Article

Evaluation of the production cost of neotropical ectomycorrhizal inoculants based on spores

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Abstract

The biological diversity in any ecosystem is fundamental for its existence and balance. Logging undoubtedly alters this diversity. An example is the modifications in the communities of ectomycorrhizal fungi, which are fundamental for the species of forest importance. Due to the above, it is essential for reforestation to carry out mycorrhization in the nursery and thereby favor the establishment of trees in the field, especially in highly disturbed or eroded areas. However, reintroducing ectomycorrhizal species is a challenge due to the ecological, biotechnological and financial implications. Particularly, there is scarce information that analyzes the production cost of ectomycorrhizal inoculants based on spores and the species or the criteria to choose the ectomycorrhizal fungus species suitable for preparation of inoculants. In the present work, the cost of production of Neotropical inoculants based on spores of edible ectomycorrhizal fungi species native to Mexico belonging to *Laccaria* spp. and *Hebeloma* spp. The production costs of the spore-based ectomycorrhizal inoculant evaluated in the present work were $2.00 Mexican pesos per gram for the powder inoculum and $0.05 pesos per mL for the liquid inoculum. Both inoculants are effective in the pine mycorrhizal colonization. We demonstrate the financial feasibility of the production of ectomycorrhizal inoculants to inoculate pine trees of forest importance.

Keywords: *Hebeloma* spp., *Laccaria* spp. and *Suillus* spp.

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Introduction

In a forest, most plants naturally mycorrhize, since they find propagules in the soil for their mycorrhization, such as spores, sclerotia, mycelium, mycorrhizal roots and rhizomorphs. One of the most studied propagules has been the spores, which have a considerable longevity since they can remain in the soil for several years and even decades before germinating (Bruns *et al*., 2009; Nguyen *et al*., 2012). In the case of plant production in nursery, the plant is outside its natural habitat and is unable to access enough fungal propagules for its mycorrhization, so it is necessary to carry out the inoculation in the nursery.

Therefore, it is essential to know the techniques of production of inocula with ectomycorrhizal fungi and the costs involved. There are three inoculation techniques with ectomycorrhizal fungi in the nursery that are: inoculation with monte soil, inoculation based on spores and inoculation with vegetative mycelium (Landis *et al*., 1989; Rossi *et al*., 2007; Duponnois *et al*., 2011). In Mexico, most traditional nurseries use the forest floor as a substrate for plant production and take advantage of it as the sole source of ectomycorrhizal fungal propagules. However, this method has a set of deficiencies, which include: i) the possibility of introducing pathogens; ii) the lack of a mycorrhization controlled by the heterogeneity of distribution of the ectomycorrhizal propagules; and iii) the erosion and destruction of the forest areas from which the soil is extracted.

The second method is the inoculation based on spores that consists in the application of the ectomycorrhizal inoculant in the irrigation water or it can also be mixed in the substrate. For the preparation of said inoculant, the ectomycorrhizal fungus is dehydrated or ground fresh and applied to the substrate. It is important to select an ectomycorrhizal species that is in abundance for the preparation of said inoculant, since large quantities of fresh fungi are required due to their high moisture content. The third method of inoculation is by means of fungal mycelium, this is based on the selection, isolation, purification and subsequent propagation of the ectomycorrhizal fungus in a carrier for example in a mixture of peat-vermiculite; which is applied directly to the substrate that will be used in the nursery.

The fungal mycelium can also be included in sodium alginate, in order to avoid dehydration and keep it in good condition until its application; this technique of preparation of inoculants has also given very good results (Pera *et al*., 1998; Oliveira *et al*., 2006). Several factors must be taken into account for the selection of the species of ectomycorrhizal fungus to be used as an ectomycorrhizal inoculant based on spores or mycelium, such as: i) the compatibility of the fungus with the host plant; ii) the efficiency of the mycobiont to promote rapid mycorrhization, greater growth and survival of the plant; iii) the shelf life of the mycobiont; iv) quality control in the production process of inoculants; and v) the methodological and financial feasibility of production of the inoculants (Brundrett *et al*., 1996a).

In particular, this last aspect has received little attention, mainly in the production of neotropical ectomycorrhizal inoculants. In the present study, an analysis of the costs of inoculants based on powder and liquid spores is described and carried out. The efficiency of the analyzed inoculants was evaluated in *Pinus patula*. Species of the genus *Laccaria* and *Hebeloma* were chosen according to previous studies which have demonstrated their abundance and biocultural importance in the center of Mexico (Pérez-Moreno *et al*., 2008).

Materials and methods

Preparation of ectomycorrhizal inoculants based on powder spores. Species sporomes of the edible ectomycorrhizal fungi belonging to the genera *Hebeloma* and *Laccaria* (Figure 1a and 2a), were acquired in the market of Ozumba, State of Mexico located at 19° 02’ 11” north latitude and 98° 47’ 48” west longitude, during the months of august and september of 2016. Once the sporomas were acquired, they were classified by species, according to the diagnostic characteristics specified by Carrasco-Hernández *et al*. (2010, 2015).

Figure 1. a) Lady collecting edible wild mushrooms in the market of Ozumba, State of Mexico; b) fresh mushrooms of *Hebeloma* spp. in the dehydrator; c) fungi of the genus *Hebeloma* spp. dehydrated; d) mill used for mushroom dehydration; e) Dehydrated inoculant of *Hebeloma* spp.; f) inoculation of *Pinus greggii* with *Hebeloma* sp.

The stipe was cut and only the pileus was dehydrated in a dehydrator type trays with steam (brand JERSA) at a temperature of 33 ±2 °C for a period of 16 hours (Figures 1b and 1c). The already dehydrated inoculum was milled in an industrial mill (Figure 1d.), with an aperture sheet of 1 mm at the outlet, to allow homogenization of the inoculum thus produced. The inoculum obtained was placed in plastic bags of 500 g capacity and 1.5 mL Eppendorf tubes (Figures 1e and 1f). The inoculant was stored at 3 °C until use. Fresh and dry weight were recorded, and these data were used for cost analysis.

Cost analysis

For the financial analysis of powder inoculum preparation, the following costs were taken into account: mushroom price, transport, manpower separation by species, use of dehydrator, grinding and storage. All costs were expressed in mexican pesos.

Cost of the fungus (Ch): the cost of fresh fungus of *Laccaria* and *Hebeloma* was 60 pesos per kilo. However, due to its high water content, the cost of one gram of dehydrated fungus was calculated which was calculated from the amount of fresh fungus acquired in kilos (Hr), the price per kilogram of mushroom (P) and the amount of dehydrated inoculum obtained in kilos (Ci). In order to obtain the price in grams, it was divided by 1 000. For this, the following model was used:

$$Ch=\left(\frac{(Hr)(P)}{Ci}\right)/1000$$

Cost of separation by species (Cse): based on previous evaluations it was determined that a person is required to separate 5 kg of mushrooms in 6 hours and a payment of $80.04 per person was considered, taking into account Mexico’s general minimum wage for the year 2017 (DOF, 2016). The cost of separation by species (Cse), was calculated with the following formula:

$$Cse=\left[\left(\frac{Hr}{5}\right) X\ 100\right]$$

Cost of transport for the acquisition of the fungus (Ct): in this case the cost of the payment of the personnel (Cp) for the acquisition of the mushroom and driver who was paid $80.04 per person was considered. The cost of gasoline (Cg) for the transfer to <the mushroom acquisition area was also considered, considering the liter of gasoline at $16.50.

$$Ct= Cp+Cg$$

Cost for dehydration (Cdh): the costs for the use of the dehydrator were as follows: 1 to 5 kg were paid $500, 5 to 30 kg were paid $1 000 and 31 to 60 kg were paid $ 1 500. Costs increased because more time was required for the use of the dehydrator.

Grinding cost (Cmo): 1 to 5 kg were paid $50; 6 to 30 kg were paid $100 and from 31 to 60 kg were paid $150. The greater the quantity of fungus, the greater the cost of electricity required by the mill.

Cost of storage (Cre): this cost was $ 50 per month of storage and a maximum storage of one year was considered, since the viability of the spores is maintained during this time (Brundrett *et al*., 1996b).

Cost of inoculant per gram (CI): The cost was calculated from the sum of the cost of the fungus and the costs of preparing the inoculant between the amount of dehydrated pileus obtained.

$$CI=\frac{Chp1+\sum Cpi}{Ps}$$

Where: Chp1= average of the average costs of the four species of *Laccaria* and of the three species of *Hebeloma*, in the four collections made.

Σ Cpi= sum of transport costs (Ct), separation (Cse), dehydration (Cdh), grinding (Cmo) and storage (Cre).

Ps = number of dry picoles obtained in g.

Preparation of ectomycorrhizal inoculant based on spores in liquid. The acquisition of the fungus for preparation of ectomycorrhizal inoculant based on spores in liquid was also carried out in the Ozumba market. Sporomes acquired from the genus Laccaria were separated by species. Three species were identified: *L. laccata*, *L. bicolor* and *L. proxima*. In the case of *Hebeloma*, three species were identified: *H. leucosarx*, *H. mesophaeum* and *H. alpinum*. Specimens of *Suillus* spp. and the following species were identified: *S. pseudobrevipes*, *S. granulatus* and *S. brevipes*.

The preparation of the spore-based liquid inoculant consisted of grinding the pileus of each species separately in a homemade blender at a dose of 30 g of fresh pileus per 100 mL of purified water (Figure 2b). Once ground, the inoculum thus obtained was stored in five liter plastic containers (Figure 2c) and refrigerated at 3 °C, until use. In order to know the spore concentration of the inoculant in liquid of each species, the spores were counted with the Neubauer chamber. The camera is divided into 9 main quadrants. To calculate the spore concentration (CE), per cm^3 or mL, the spores of the five main quadrants were counted: A, B, C, D and E (Figure 2e) and the following formula was used:

$$CE= ((\sum A, B, C, D, E))\ 2000$$

Cost analysis. For the financial analysis of the ectomycorrhizal liquid inoculant preparation the following costs were taken into account: fungi, transport, water, containers, use of blender, electric power, fungus separation by species and storage. As for the preparation of powdered ectomycorrhizal inoculant, the price of the mushroom kilo at 60 pesos was considered and the same variables were taken for the analysis of transport cost and separation by species. To calculate the cost of the final inoculant, all costs were added and divided by the amount of inoculum obtained in milliliters.

Evaluation of the liquid ectomycorrhizal inoculant: the seeds of *P. patula* were put to germinate in a substrate of pine bark, river sand and forest floor in a ratio of 2:2:1, previously sterilized and inside a plastic tube with a capacity of 350 mL. One month after having germinated, the seedlings were applied the first inoculation with 5 mL of the inoculum (Figure 1d) and after two weeks another dose of 5 mL of the spore-based inoculants was applied in liquid of *L. proxima*, *H. mesophaeum* and *S. pseudobrevipes* separately in 20 pines per fungus species.

In addition, 20 uninoculated pine trees were maintained that corresponded to the controls. These fungal species were chosen because they were collected in greater quantity in 2016. After maintaining the plants in the greenhouse, the percentage of external mycelium was evaluated (Figure 2f) and the interval was found: 0 to 25%, from 25 to 50%, from 50 to 75% and from 75 to 100%. (Table 4), as an indicator of the percentage of ectomycorrhizal colonization. During this period they were irrigated every third day and no fertilizers or fungicides were applied.

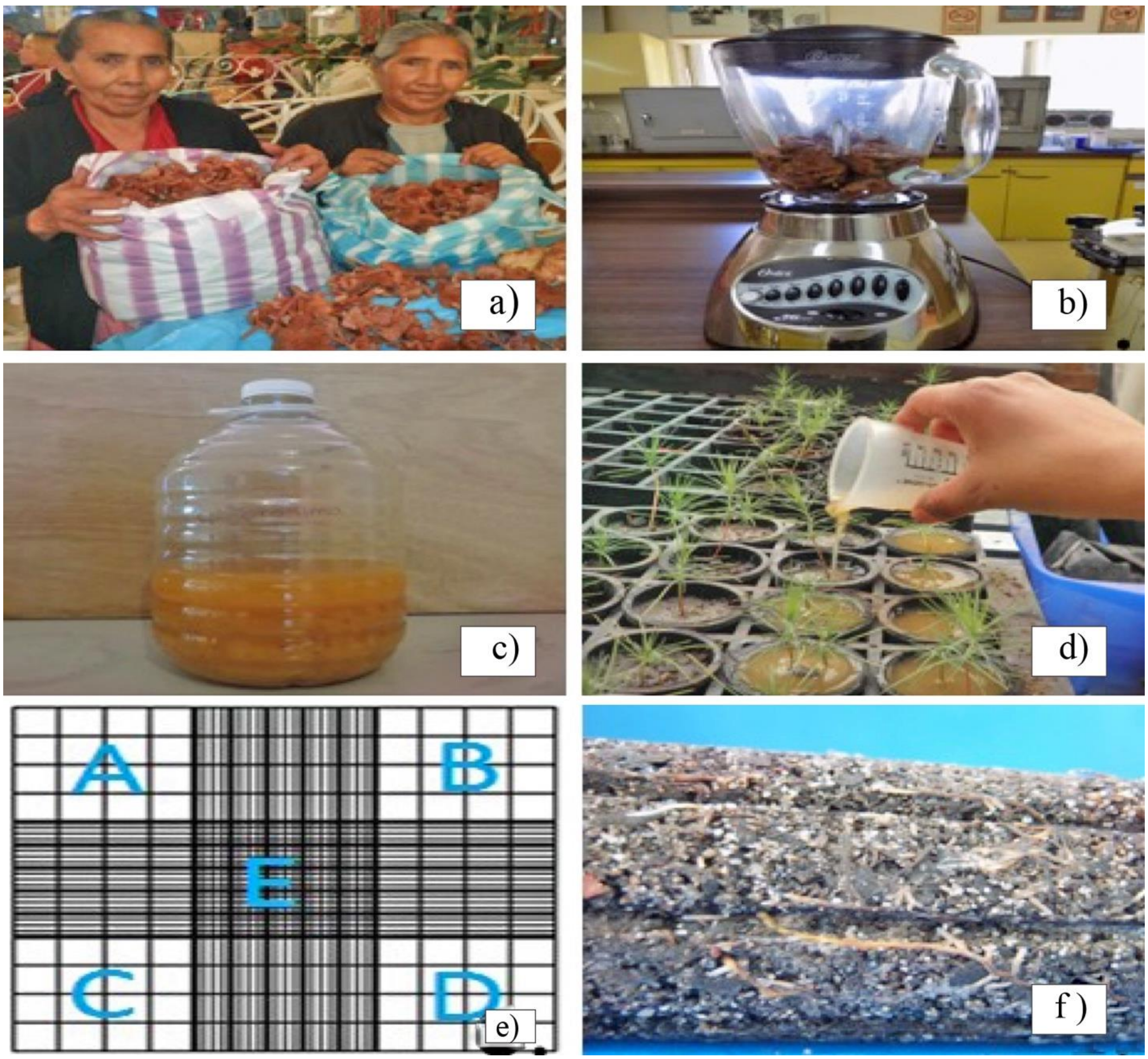

Figure 2. a) Wild edible mushroom collectors in the market of Ozumba, State of Mexico, also called "hongueras"; b) grinding of *L. proxima* for preparation of ectomycorrhizal inoculant in liquid; c) inoculant in *Laccaria proxima* liquid; d) application of liquid inoculant of *L. proxima*; e) quadrants of the Neubauer camera; f) external mycelium of *L. proxima* in *Pinus patula*.

Results and discussion

One of the greatest impacts of logging is the change in the composition of the community of ectomycorrhizal fungi, since these are altered by the chemical and biological changes that occur in the soil after deforestation (Bradley, 2001; Jones *et al*., 2003). It should be noted that the mycorrhizal association according to Dupponnois *et al*. (2011), 95% of native vegetation areas are present that have not been disturbed, while this is less than 1% in disturbed sites.

Due to the above, it is essential that in the nursery the appropriate ectomycorrhizals are introduced that adapt to the plant species and also to the new conditions of the area that is to be regenerated, especially in highly degraded areas. In this sense, it is very important to know the technologies for preparation of ectomycorrhizal inoculants and the costs that would imply for their application in a large-scale nursery.

In the species studied, the average humidity percentage varied from 89 to 91% for the *Laccaria* case and from 91.6 to 94.4% for the *Hebeloma* species. The remaining percentage corresponds to dry matter. In the case of *Laccaria*, an average of 4.8 to 5.5% was obtained for the pileus and 2.6 to 5.3% for the stipe of dry matter. Regarding the *Hebeloma* species, the dry matter pile percentage was 4.3 to 5% and from 1.2 to 2.7% for the stipe. In both cases the highest percentage of dry matter was recorded in the pileus.

It should be noted that only the pileus is used for preparation of ectoomycorrhizal inoculant of *Laccaria* spp. and *Hebeloma* spp. because in the sheets is where the spores are, which are the reproductive structures from which the mycelium with ectomycorrhizal colonization capacity is produced. The data in Table 1 and 2 show the amount of fresh fungi collected for *Laccaria* spp. and *Hebeloma* spp. respectively, as well as the quantities of dry pileus (inoculum) used for the cost analysis. The final cost of producing the ectomycorrhizal powder inoculant for the species evaluated was 2 pesos per gram of ectomycorrhizal inoculant.

In previous works this type of inoculant (3g seedling^{-1}), in *P*. *greggii*, *P*. *patula* and *P*. *pseudostrobus* has been applied at a spore concentration of 10^6 to 10^8 per gram and high efficiency results have been obtained with percentages high mycorrhization that have varied from 70 to 90% (Carrasco-Hernández, 2011; Mendez-Neri *et al*., 2011; Martínez-Reyes, 2012). If 3 g of inoculant powder-based inoculant are applied per plant, the cost of inoculation per plant is $6. The cost of producing one kilogram of inoculant ectomycorrhizal powder is $2 000, if you consider applying 3 g by plant this would reach to inoculate 333 plants. However, if the dose is reduced by half 1.5 g per seedling, the cost of the inoculant would be $3, which has also been shown to be effective (Rendón *et al*., 2014). The cost of production of *P*. *greggii* and *P*. *pseudostrobus* is 2.72 and 2.98 pesos respectively in the nursery of the Autonomous University of Chapingo.

The plant that is offered at this price is a 10-month plant for *P*. *greggii* and 12 months for *P*. *pseudostrobus* and are produced in plastic tubes (black virgin polypropylene containers with a capacity of 140 mL) in a peat substrate-vermiculite-perlite in a proportion of 50, 30 and 20 respectively. To these plants were applied eight fertilizations and four fumigations per month. The fertilizers used and the doses of N varied according to the stage of the pine and were the following: as initiator: 9-45-15 to 50 ppm of N, in its growth stage: calcium nitrate combined with 20-20-20 to 200 ppm of N and as finalizer: 4-25-35 to 50 ppm of N.

Table 1. Fresh and dry weight (kg) of *Laccaria* spp. in the five collections made in the market of Ozumba, State of Mexico.

Characteristics		*L. proxima*	*L. laccata*	*L. proximella*	*L. bicolor*
		A1			
Fresh weight	Pilea	39	0.789	2.8	0.643
	Stipe	13	0.424	2.2	0.302
	Total	52.6	1.2	5	0.945
Dry weight	Pilea	2.5	0.071	0.283	0.085
	Stipe	1.3	0.064	0.167	0.054
	Total	3.8	0.135	0.45	0.139
		A2			
Fresh weight	Pilea	5.9	4.4	0.45	0.43
	Stipe	2.7	2.5	0.17	0.42
	Total	8.6	6.9	0.62	0.85
Dry weight	Pilea	0.473	0.323	0.038	0.035
	Stipe	0.289	0.202	0.018	0.04
	Total	0.762	0.525	0.056	0.075
		A3			
Fresh weight	Pilea	29.8	0.101	3.5	0.069
	Stipe	11.5	0.074	2.8	0.089
	Total	41.3	0.175	6.3	0.158
Dry weight	Pilea	2.2	0.01	0.369	0.005
	Stipe	0.704	0.008	0.201	0.007
	Total	2.9	0.017	0.57	0.012
		A4			
Fresh weight	Pilea	1.4	1	0.068	0.026
	Stipe	0.689	0.66	0.079	0.039
	Total	2	1.66	0.147	0.065
Dry weight	Pilea	0.106	0.075	0.005	0.002
	Stipe	0.056	0.058	0.007	0.004
	Total	0.162	0.133	0.012	0.006

A1= acquisition of mushrooms on the date August 23, 2016; A2= acquisition of mushrooms on the date August 30, 2016; A3= acquisition of mushrooms on the date September 6, 2016; A4= acquisition of mushrooms on the date September 13, 2016.

Table 2. Fresh and dry weight (g) of *Hebeloma* spp. in the five collections made in the market of Ozumba, State of Mexico.

Characteristics		*H. leucosarx*	*H. mesophaeum*	*H. aff. alpinum*
		A1		
Fresh weight	Pilea	669	439.5	287.3
	Stipe	223	133.3	108.6
	Total	892	572.8	395.9
Dry weight	Pilea	54.2	28.5	20.5
	Stipe	24.5	9.8	9.1
	Total	78.7	38.3	29.6
		A2		
Fresh weight	Pilea	435	60	440
	Stipe	200	20	190
	Total	635	80	630
Dry weight	Pilea	35.7	3	33
	Stipe	18	1	18
	Total	53.7	4	51
		A3		
Fresh weight	Pilea	182.2	205.8	335
	Stipe	70.8	67.1	98.1
	Total	253	272.9	433.1
Dry weight	Pilea	14.4	11.3	19.3
	Stipe	6.7	2.7	5
	Total	21.1	14	24.3
		A4		
Fresh weight	Pilea	40	775	67.5
	Stipe	15	250	33
	Total	55	1025	100.5
Dry weight	Pilea	3	45.7	5
	Stipe	1.5	10.3	3
	Total	4.5	56	8

A1= acquisition of mushrooms on the date August 23, 2016; A2= acquisition of mushrooms on the date August 30, 2016; A3= acquisition of mushrooms on the date September 6, 2016; A4= acquisition of mushrooms on the date September 13, 2016.

From the above it is deduced that the cost of a mycorrhized plant with the powder inoculum, would have a price of $5.72 for *P. greggii* and of $5.98 for *P. pseudostrobus* at a dose of 1.5 g of inoculum per plant. It is important to mention that studies are needed related to the minimum doses of ectomycorrhizal inoculant powder, which can reach levels of mycorrhization abundant (higher than

70%) and reduce costs. It should be noted that CONAFOR (2010) takes into account mycorrhiza as an important quality index and that it must cover at least 40% of the root ball so that it is considered an optimum quality plant to be taken to the field.

An alternative form of preparation of ectomycorrhizal inoculant is to take the fresh pileus and grind it for its application as a liquid inoculant. The cost per ml of liquid ectomycorrhizal inoculant was $0.05 per mL and $50 per liter. The concentrations of spores varied according to the species (Table 3). The ectomycorrhizal inoculant based on spores in liquid prepared in the present study was inoculated in *Pinus patula* to test its effectiveness. The fungi inoculated were: *L. proxima*, *H. mesophaeum* and *S. pseudobrevipes* and the majority of pines presented high percentages of mycorrhization (75% to 100%), which demonstrates their effectiveness (Table 4).

Table 3. Concentration of spores per cm^3 or mL of the inoculant in prepared liquid.

Spores	Number of spores cm^{-3}
L. laccata	7.9×10^5
L. proxima	8.7×10^5
L. bicolor	4.66×10^5
H. mesophaeum	5.5×10^6
H. leucosarx	7.2×10^6
H. alpinum	3.5×10^6

Table 4. Percentage of external mycelium in Pinus patula inoculated with three species of edible ectomycorrhizal fungi one year after the application of the ectomycorrhizal inoculant in liquid.

Percentage	Control	*L. proxima*	*H. mesophaeum*	*S. pseudobrevipes*
0 a 25	20	1	0	0
25 a 50		7	5	2
50 a 75		5	2	2
75 a 100		7	13	16

According to evaluations developed in the present work with 10 mL of liquid inoculum per plant, optimal mycorrhization values are obtained and the price would be $0.50 per plant, which substantially reduces the cost compared with the ectomycorrhizal powder inoculant.

The cost of the powder ectomycorrhizal inoculant evaluated in the present work was of $2 000 kg^{-1}, which is lower compared to the Ecto-Rhyza product of the PHC company, although if the amount of inoculum needed per plant is considered, the product is more expensive than those handled by this company (Table 5). It should be noted that the inoculum evaluated in the present work has not been tested in smaller quantities, which would substantially reduce the cost per plant. In addition to this, the PHC company does not show greenhouse bioassays on the label of its products where it can be confirmed that the concentrations they manage can reach high mycorrhization percentages (greater than 70%).

The cost of production of the inoculum in liquid evaluated in the present work is $0.05 per mL and $50 pesos per liter, which would reach 100 plants in a dose of 10 mL per plant. This price was similar to the product Mycogrow soluble and PHC Ectorhyza and slightly lower in cost to the product Ectoplant irrigation (Table 5).

Table 5. Cost of ectomycorrhizal inoculants.

Name of the company and location	Product	Content	Cost in mexican pesos	Cost of inoculum per plant	Source
Forest and applied mycology Spain	Ectoplant Irrigation	Esporas de *Pisolithus tinctorius*, *Scleroderma* spp., *Rhizopogon* spp.,	$23 423.85 L^{-1} Enough to 25 000 plants	$0.93	1
	Ectoplant tablets	*Rhizopogon* spp., *Pisolithus tinctorius*, *Scleroderma verrucosum* y *Suillus* spp.	500 tablets per $1 247.64 Enough to 500 plants	$2.4	
MycoGrow® United States	MycoGrow™ Micronized Endo/Ecto	Mix of spores of 4 different species of endomycorrhizal fungi and 7 ectomycorrhizal fungi.	28 g per $88.15 Enough to 453.592 g of seed	The cost is variable since it will depend on the size of the seed	2
	MycoGrow™ Soluble	Mixture of spores of 9 different species of endomycorrhizal fungi and 10 ectomycorrhizal fungi as well as two disease-inhibiting species and 12 beneficial bacteria.	453.592 g per $1 423.73 It can reach from 2 000 to 4 000 plants, which will depend on the size of the plant and method of application.	From $0.30 to $0.70 if considered for 2 000 and 4 000 plants respectively	
	Plant Success™	Tablets compressed with concentrate of 7 spores of endomycorrhizal fungi and 5 spores of ectomycorrhizal fungi.	150 tablets per $444.02 Enough to 150 seedlings	$ 2.9	
Planth Health Care of Mexico Mexico	PHC® Ecto-Rhyza®	250 million cfu g of spores of Pisolithus *tinctorius* and 4 strains of *Trichoderma harzianum* (5x10) cfu g^{-1}.	$ 5300 kg^{-1} Enough to 100 000 plants	$0.05	3
	MycorTree® Ecto-Injectable ®	Spore of *Pisolithus tinctorius*, *Scleroderma citrinum*, rhizobacteria and cassava extracts.	226 g $1 400 Enough to 15 000 plants	$0.09	

1= http://micofora.com/; 2= http://www.fungi.com/shop/fungi-for-healthy-gardens.html; 3= http://www.phcmexico.com.mx/phcmicorrizas.html.

Conclusions

The ectomycorrhizal inoculant based on liquid spores has a lower cost when compared to the ecomicorrízico inoculant based on spores in powder.

The ectomycorrhizal inoculant based on spores in liquid is effective and also has the potential to originate high percentages of mycorrhization as well as with the ectomycorrhizal inoculant based on powder spores.

The economic feasibility, comparable with the current prices of commercial inoculants existing in the international market, of the production of inoculants based on powder or liquid spores produced for neotropical tree species is demonstrated.

It is necessary to encourage the creation of national companies that produce ectomycorrhizal inoculants based on fungi native to Mexico, since acquiring and introducing species from other countries carries the risk of serious ecological consequences, such as the possible displacement of native species of the country and economic and social benefits as the creation of local and regional jobs.

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