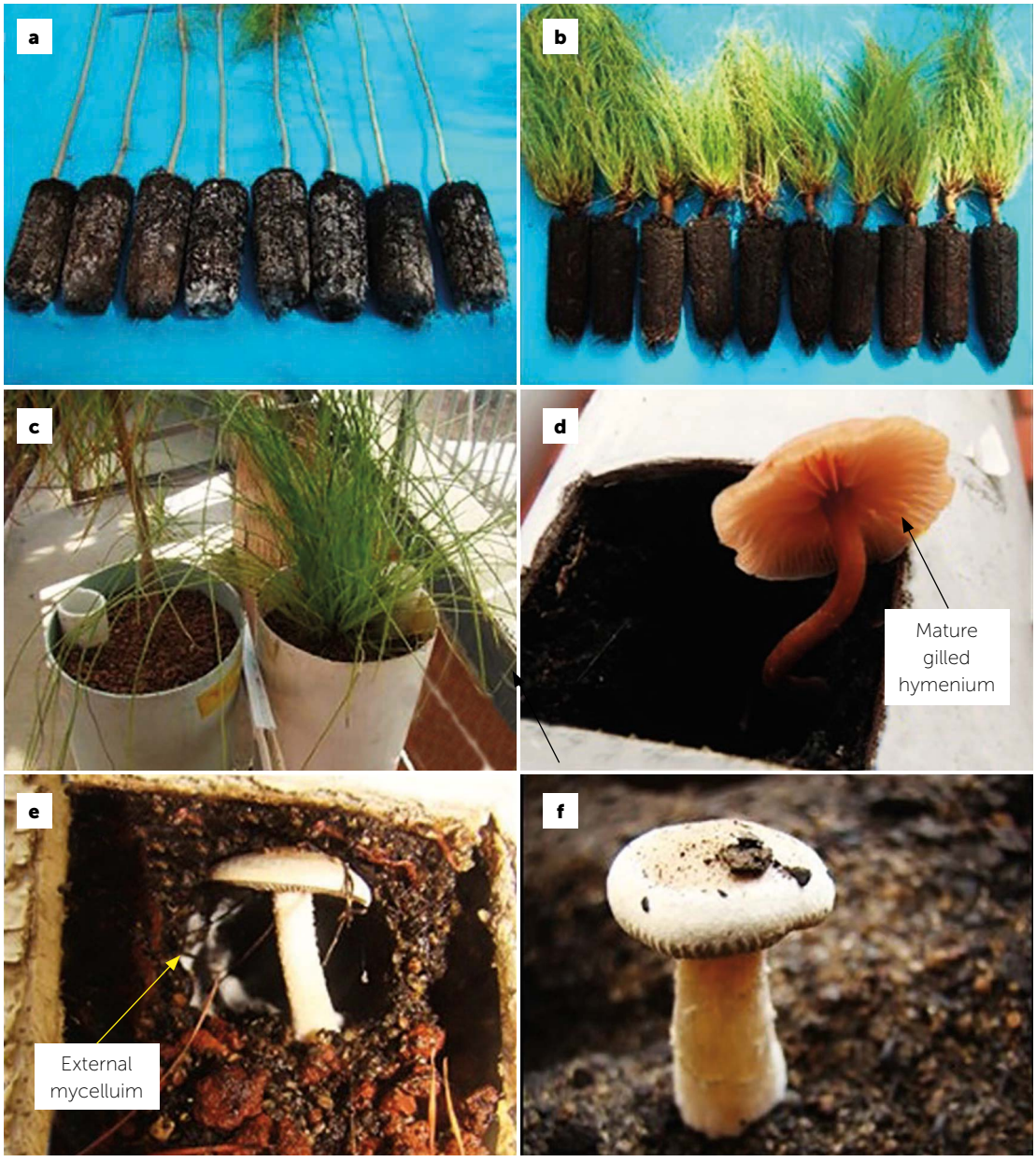

Figure 1. General aspects of the bioassay. a) *Pinus greggii* root balls showing abundant external hydrophobic mycelia of *Hebeloma leucosarx* (Hl); b) *P. montezumae* inoculated with *L. laccata* (Ll), showing hydrophilic mycelia in the root balls; c) General view of the experiment in PVC tubes; to the left *P. gregii* and to the right *P. montezumae*; d and e) Formation of mature sporomes of Ll (d) and Hl (e) in PVC containers, with mycelial disturbance. F) Mature sporome of Hl without mycelial disturbance.

mesh was placed, although the soil was not extracted so the mycelial disturbance was not made. All the plants were maintained during two more years to carry out the evaluations in the greenhouse, so the total duration of this experiment was 5 years.

Experimental design and statistical analysis

The experimental design used was completely randomized with 4 treatments and 6 repetitions for each pine species (*P. greggii* and *P. montezumae*). Then, there were 48 experimental units in total, each one consisting of an inoculated tree. The treatments were the following: i) Inoculated plants (IP) with *H. leucosarx* (Hl) without mycelial disturbance; ii) IP with Hl with mycelial disturbance; iii) IP with *Laccaria laccata* (Ll) without mycelial disturbance; and iv) IP with Ll with mycelial disturbance. A variance analysis was carried out and then Tukey's test was used out to compare means with $p \leq 0.05$ (SAS, 2002).

Sporome record and mycorrhization

Mycelial disturbance was carried out at the beginning

of April and the formation of sporomes was recorded daily in the PVC containers three months later, when the sporomes began to appear. This record was made during July to August, for 2 consecutive years, 2018 and 2019. The formation of sporomes was registered photographically (Figure 1d-f) with a Full HD 1080 digital camera (SONY Corporation, Japan). Only those sporomes that reached their state of maturity were counted, for which a slide was placed to collect the spores and carry out a microscopic evaluation of these (Figure 2c, f). The spores were photographed in a microscope Olympus BX51 model U-LH100H. At the end of the experiment a morphoanatomical evaluation of the mycorrhizal root tips in all of the experimental units was carried out in order to assess the presence of mycorrhizae belonging to the evaluated mycobionts, finding a minimal mycorrhization of 90% in all cases (Figure 2a, b, d, e).

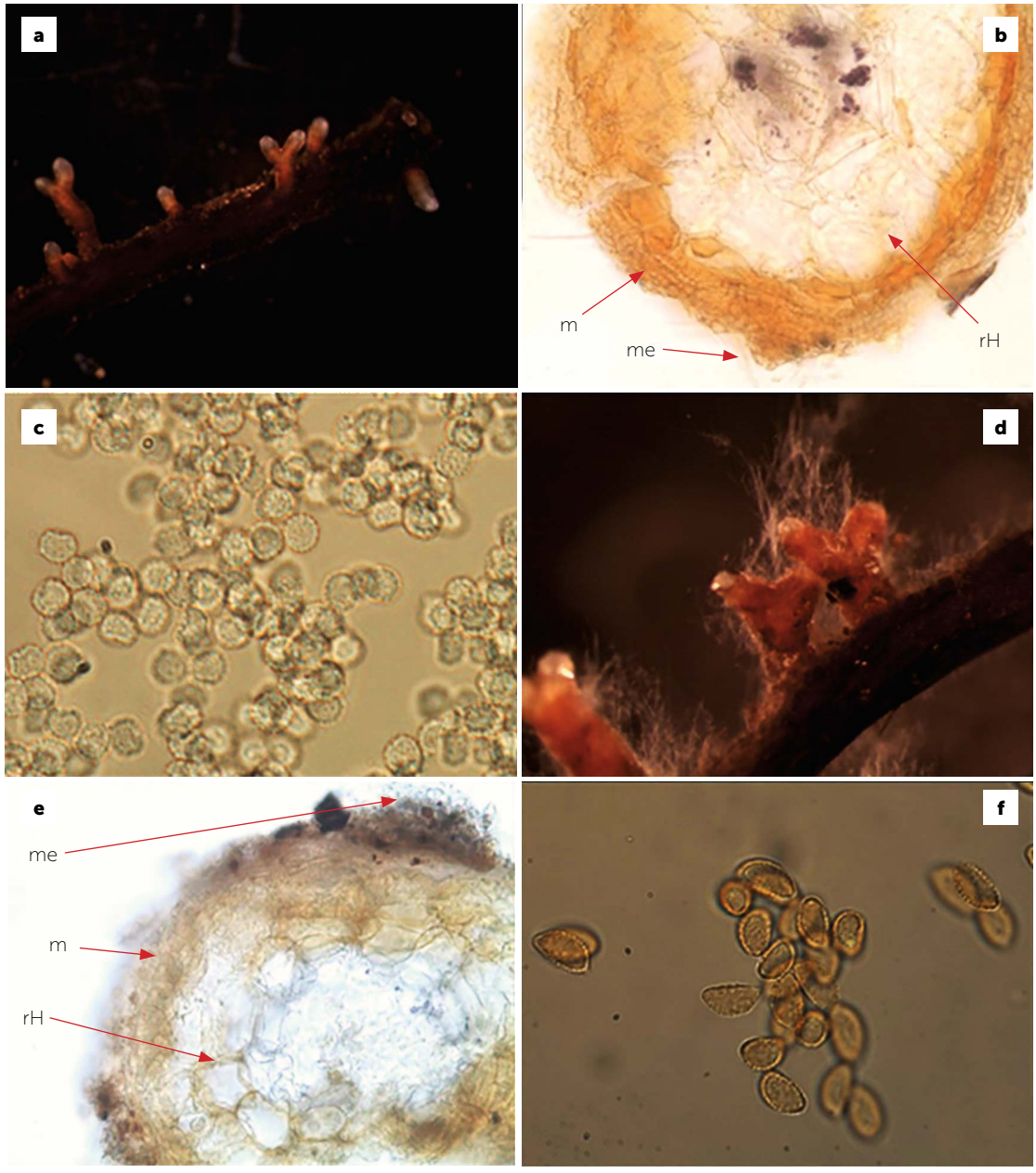

Figure 2. Microscopy of ectomycorrhizae of *Pinus greggii* with *Laccaria laccata* (Ll) and *Hebeloma leucosarx* (Hl) and morphology of mature spores. (a) Simple and dichotomous Ll mycorrhizal roots; (b) Cross section of Ll ectomycorrhiza showing Hartig net (rH), mantle (m) and external mycelium (me); (c) Echinulate globose spores characteristic of Ll produced by mature sporomes in the bioassay; (d) Hl mycorrhizal root with abundant loose emanating hyphae, white in color; (e) Cross section of Hl ectomycorrhiza showing abundant external mycelia (me), mantle (m) and Hartig net (rH); (f) Amygdaliform spores with verrucose ornamentation collected from mature sporomes of Hl produced in the bioassay.

RESULTS AND DISCUSSION

Mycelial disturbance and sporome number

The mycelial disturbance induced the sporome formation of *L. laccata* and *H. leucosarx*, regardless of the host plant (Figures 1d-f). The sporome number of *H. leucosarx* was twice higher when the plants were exposed to the mycelial disturbance compared to those that did not have mycelial disturbance, in *P. montezumae* and *P. greggii*, respectively. Meanwhile, in the case of *L. laccata* the sporome number recorded was three times higher (Figure 3).

Likewise, mycelial disturbance promoted a higher formation of sporomes of *L. laccata* (7%) and *H. leucosarx* (81%) compared to the experimental units without mycelial disturbance. It was observed that in the first year of evaluation, sporomes were formed with completely exposed lamellae from 90 to 150 days after the transplant to PVC tubes. From the mature sporomes, 57% and 43% corresponded to *H. leucosarx* and *L. laccata*, respectively. *P. montezumae* produced 20 sporomes, while *P. greggii* produced only 15 sporomes. The first sporomes of *H. leucosarx*, associated to *P. montezumae* were recorded 90 days after the transplant. In the second year after the mycelial disruption, 110 mature sporomes of *L. laccata* and *H. leucosarx* with completely exposed lamellae were found. Seventy-one percent corresponded to *H. leucosarx* and 29% to *L. laccata*. Sixty five sporomes were formed associated with *P. greggii*, and in contrast only 26 were formed with *P. montezumae*.

The sporome longevity, from a visible globose primordium of at least 3 mm of diameter until senescent sporomes, was on average 18 days. In contrast, the time elapsed from the beginning of the mycelial aggregates of 500 μm until the senescence of sporomes was 36 days. The sporomes of *L. laccata* and *H. leucosarx* produced abundant spore prints white in color 10 and 14 days, respectively, after the appearance of visible primordia. The microscopic analysis of these spores confirmed the identity of the fungal evaluated species. All the primordia of *L. laccata* and *H. leucosarx* were developed in the orifices that were made on the PVC tubes, with or without mycelial disturbance. However, there were clear differences in the sporome number between the experimental units with or without mycelial disturbance. All the sporomes presented positive geotropism, and they reached heights of 5 to 7 cm, which are similar to their heights recorded under natural conditions in forest areas (Figure 1d,e).

Diagnostic description of the sporomes of *H. leucosarx* and *L. laccata*

The sporomes of *H. leucosarx* pilei were cream-colored, sub-globose to hemispheric, changing to flat-convex with age, lamellae were concolorous with the pilei and turned light brown color when mature. The spores had amygdaliform shape (Figure 2f). The morphological characteristics of the spores of *H. leucosarx* recorded in this study agree with those described by Villegas-Olivera (2017). Meanwhile, the sporomes of *L. laccata* presented convex pilei salmon pink in color to brown orange, and cylindrical stipes concolorous with the pileus or slightly darker, with free, pale pink lamellae. The spores had globose shape and echinulate ornamentation (Figure 2c). The characteristics of the sporomes of *L. laccata* agreed with those described by Mueller (1985).

The factors that promote the sporome formation by ectomycorrhizal fungi associated to their plant symbionts have not been fully understood. However, it is reasonable to hypothesize that these factors are differential among the huge diversity of ectomycorrhizal fungi known worldwide. In this study it was found that the mycelial disturbance promoted a higher formation of sporomes of *L. laccata* and *H. leucosarx*. A greater

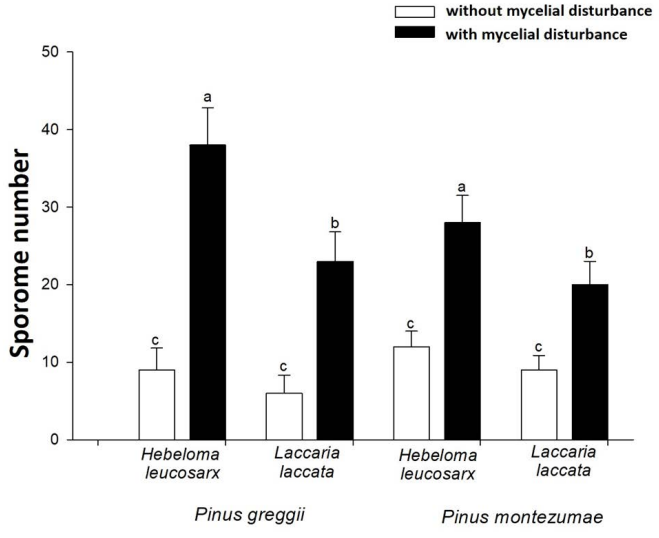

Figure 3. Production of sporomes during a 3-month period (from July to August) of two edible ectomycorrhizal fungi associated with 2 species of 5-year old Neotropical trees, without mycelial disturbance (hollow columns) and with mycelial disturbance (full columns). The bars represent average values and the lines over them the standard error of the mean. n=6. Bars with the same letter for each tree species are equal according to Tukey's means comparison test ($p \leq 0.05$).

induction of the formation of sporomes of *L. laccata* and *H. leucosarx* was observed with *P. montezumae,* compared to *P. greggii*, under the same environmental conditions. Previously, Godbout and Fortin (1990) had found that *L. bicolor* formed more sporomes with *P. strobus* than with *P. taeda* and *Picea glauca*. In addition, it has been found that environmental conditions such as temperature, carbon dioxide concentrations in the environment and salinity are determinant in the formation of sporomes by ectomycorrhizal fungi (Kües and Liu, 2000). The influence of the fertilization regimes and of the relative humidity in the formation of sporomes have also been demonstrated (Godbout and Fortin 1990). It has also been shown that the application of electrical impulses promotes the formation of sporomes, probably associated to the fact that the cracks that they generate in the mycelium modify the enzymatic activity, mainly that of laccases and proteases (Ohga *et al*., 2001; Ohga and Ida, 2001; Islam *et al*., 2013; Takaki *et al*., 2014). Ohga *et al*. (2001) recorded the formation of sporomes of *L. laccata* associated with *P. densiflora* when using electrical impulses, and Islam *et al*. (2013) and Ferzana and Shoji (2012) found the same phenomenon when applying electrical discharges in *T. matsutake*, associated to *P. densiflora* in the field. Similarly, it has been shown that the application of electrical impulses stimulates a greater formation of sporomes of edible saprotrophic fungi such as *Lentinus edodes*, *Pholiota nameko* and *Lyophyllum decastes* (Ohga *et al*., 2001; Takaki *et al*., 2014). In this study an interesting observation was the difference in the sporome size that had been previously found in bioassays in forest tubes in various species of *Laccaria* and *Hebeloma* (Pérez-Moreno *et al*., 2020), compared to those recorded in this study in the containers with larger volume. The sporomes in forest containers of 140 cm^3, are maximum 3 cm in height, while the sporomes formed in the PVC tubes of 6 kg were up to 8 cm in height. A reason that could explain the reduced size of sporomes in the containers of smaller size might be a reduced supply of nutrients and water, as pointed out by Godbout and Fortin (1990).

CONCLUSIONS

This study demonstrates for the first time that mycelial disturbance stimulates the formation of sporomes of Neotropical ectomycorrhizal fungi. Two- to three-fold increase in the number of sporomes of *H. leucosarx* was recorded when the plants were exposed to mycelial disturbance compared to those that did not have hyphal ruptures, both in *P. montezumae* as in *P. greggii*. In addition, there were differences in the sporome number produced when comparing the species of fungi and trees evaluated. *H. leucosarx* produced a higher amount of sporomes compared to *L. laccata* when their mycelia were disturbed regardless of the associated phytobiont. The sporome number produced by *P. greggii* was higher when compared to *P. montezumae*. This study shed some light on the comprehension of the factors involved in the formation of edible ectomycorrhizal fungi, which is valuable information for the domestication and production of wild edible fungi.

ACKNOWLEDGEMENTS

We are grateful for the support granted by the National Science and Technology Council (CONACYT), as well as Colegio Postgraduados to perform a doctoral stay.

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