Article

Mycelial colonization of *Flammulina mexicana* from solid and liquid inoculum in agroforestry residues

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Abstract

The production of inoculum is a process that requires its optimization, since, to a great extent, it depends on generating a greater biological efficiency in the cultivation of mushrooms, reducing the economic costs, as well as problems of contamination and time in the cultivation cycles. In the present investigation, during 2016, the colonization of four substrates was evaluated (corn stubble, *Quercus* sp. sawdust, *Senecio cinerarioides* sawdust and maize stubble in combination with *S. cinerarioides* sawdust) with liquid and solid inoculum of *F. mexicana* (strains IE 974, IE 984, IE 985, IE 986). As results, it was obtained that the colonization rate of the different substrates varied among the four strains ($p\leq 0.0001$). Substrates with solid inoculum showed lower growth rates compared to liquid inoculum, substrates with liquid inoculum were colonized in a period of 17 days and with solid inoculum took 50 days and in some cases, there were parts that were not colonized; which was also related to the substrate used, presenting an interaction ($p\leq 0.0001$) between the type of inoculum and the substrate that affects the growth rate. The incorporation of liquid inoculum in the cultivation of fungi allows to increase the density and speed of mycelial growth, as well as the early appearance of primordia, which contributes to reduce the time in the cultivation cycle.

Keywords: corn stubble, growth speed, pellets, primordia, wheat.

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Introduction

The genus *Flammulina* ranks fifth among edible fungi grown worldwide. The cultivated species of this genus of which so far is reported are *F. velutipes* with a production of 300 000 tons per year (Psurtseva, 2005), *F. populicola* cultivated in the United States of America and Canada (Stamets, 2000, 2005; Tradd, 2014) and *F. mexicana* (Arana-Gabriel, 2018), the latter is in the experimental phase; however, it could mean an important alternative in the diversification of species cultivated in Mexico.

Flammulina velutipes is a cosmopolitan species, tolerant to temperatures below 18 ºC, is commonly known as enokitake, enoki, winter mushroom or golden needle mushroom in countries such as China, Japan, Siberia, United States of America, Australia and Taiwan. In Asia, it is a mushroom highly appreciated for its medicinal properties and high nutritional value; in addition, it is considered a gourmet quality product (Chang and Miles, 2004; Sharma *et al*., 2008; Hasan *et al*., 2012).

In Mexico, *F. velutipes* is marketed only in supermarkets, mainly in the central region of the country (Mayett and Martínez-Carrera, 2010). On the other hand, *Flammulina mexicana*, is a wild species endemic to Mexico that develops on stems and branches of the rockrose (*Senecio cinerarioides*) in the forest areas of *Pinus* and *Abies* of central Mexico at higher altitudes of 2 700 m and a temperature mean less than 18 ºC (Redhead *et al*., 2000; Franco *et al*., 2012; Arana-Gabriel *et al*., 2014). It is usually extracted for self-consumption and is among the 20 species of fungi of greater cultural importance in some localities of the Nevado of Toluca, Mexico (Franco *et al*., 2012; San Roman, 2014; De la Garza, 2017).

In recent dates the experimental culture of *F. mexicana* has been developed on diverse substrates such as the rockrose (*S. cinerarioides*), maize stubble (*Zea mays*) and oak sawdust (*Quercus* sp.) (Arana-Gabriel, 2018). The mushroom cultivation process involves different phases; substrate preparation, inoculum preparation, inoculation of substrates and induction of fruiting bodies. The proper execution and sequence of each one of these phases, at the end of the process represents a successful production or not of fruiting bodies. One of the stages in which special care must be taken is the preparation of inoculum, since it directly affects colonization time, mycelial density and biological efficiency (Kawai *et al*., 1996; Rosado *et al*., 2002; Abdullah *et al*., 2013).

The production of inoculum is a process that is based, mainly, on solid fermentation in grains of grasses such as wheat, sorghum, barley and millet. On an industrial scale, this process is expensive given the use of sterilization and incubation equipment and materials such as type of grain used and supplements (Rosado *et al*., 2002). An alternative to reduce the production costs involved in solid inoculum is the substitution of solid inoculum for liquid inoculum. The use of liquid inoculum allows a uniform distribution of the mycelium in the substrate, in short periods of time, which also reduces contamination problems (Frieal and McLoughlin, 2000; Abdullah *et al*., 2013; Ma *et al*., 2016).

Some species of fungi in which liquid inoculum production has been carried out are *Pleurotus* spp. (Rosado *et al*., 2002; Silveira *et al*., 2008; Abdullah *et al*., 2013), *Agaricus bisporus* (Frieal and McLoughlin, 2000), *F. velutipes* (Hassan *et al*., 2012), *Sparassis latifolia* (Ma *et al*., 2016) and

Lentinula edodes (Kawai *et al*., 1996) as most of the biomass production research in submerged fermentation is for the production of secondary metabolites and polysaccharides (Petre *et al*., 2010; Elisashvili, 2010).

Given the advantages of using liquid inoculum and that the species, strain and type of substrate affects the speed of mycelial colonization of the substrates; the objective of the present investigation was to compare the efficiency in the colonization of substrates between liquid and solid inoculum of four strains of *F. mexicana*, a species that has shown to have commercial cultivation potential.

Materials and methods

Biological material

Four strains of *F. mexicana* were used: IE 974, IE 984, IE 985, IE 986 from the strain collection of the Institute of Ecology (INECOL) of Xalapa, Veracruz, Mexico; which were previously obtained; from fruiting bodies of the high mountain region of the Nevado of Toluca in central Mexico (Arana-Gabriel *et al*., 2014; Arana-Gabriel., 2017). Strains were re-isolated in agar medium (CA) (15 g of agar, 15 g of croquettes for dog Ganador®, 2 g of yeast extract, 1 g of gelatin peptone in one liter of distilled water) (Stamets, 2000; Arana-Gabriel *et al*., 2014) at 18 °C and characterized by Cruz-Ulloa (1995) and the color code 'HTML color codes' (html-color-codes.info/).

Production of solid inoculum

Primary and secondary inoculum was prepared with wheat grains. To prepare the primary inoculum, the wheat grains were cleaned, washed and hydrated for 24 h, then boiled for 10 min. Once drained and with a humidity of 70%, 5 bottles were prepared with 200 g of grains for each of the four strains used, to which were added lime [Ca(OH)2] (0.7 g of lime per bottle) leaving the pH of the wheat in 8.5, later underwent sterilization in an autoclave at 121 °C/15 lb of pressure for one hour (Guzmán *et al*., 2013). Once the bottles were cooled with wheat, they were inoculated with three fragments of 1 cm^2 of mycelium from the four strains. For the secondary inoculum, the wheat was subjected to the procedure described above, but now the grain was inoculated with 5% of the primary inoculum. All the treatments were incubated in dark at 18 ºC until the mycelium invaded 100% the grains of wheat to be able to inoculate the different substrates.

Production of liquid inoculum and micellar characterization

The liquid inoculum was prepared using croquettes added with peptone and yeast (C-PL) (15 g of agar agar, 15 g of dog croquettes Ganador®, 2 g of yeast extract, 1 g of gelatin peptone in one liter of distilled water) (Stamets, 2000; Arana-Gabriel *et al*., 2014). The medium was sterilized in an autoclave at 121 °C/15 lb. pressure for fifteen minutes and 0.05 g of Chloramphenicol (SIgma®) was added as an antibiotic.

100 ml samples of culture medium (C-PL) were prepared in 250 ml Erlenmeyer flasks with five replicates. The media were inoculated with 0.5 cm diameter of each strain and incubated in dark for 20 days at 18 °C and 120 rpm in a PolyScience® agitated water bath.

After the incubation period, the pH of the medium was measured and the biomass was characterized both morphologically (shape and size) and microscopically to report the mycelial behavior under agitation conditions. The color of the mycelium was reported with the color code 'HTML color codes' (html-color-codes.info/). For the microscopic characterization, small samples of mycelium were taken with which temporary preparations were made with 10% Congo red, which allowed to observe the presence of fibulas and rule out that the mycelium produced was contaminated. Likewise, the diameter of the hyphas was measured at 100 x (20 hyphas per treatment) with the help of the Motic digital Microscope DMB3-223 program (Motic China Group Co., Ltd., 2001-2004).

Subsequently, the biomass was filtered, washed with sterile distilled water to avoid that the culture medium was a factor in the colonization of the substrates and homogenized in a Magic Bullet Deluxe® food processor for 10 s with 100 ml of sterile distilled water (modified Stamets, 2000). In the same way, the dry biomass was quantified after the incubation period, the biomass was filtered, washed with sterile distilled water and put to dry in an oven at 80 °C for 24 h for its subsequent weighing.

Evaluation of mycelial growth in different substrates

To determine the efficiency of the liquid and solid inoculum with the different strains and substrates with respect to colonization time, mycelial density and percentage of contamination, four different formulations were used for the substrates: 1) Oak sawdust (*Quercus* sp.) 100% (AQ); 2) Rockrose sawdust (*S. cinerarioides*) 100% (ASC), 3) Corn stubble (*Zea mays*) with sawdust from *S. cinerarioides* 50:50 (ASC+RM); and 4) 100% corn stubble (RM) (Arana-Gabriel, 2018). The different substrate combinations were hydrated at 70% (for each kilogram of substrate, 1.4 l of water was added) and 0.1% lime [$Ca(OH)_2$] and 0.1% gypsum ($CaSO_4$) were added. The duly homogenized mixture was placed in test tubes (2.5 x 15 cm) at 12 cm of the volume of the tube (Carreño-Ruiz *et al*., 2014) from its base (equivalent to 20 g wet weight). Five tubes were prepared per treatment with a factorial analysis of: 4 x 4 x 2 x 5 (4 strains x 4 substrates x 2 types of inoculum x 5 replicates).

The test tubes with the substrate were sterilized for one hour at 121 °C and 15 pounds of pressure; subsequently they were inoculated with 5% (wet weight of the substrate) of secondary inoculum or 1 ml of liquid inoculum; they were covered with sterile cotton to allow gas exchange and incubated at 18 °C under dark conditions until the mycelium completely invaded the different substrates; making observations every third day to monitor mycelial growth. The growth rate (VC) in the different substrates was calculated by dividing the substrate length (12 cm that the substrates had in the tubes) between the number of days it took the mycelium to invade it (modified by Huerta *et al*., 2009). Once the substrate was covered by the mycelium, the density of this was classified visually according to Shrestha *et al*. (2006), (-) = extremely scarce, (+) = scarce, (++) = moderate, (+++) = abundant.

Statistical analysis

The statistical analysis was carried out with the Statgraphics® Centurion XVI program (Statpoint Technologies, Inc., 2009), the mycelial growth rate data were subjected to an analysis of variance (Anova) to determine whether or not there were differences between strains, substrates and type of inoculum (solid and liquid) and a multivariate analysis of variance (Manova) to determine if there was interaction between the substrates and the type of inoculum that affected the growth rate. The significant differences were determined with a Tukey multiple range test ($p \leq 0.05$).

Obtaining fruiting bodies

Once all the treatments had 100% colonization, the production of fruit bodies was stimulated, transferring the test tubes to a room with light (100 lux), wet cotton was placed with sterile distilled water on top of the substrate, the cotton remained moist and was removed by observing the appearance of primordia; from that moment, two water irrigations per day were applied.

Results and discussion

Biological material

The reisolated strains IE 974 and IE 986 in medium AC took 14 days to invade the Petri dish of 9 cm and strains IE 984 and IE 985 did so in 16 days. The four strains developed a uniform, aerial growth of white color (#FFFFFF), with cottony texture, fringed margin and without the presence of exudates. Microscopically, the hyphae were smooth, branched, septate and with abundant fibulas.

Production of solid inoculum

For the production of primary inoculum, the wheat seeds took 20 days to present an abundant mycelial colonization (+++) of 100%. With secondary inoculum colonization time was 15 days (100% invaded grain and abundant mycelium), time that is within the range reported for species of the genus *Pleurotus* where mycelial growth takes 20-26 days (Grison *et al*., 2008). The time of colonization and density of the mycelium is a function of the strain, substrate used, pH, percentage of humidity and incubation temperature. In the production of solid inoculum, another type of supplemented seed or sawdust could be used; however, its use depends on the availability and costs of each region. In this case, wheat is one of the grains with more availability in terms of production and costs in the State of Mexico; it is also considered a good vehicle for the propagation of the mycelium; the humidity of 70% and pH of 8.5 are values that are within the ranges reported for mycelial growth since they facilitate the availability of nutrients (Ríos *et al*., 2010).

Production of liquid inoculum and micellar characterization

The biomass production of the four strains IE 974, IE 984, IE 985 and IE 986 in liquid medium C-PL was, on average, 7.9 g L^{-1} after 20 days of agitation. The mycelium formed globose pellets with a fibrous surface with a size of 1.5 to 3 cm in diameter. Microscopically, the hyphae were smooth with a diameter of 1.1-3.3 µm, branched, septate and with abundant and frequent fibulas. The morphology of the mycelium (free filaments or pellets) and the size of the pellets depend on the intensity of agitation; in terms of the amount of biomass produced depends on the species, strain, carbon source of the culture medium, pH and dissolved oxygen (Cui *et al*., 1997; Frieal and McLoughlin, 2000; Grison *et al*., 2008; Ma *et al*., 2016). For example, for *Sparassis latifolia*, which has been obtained to obtain biomass in liquid medium, with a production of 11.96 g L^{-1} after 15 days (Ma *et al*., 2016) or for *Pleurotus pulmonarius* where biomass was obtained in three days from an automated bioreactor (Abdullah *et al*., 2013).

Evaluation of mycelial growth on different substrates

The colonization rate of the different substrates varied among the four strains ($F_{3,159}$= 15.54, $p\leq$ 0.0001), with IE 974 and IE 986 being the ones that colonized the RM and ASC+RM substrates more rapidly ($F_{4,159}$= 5.09, $p\leq$ 0.0007) with liquid inoculum ($F_{1,159}$= 8.72, $p\leq$ 0.0036) (Table 1). Strain IE 974 showed a mycelial growth rate of 0.71 cm day^{-1}, taking an average of 17 days to invade the substrate of ASC+RM with abundant density (Figure 1d) of mycelium as well as EI 986 in RM and ASC+RM (Figure 1a).

In contrast, the RM substrate inoculated with solid inoculum in the four strains showed the lowest growth rate, taking on average up to 50 days (0.24 cm day^{-1}) to colonize 100% of the substrate; however, despite the slow growth rate, the mycelial density was abundant (Figure 1e-h). The AQ substrate in three of the four strains with solid inoculum showed moderate density, with the strain IE 986, the density was scarce and with liquid inoculum the density was moderate to abundant, except for strain IE 984 where it was extremely scarce, with a colonization time of 27-29 days (0.13-0.26 cm day^{-1}). Mycelial growth was more uniform in the substrates inoculated with liquid inoculum, in contrast to the substrates with solid inoculum that presented sites that were not completely colonized (Table 1, Figure 1).

Table 1. Mycelial growth rate in different substrates.

Strain	Substratum	Type of inoculum	Mycelial growth rate (cm day^{-1})	Mycelium density
IE 974	AQ	S	0.41 defghi*	++
	ASC	S	0.42 cdefgh	+++
	RM	S	0.29 fghijkl	+++
	ASC+RM	S	0.38 defghij	+++
	AQ	L	0.44 bcdefg	++
	ASC	L	0.45 bcdef	+++
	RM	L	0.42 cdefgh	+++
	ASC+RM	L	0.71 a	+++
IE 984	AQ	S	0.41 defghi	++

Strain	Substratum	Type of inoculum	Mycelial growth rate (cm day^{-1})	Mycelium density
	ASC	S	0.46 bcde	+++
	RM	S	0.26 ijkl	+++
	ASC+RM	S	0.27 hijkl	+++
	AQ	L	0.26 ijkl	-
	ASC	L	0.13 l	-
	RM	L	0.22 jkl	-
	ASC+RM	L	0.21 kl	+
IE 985	AQ	S	0.41 defghi	++
	ASC	S	0.44 bcdefg	+++
	RM	S	0.28 ghijkl	+++
	ASC+RM	S	0.35 efghijk	+++
	AQ	L	0.41 defghi	++
	ASC	L	0.58 abc	+++
	RM	L	0.59 ab	+++
	ASC+RM	L	0.53 bcd	+++
IE 986	AQ	S	0.44 bcdefg	+
	ASC	S	0.48 bcde	+++
	RM	S	0.24 jkl	+++
	ASC+RM	S	0.38 defghij	+++
	AQ	L	0.44 bcdefg	++
	RM	L	0.71 a	+++
	ASC	L	0.26 ijkl	+++
	ASC+RM	L	0.69 a	+++

AQ= Sawdust of *Quercus* sp.; ASC= sawdust from *S. Cinerarioides*; RM= corn stubble; ASC+RM= sawdust of *S. cinerarioides* and corn stubble; L= liquid inoculum; S= solid inoculum; -= extremely scarce; += scarce; ++= moderate; ++ = abundant. *= different letters in the same column indicate significant differences (Tukey, $p \leq 0.05$).

In Table 1, differences in growth velocity between the two types of inoculum and different substrates are shown according to Tukey's multiple range test. Strain IE 984 was the only one that showed the lowest growth rate in the four substrates with liquid inoculum, and the density of the mycelium was extremely scarce (Figure 1c). In general, the solid inoculum showed lower growth rates than the liquid inoculum, which was also related to the substrate used, presenting an interaction between the type of inoculum and the substrate that affects the growth rate ($F_{4,159}$= 18.94, $p \leq 0.0001$).

The rapid colonization of substrates and early maturation of the mycelium with liquid inoculum has also been reported for species such as *L. edodes* (Kawai *et al*., 1996), *Pleurotus ostreatoroseus* (Rogeiro *et al*., 2002) and *P. pulmonarius* (Abdullah *et al*., 2013). This is due to the fact that the liquid inoculum is used directly in the substrate (Frieal and McLoughlin, 2000; Gregori *et al*., 2007) increasing the number of invasion points where the mycelial growth resumes, beginning to feed directly from the substrate. In the inoculum in wheat, on the contrary, the invasion points decrease since it starts at the top of the tubes and in this case, the mycelium first began to feed on the seed, thus delaying the colonization of the substrates.

Figure 1. Mycelial growth of four strains of *F. mexicana*: a,e) IE 986; b,f) IE 985; c,g) IE 984; d,h) IE 974 on different inoculated substrates. Liquid inoculum (a-d); solid inoculum (e-h). From left to right the test tubes with the substrates correspond to RM (corn stubble); AQ (sawdust from *Quercus* sp.); ASC (sawdust from *S. cinerarioides*) and ASC+RM (sawdust of *S. cinerarioides* and corn stubble).

Another disadvantage of using solid inoculum, is that, when a culture medium with a simple source of carbohydrates is continuously used, the fungi become less efficient in the colonization of substrates and the production of fruiting bodies, besides that they are not viable metabolically (Rogeiro *et al*., 2002).

The use of liquid inoculum in the present investigation had favorable results from the beginning of the colonization of the substrates on the third day; in contrast to inoculation with wheat that began on the fifth day, a trend also reported for *L. edodes* (Kawai *et al*., 1996). However, despite the efficiency in the colonization of the substrates with liquid inoculum, Ma *et al*. (2016); Silveira *et al*. (2008) report that there are no differences in biological efficiency in the production of fruiting bodies between different types of inoculum.

The success in the use of liquid inoculum is related to the efficiency when transferring it to the substrate, allowing rapid colonization, which translates into a reduction in the duration of the production cycle. The more uniform distribution of the inoculum in the substrate, provides a

homogenous growth of the mycelium, which reduces the costs of both labor and materials; as well as the considerable reduction of pollution problems by antagonist organisms (Frieal and McLoughlin, 2000; Rogeiro *et al*., 2002; Abdullah *et al*., 2013; Ma *et al*., 2016). The appearance of antagonistic fungi can inhibit the growth of the mycelium and reduce or cancel the production of fructifications; which represents one of the fundamental needs in the edible mushroom growing industry (Mata *et al*., 2011).

The ability of fungi to grow on a substrate is not only related to the inoculum used, but also to the vigor of their mycelium, the ability of the strains to properly exploit the nutrients of the substrate and have a greater chance of competing against antagonists, the production of lignocellulolytic enzymes, incubation temperature, as well as the particle size, chemical composition and pH of the substrates (Staments, 2000; Rogeiro *et al*., 2002; Mata *et al*., 2011).

Obtaining fruiting bodies

The first substrates that formed primordia were RM and RM+ASC with liquid inoculum of the strain IE 986 after 5 days of having induced the formation of fructifications (Figure 2), the rest of the treatments took from 7 to 10 days, except of the four substrates inoculated with liquid inoculum of IE 984 and the AQ substrate with both liquid and solid inoculum in the four strains where there was no formation of primordia.

The formed primordia showed cespitose growth, the pinillos were small yellow (# F2F5A9) with long, smooth stipes of yellow color (# F2F5A9, # F7D358) with a lighter shade near the pileus (Figure 2). The appearance of fruiting bodies is an indication that the strains and substrates used are viable. The appearance of fructifications in a shorter period of time with liquid inoculum have also been reported for *L. edodes* (Kawai *et al*., 1996) who mention that the early appearance of primordia is a hereditary phenomenon, which could be induced with the liquid inoculum and delayed in the solid. The use of liquid inoculum together with the method used in this study for the induction of fructification can be used to test at a small scale the viability of the strains, substrates and environmental conditions that allow the cultivation of fungi, before being taken to the level of greenhouse.

Figure 2. Primordia of *F. mexicana*.

Although, in the present investigation, the production of biomass in liquid medium was carried out in Bach system, this procedure can be scaled at the bioreactor level for its optimization and automation; which would allow to increase the biomass produced and considerably reduce the inoculum production and the cultivation cycle. On the other hand, the use of native germplasm can diversify edible cultivated species with functional properties, as well as work with species of cultural importance in each region and use the substrates available and suitable for cultivation (Morales *et al*., 2010).

Conclusions

Generally, the submerged culture or liquid is carried out to obtain bioactive substances, but by generating high amounts of biomass in short periods of time and small spaces, it can be used as an inoculum on a solid substrate. The results were favorable using liquid inoculum based on dog croquettes added with yeast extract and gelatin peptone, obtaining on average 7.9 g L^{-1} of dry biomass in the four strains used of *F. mexicana*. The efficiency between solid and liquid inoculum was measured based on the speed of colonization of the substrates and density of the mycelium. Corn stover and corn stubble with rockrose sawdust were the substrates where the growth rate was higher with strains IE 974 and IE 986 of *F. mexicana* with liquid inoculum. Both the strains and the type of substrate and inoculum interfere in the early colonization of substrates and micellar density, as well as in the production of fruiting bodies. Performing this type of research allows identifying and modifying the factors that should be considered for the cultivation of commercial species or determining the viability of strains of wild species, before being taken to an experimental, commercial or industrial scale culture. Both the strains and the type of substrates and inoculum interfere in the early colonization and mycelial density as well as in the production of fruiting bodies.

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