Lyophilized biopolymeric beads of chitosan-xanthan with edible fungus *Laccaria laccata* (Scop.) Cooke as forest ectomycorrhizal biofertilizers

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ABSTRACT

Objective: To evaluate whether or not the spores of the edible fungus *Laccaria laccata* (Scop.) Cooke encapsulated in a lyophilized biopolymeric matrix of chitosan-xanthan can cause ectomycorrhization in *Pinus greggii* Englem. trees under greenhouse conditions.

Methodology: Spores of the edible ectomycorrhizal fungus *L. laccata* were encapsulated in beads made with the chitosan-xanthan biopolymer. The embedded spores were analyzed using scanning electron microscopy to evaluate possible structural damage. Next, these beads were used as biofertilizers in a greenhouse bioassay using *Pinus greggii* plants to evaluate their ability to be ectomycorrhized. The bioassay lasted 270 days. Subsequently, stereoscopic and bright field microscopy was used to determine if the roots of the pines had been subjected to an ectomycorrhizal colonization. Additionally, the growth of inoculated plants was evaluated compared to non-inoculated plants, 180 and 270 days after sowing.

Results: The spores of *L. laccata* encapsulated in the biopolymeric matrix formed ectomycorrhizae in the roots of *P. greggii*. The percentages of ectomycorrhizal colonization in the plants ranged from 80 to 90%, demonstrating that the production of chitosan-xanthan biopolymeric beads can maintain the viability of the spores of the ectomycorrhizal fungus evaluated and extensively colonize the roots of *Pinus greggii*.

Study Limitations/Implications: The biopolymeric matrix beads that contain spores of the fungus *L. laccata* can induce ectomycorrhization in trees of forest importance.

Conclusions: The spores of an edible ectomycorrhizal fungus encapsulated in the chitosan-xanthan biopolymer have potential as a forest biofertilizer, which opens the opportunity to scale its use up to an industrial level.

Keywords: Edible ectomycorrhizal fungi, biofertilizers, forest production, Mexican pines.

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INTRODUCTION

Some fungi found in the soil form mycorrhizae, which provides many benefits to the plant (Jamiołkowska *et al.*, 2021; Rigueiro-Messa and Rodrigues-Savioli, 2021; Nairet *et al.*, 2022; Milton *et al.*, 2021). The technological challenge is to inoculate mycorrhizal fungi

in order to produce ectomycorrhized plants; therefore, the spores of the said fungi have been used on different commercial substrates. However, one limitation is the development of techniques that prolong the shelf life of mycorrhizal inoculants (Salomon *et al.*, 2022).

There are different types of mycorrhizae, including ectomycorrhizal, which is a mutualism established between plant species, mainly trees (angiosperms and gymnosperms) and fungi, mainly Basidiomycetes and Ascomycetes (Smith and Read, 2008; Hall *et al.*, 2003). Ectomycorrhizal fungi can improve the nutritional conditions associated with the poor environments —especially nitrogen- and phosphorus-deficient ecosystems— in which their host plants grow. This property has been used in reforestation to promote the growth of plants in the field. Currently, one of the selection criteria of the fungi to be used to inoculate trees is their edibility. Mexico ranks second in the world with the greatest biodiversity of edible wild fungi —500 species which include around 200 ectomycorrhizal species (HSCE). These fungi are sold in local markets and some of them have a high value in international markets (Villarreal and Pérez-Moreno, 1989; Gómez-Vázquez *et al.*, 2019). The survival of trees in the field depends, to a large extent, on the formation of ectomycorrhizae, which can be evaluated in greenhouse-inoculated plants (Tateishi *et al.*, 2003; Bernaola-Paucar *et al.*, 2022; Salcido-Ruiz *et al.*, 2021).

Due to their low cost and relative ease of handling, the use of spores from fungal species involved in ectomycorrhizal symbiosis has enormous biotechnological potential for the production of forest inoculants. Currently, the production of ectomycorrhizal fungi-based inoculants has gained enormous importance in countries with a forest tradition (Pérez-Moreno *et al.*, 2008). However, no ectomycorrhizal fungi-based commercial inoculant made from native species is available in Mexico. Consequently, the objective was to encapsulate spores of the fungus *Laccaria laccata* (an HSCE) in a chitosan-xanthan biopolymeric matrix, in order to maintain its viability and verify their capacity to generate ectomycorrhization in *Pinus greggii* Englem plants under greenhouse conditions.

MATERIALS AND METHODS

Location

The research was carried out in the Microbiology greenhouse of the Edaphology Program, at the Colegio de Posgraduados, Campus Montecillo, Mexico-Texcoco Highway, Km 36.5, Montecillo, Texcoco, State of Mexico.

Establishment of the greenhouse

The seeds of *P. greggii* used in this work were collected at the natural forest of Chignahuapan, in the municipality of Puebla, State of Puebla, Mexico. During the pre-germination treatment, the seeds were soaked in running water for 24 h and were subsequently disinfected with hydrogen peroxide (H_2O_2) at 30% v/v in water for 20 min. Finally, the seeds were rinsed twice with sterile distilled water, the excess water was removed, and the seeds were sown.

The sowing was carried out on September 22, 2020. Three seeds were placed in every140-cm^3 black plastic forestry tube with internal root guides at a 2-cm depth. The tubes were placed in tube-stock trays with 48 cavities. The substrate used consisted of

a 2:2:1 mixture of river sand, pine bark, and forest soil. The substrate was previously subjected to steam sterilization, at a pressure of 1.3 kg/cm^2 and a temperature of 125 °C for 5 h. It was left to rest for two days and then sterilized again for 5 h.

To avoid cross contamination, the tube-stock trays were placed in a wooden container, covered with a plastic film. This container was located inside the greenhouse, which has a metal structure and side windows. A 2-mm black shade net was placed over the metal structure.

The seeds sown were irrigated every third day with bottled water for 90 days. Subsequently, the seeds sown were irrigated with running water. The amount of water in each irrigation reached field capacity.

The Captan 50 WP (Captan: N-trichloromethylthio-4-cyclohexene-1,2-dicarboximide) fungicide was used to prevent damage from damping off fungi. The seedlings were watered with a 2-g/L solution of this fungicide, up to three months after sowing.

Synthesis and characterization of encapsulated spores

The fungal material used was acquired in the Ozumba market, State of Mexico, located at 18° 15' 00" N and 98° 46' 30" W. The said material had been collected in the surrounding pine forests. The inoculum was obtained from fresh *L. laccata* sporocarp, separating the pileus from the stipe. The pileus was dehydrated at 35 °C for 48 h in a Jersa® fruit dehydrator. Subsequently, these pilei were ground and sieved with a 1.19-mm mesh, in order to homogenize the particle size. The spores were counted using a Neubauer chamber (Marienfeld, Lauda-Königshofen, Germany) to determine their concentration.

The *L. laccata* inoculum was encapsulated in a bead-shaped chitosan-xanthan biopolymeric matrix for its application on *P. greggii* seeds. High-density, industrial grade, America Alimentos® chitosan (Q) from lot K1202029 and Sigma® xanthan (X) from lot G1253-500G were used for this matrix.

The chitosan-xanthan beads (Q-X beads) were synthesized following the procedure described below. Two g of Q were dissolved in 100 mL of 0.4 M acetic acid, 24 h after the Q had been placed on the acid solution for its hydration. Subsequently, 0.6 g of X were added. An Ultra-Turrax® homogenizer (IKA) was used to homogenize the suspension, at 25 °C and 7,000 rpm for 30 min. Afterwards, 80 g of *L. laccata* inoculum were added and the mixture was homogenized again for another 30 min. It was then sonicated in an ultrasonic bath (Branson 2510MT Ultrasonic Cleaner). Once its pH was determined, the mixture was dripped at a 3-mL/min flow, in a 1 M sodium hydroxide solution at 25 °C, using a Masterflex® L/S peristaltic pump (Cole Parmer, model 7523 80) with a 2-mm internal diameter nozzle. The resulting Q-X beads with inoculum were left to mature for 2 h. Finally, they were washed once with distilled water (DW) and dehydrated using a FreeZone Plus® 2.5-L lyophilizer (Labconco, Cascade Benchtop Freeze Dry System), before they were stored at room temperature awaiting their characterization and subsequent use.

After the beads were lyophilized and covered with a gold bath for 30 s, they were characterized through a morphological analysis, using a JSM-6610LV scanning electron microscope (JEOL).

Experimental design and treatments

A completely randomized experimental design was used to evaluate the potential ectomycorrhization of the beads of chitosan-xanthan containing the spores of the fungus *L. laccata*. There were two treatments: the control and the plants inoculated with Q-X beads with the fungus *L. laccata*. Each treatment consisted of 22 plants; therefore, 44 experimental units were established, each consisting of one plant. For the inoculation of the plants, the seeds were covered with 10 g of substrate; the 0.15-g Q-X beads were placed on top of the substrate and they were covered with approximately 1 cm of substrate. In total, each plant was inoculated with 10^7 to 10^8 spores of *L. laccata* embedded in the biopolymeric matrix.

Evaluation

Two morphometric evaluations were performed at 180 and 270 days after sowing (das). The height was measured from the base of the stem to the apical bud with a graduated ruler, while the diameter was measured at the base of the stem of each plant, with a Mitutoyo digital vernier caliper.

The percentage of mycorrhizal colonization (PMC) was evaluated in three plants per treatment. The said evaluation took 4 to 5 days per plant. The plants were randomly selected at 270 das. The root balls of the plants were extracted from the forestry tubes and the root part was washed under a low-pressure water jet, using three Duvesa and FIICSA sieves (1.19-, 0.180-, and 0.0850-mm opening diameters), to avoid the loss of short roots. The number of mycorrhized apices was determined by direct observation with a EZ4 HD stereoscopic microscope (Leica, Switzerland) and the PMC was calculated.

$$PMC = \left(\frac{mycorrhized\ apices}{mycorrhized\ apices + non - mycorrhized\ apices} \right) 100 \quad (1)$$

To verify the existence of colonized or ectomycorrhizal roots, cross sections and preparations for anatomical characterization were made, in order to find the ectomycorrhizal structures (mantle, Hartig net, and external mycelium).

RESULTS AND DISCUSSION

The spores were satisfactorily encapsulated in the chitosan-xanthan biopolymeric matrix, generating brown beads with a robust consistency to the touch and handling (Figure 1b). These beads were lyophilized prior to their use (Figure 1c). Figures 1 d and e show the images obtained by the scanning electron microscopy (SEM) of the spores of *L. laccata*, both in the inoculum and in the beads where they were encapsulated. The said spores remained turgid, did not show ruptures, or evident morphological modifications after the encapsulation and lyophilization processes.

Table 1 shows the results of the morphometric evaluation of the treatments at 180 and 270 das. The average height is higher in the treatment inoculated with Q-X beads,

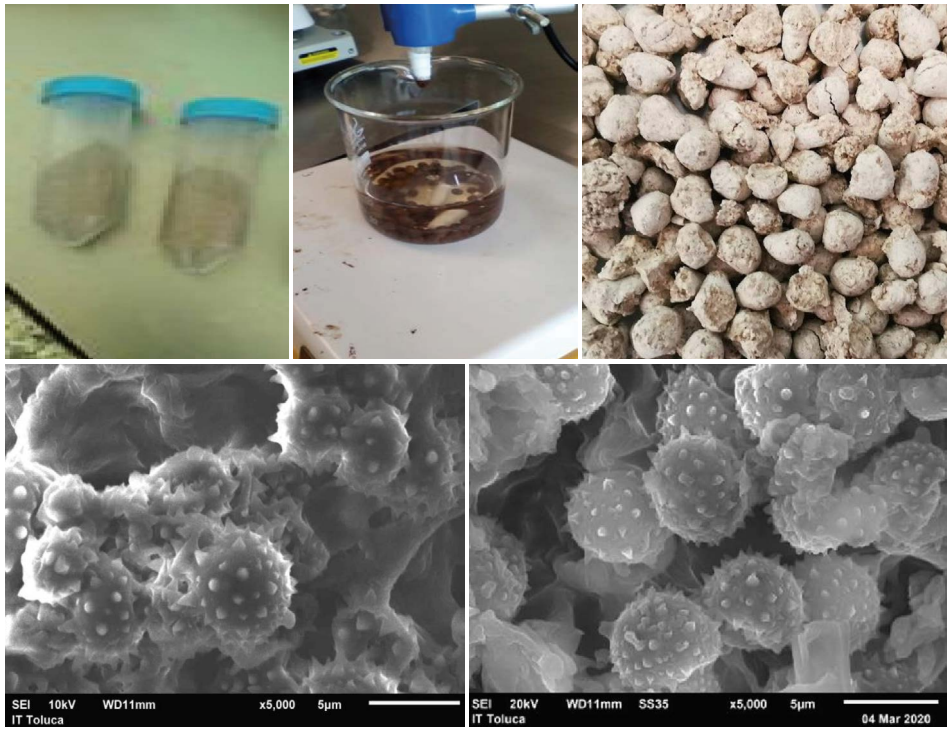

Figure 1. a) *L. laccata* inoculum; b) Q-X beads with encapsulated spores; c) lyophilized beads; d) Micrograph of spores in the inoculum; e) Micrograph of the spores encapsulated in the chitosan-xanthan biopolymeric matrix (Q-X beads).

both at 180 and 270 das. Likewise, the average diameter was also greater in the treatment inoculated with Q-X beads, both at 180 and at 270 das. This shows that the inoculation was favorable for mycorrhization, and therefore that the encapsulated spores remained viables. The high PMC recorded at 270 das also accounts for the viability of the spores encapsulated in the beads of the chitosan-xanthan biopolymeric matrix.

A high percentage of mycorrhization (mantle, Hartig net, and external mycelium) was observed in the inoculated plants and ranged from 80 to 90% in the roots of the ectomycorrhized plants. The results of the anatomical characterization are shown in Figure 2. The presence of the mycorrhization can be clearly seen in the treatment inoculated with Q-X beads (images a, b, and c). Figure 2d clearly shows the effect of mycorrhization on the height of the treatment where it was inoculated.

Table 1. Results of the morphometric and PMC evaluation in the experimental design.

Parameter	180 das		270 das	
	Control	Q-X	Control	Q-X
Height (cm)	7.830±1.119	7.865±1.166	18.890±2.750	21.800±3.430
Diameter (mm)	0.954±0.074	1.060±0.127	1.440±0.100	1.600±0.155
PMC	-	-	0.00	83.01±12.48

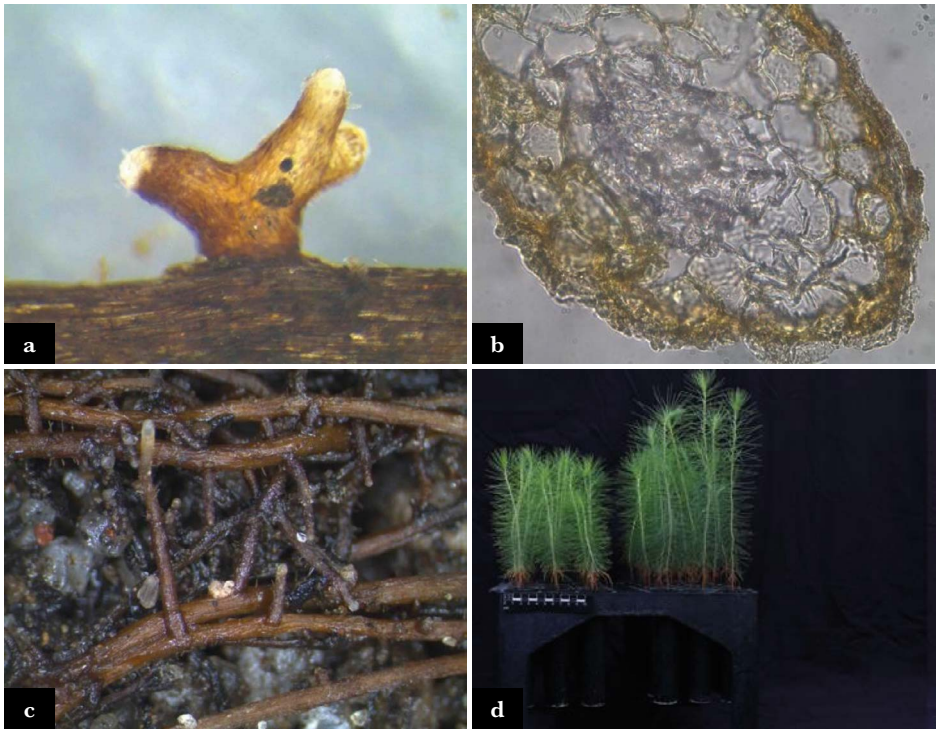

Figure 2. Images obtained from the treatment inoculated with Q-X beads. a) Tetrapod-shaped mycorrhized root; b) Cross section in which the fungal mantle, Hartig net, and external mycelium can be observed; c) Root ball; d) Differences in the growth of both treatments: control (left) and the treatments inoculated with Q-X beads (right).

CONCLUSIONS

Scanning electron microscopy showed that the spores of *L. laccata* did not suffer harmful structural modifications when they were encapsulated in the chitosan-xanthan biopolymeric matrix. The spores of *L. laccata* encapsulated in the biopolymeric matrix under study caused high mycorrhizations (80-90%) in *P. greggii* plants inoculated under greenhouse conditions after 270 das. In contrast, non-inoculated plants lacked mycorrhization. This research shows that the spores of the ectomycorrhizal fungus *L. laccata* encapsulated in chitosan-xanthan biopolymeric beads can be used to produce a biofertilizer for inoculation purposes in plants of forest interest. Consequently, this research proves that there is potential both for increasing the shelf life of the ectomycorrhizal inoculum and for scaling it up to an industrial level.

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