Vol. 21, No. 1(2022) Bio2735
Revista Mexicana de Ingeniería Química

Evaluation of bagasse *Agave salmiana* as a substrate for the cultivation of *Pleurotus djamor*

Evaluación del bagazo de *Agave salmiana* como sustrato para el cultivo de *Pleurotus djamor*

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Received: February 7, 2022; Accepted: March 21, 2022

Abstract

The objective of this study is to evaluate the potential of agave bagasse supplemented with urea for use as an alternative substrate in the cultivation of a wild fungus isolated from *Agave salmiana* (*A. salmiana*), determining the biological efficiency, production rate, morphological characteristics and nutritional. The fungus was identified by molecular methods amplifying the ITS region and subsequently cultivated on *A. salmiana* bagasse supplemented with urea (0.77, 0.95, 1.14, 1.32 and 1.5% of total nitrogen (TN)). For the cultivation of the fungus, two types of inoculum were used: in the form of grain and in the form of granules. The fungus was identified as *Pleurotus djamor*. A biological efficiency (BE) of 70% was obtained in the sample inoculated with grain at 1.32% TN, at 1.5% it produced malformations in the fruiting bodies. Chemical analysis of the sporocarps shows a crude protein content of 15-26%. This is the first report of the isolation of *P. djamor* from *A. salmiana* as an atypical substrate, which represents an opportunity for its commercialization and the bagasse, due to its chemical composition and being supplemented with urea, allows the growth of the fungus without alter its nutritional composition of this genus.

Keywords: basidiomycete fungus, maguey, nutritional composition, phylogenetic tree, production parameters.

Resumen

El presente estudio tiene como objetivo evaluar el potencial del bagazo de agave suplementado con urea para su uso como sustrato alternativo en el cultivo de un hongo silvestre aislado de *Agave salmiana* (*A. salmiana*), determinando la eficiencia biológica, tasa de producción, características morfológicas y nutricionales. El hongo fue identificado por métodos moleculares amplificando la región ITS y posteriormente cultivado sobre bagazo de *A. salmiana* suplementado con urea (0.77, 0.95, 1.14, 1.32 y 1.5% de nitrógeno total (TN)). Para el cultivo del hongo se utilizaron dos tipos de inóculo: en forma de grano y en forma de gránulos. El hongo fue identificado como *Pleurotus djamor*. Se obtuvó una eficiencia biológica (BE) del 70% en la muestra inoculada con grano al 1.32% de TN, a 1.5% produjó malformaciones en los cuerpos fructíferos. El análisis químico de los esporocarpos indicó un contenido de proteína cruda de 15-26%. Este es el primer reporte del aislamiento de *P. djamor* a partir de *A. salmiana* como sustrato atípico, por lo que representa una oportunidad para su comercialización y el bagazo debido a su composición química y al ser suplementado con urea permite el crecimiento del hongo sin alterar su composición nutricional de este género.

Palabras clave: hongo basidiomiceto, maguey, composición nutricional, árbol filogenético, parámetros productivos.

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https://doi.org/10.24275/rmiq/Bio2735
ISSN:1665-2738, *issn-e*: 2395-8472

1 Introduction

The genus *Pleurotus* (basidiomycetes) are a cosmopolitan group of fungi with high nutritional value, therapeutic properties, and a wide range of biotechnological and environmental applications, causing its cultivation to increase worldwide (España-Rodríguez *et al*., 2021). It is the second-most widely cultivated mushroom on the planet with an estimated production of 6.46 x 109 kg (Sardar *et al*., 2017). Its cultivation requirements and nutraceutical and biodegradative properties have attracted interest, and it is now a promising agroindustrial product with expanding cultivation in Latin America and other areas of the world (Salmones, 2017).

In Mexico, the species with the highest production volumes are *Pleurotus ostreatus*, *Pleurotus djamor* (*P. djamor*), and *Pleurotus pulmonaris* (*P. pulmonaris*) (Salmones, 2017). These species develop naturally in the bark of trees, dead organic matter and, in general, on lignocellulosic residues (Royse and Sánchez, 2017). Due to the ability of the genus *Pleurotus* to grow in various substrates, they have been found in agave plants where it develops mainly in shoots near the ground around the base of the plant and in the cavity of depleted magueys, identifying as *Pleurotus opuntiae* (*P. opuntiae*) (Heredia-Solís *et al*., 2017). However, it has been proven that the fungi that grow on these plants not only correspond to this species, but to a wide variety, including *P. djamor*, *Pleurotus flabellatus*, *P. opuntiae* , *Pleurotus ostreatoroseus* (*P. ostreatoroseus*), *Pleurotus parsonsiae* (*P. parsonsiae*), *Pleurotus salmoneostramineus* (*P. salmoneostramineus*), *Pleurotus dryinus* (Zervakis *et al*., 2019).

To increase the production and nutritional value of these fungi, they have been cultivated on a broad range of agroforest by-products: rice chaff, oats, wheat, cotton, palm oil, scrub, sawdust, pulps, fruit peels, jute waste, bagasse, and other kinds of chaff, all of which are rich in the carbon, nitrogen, sulfur, and phosphorus necessary for the development of mushroom biomass (Bellettini *et al*., 2019). In this sense Agave bagasse has these same properties, is obtained during the production process of mezcal, pulque, inulin and agave honey (España-Rodríguez *et al*., 2021). Composed of cellulose (31-43%), hemicellulose (11-22%) and lignin (11-20%); It presents in its structure polysaccharides / oligosaccharides soluble in water, inulin, oligomers, sucrose, glucose, galactose and fructose that make it an alternative substrate for the cultivation of fungi and usually also contains calcium oxalate. Estimates indicate that this material is produced at a rate of 4'709,000 tons per day. Accumulation of this bagasse causes environmental problems and contributes to the proliferation of rodents, insects, bacteria, and fungi (Nava-Cruz *et al*., 2015). Given the above, these properties can be used for the cultivation of the genus *Pleurotus*.

However, the above is not the only factor that is important to select the substrate, it must also be taken into account that the growth and development of fungi as well as the quality and quantity aspects (productivity and biological efficiency) are closely related to the type of nutrients of the substrate, as well as the conditions of the crop. In addition, it has been shown that the substrate has a direct influence on the mineral composition because the hyphae take from it the essential elements for them. The substrates on which *Pleurotus* naturally grows tend to contain low nitrogen values (in the range of 0.03% to 1.0%) and still form fruiting bodies with nitrogen concentrations higher than those of the substrate (Bellettini *et al*., 2019). A good substrate must promote a satisfactory performance of the fungus and must contain the adequate amount of nitrogen and carbohydrates to promote the growth of the fungus, in most cases the substrate has low nitrogen contents to satisfy the demands of the development of the fungus, of hence the need to supplement the substrate with nitrogen sources that promote better growth (Ogundele *et al*., 2014) so it is important to take care that the C / N ratio is balanced (28-30% carbon and 1% nitrogen). Although supplementing the substrate leads to greater growth of the fungus, there are limitations to its use, since high supplementation can lead to contamination and reduced yield. (Fanadzo *et al*., 2010). Therefore, when a substrate is proposed for growing mushrooms it is important to evaluate these parameters. Some of the well-known supplements include but are not limited to urea, ammonium sulfate, chickpea flour, molasses, mustard cake, soy flour and cottonseed cake (Mkhize *et al*., 2016).

The objective of this study was to evaluate the potential of agave bagasse supplemented with urea for its use as an alternative substrate in the cultivation of a wild fungus isolated from *Agave salmiana* (*A. salmiana*), determining the biological efficiency, production rate, and morphological and nutritional characteristics. With the purpose of valorizing these agroindustrial residues by cultivating fungi that grow

in these substrates. Therefore, a nutritious functional food or food additives could be produced using residues of *A. salmiana*.

2 Materials and methods

2.1 Isolation and cultivation

The wild mushroom was isolated from a fruiting body that developed on *A. salmiana* in the municipality of Chilcuautla, Hidalgo, Mexico (20°20'00"N 99°14'00"Or). The hymenia were cut in 1-cm^2 fragments, subjected to treatment (5 min in sterile distilled water, 5 min in a hypochlorite solution at 5%, and washing with sterile water), and then seeded on plates in potato dextrose agar (PDA) medium for 7 days at 28ºC until the mycelium characteristic of the mushroom was obtained. Re-seedings were done periodically to achieve purification.

2.2 Molecular identification

2.2.1 DNA extraction

The mushroom's genomic DNA was obtained from 0.750-g pellets recovered by filtration, frozen, and then lyophilized from a liquid culture in Kirk medium for 7 days, utilizing the protocol proposed and modified by Huanca-Mamani *et al.* (2014). The concentration and purity of the DNA were measured in a NANODROP 2000 (Thermo Scientific^{TM}), and integrity was verified by electrophoresis in agarose gel (Sigma Aldrich, México) at 1% (30 min, 80V, electrophoresis chamber).

2.2.2 Amplification and sequencing

The ITS1-5.8-ITS2 region of the DNA of the mushroom was amplified by the polymerase chain reaction (PCR) using the universal oligonucleotides ITS1 and ITS4 (Biorad Laboratories Inc., Hercules, CA, USA), following Díaz *et al.*'s (2014) modified protocol. The product of PCR was analyzed by electrophoresis (Equipment Bio-Rad) in agarose gel at 1%. The base pairs were determined by comparison to the 1 Kb marker. The Wizard® S.V. Gel and PCR Clean Up System kit (Thermo Scientific^{TM}) was utilized to purify the PCR products. Sequencing was performed at the *Unidad de Síntesis y Secuenciación de AND* at the *Instituto de Biotecnología* of the National Autonomous University of Mexico (UNAM).

2.2.3 Analysis of the sequence

The sequence was compared to the NCBI's DNA database (www.ncbi.nlm.nih.gov) using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1990). A total of nine sequences of *Pleurouts* were recovered (*Pleurotus opuntiae* - MN477934, MK757594, KY214255; *Pleurotus djamor* - MF574728, JN637828, MG328900, MN398667, GU722273, GU722271, GU722265; *Pleurotus ostreatoroseus* - MG282434; along with one sequence of *Fusarium phyllophilum* - KR909430, to create the phylogenetic tree. The evolution model employed was GTR I+G, determined by ModelTest v. 2.3 (Nylander, 2004). The tree was generated on the Phylogeny.fr platform (https://www.phylogeny.fr/; Dereeper *et al.*, 2008) using the ClustalW, Gblocks, Mr.Bayes, and TreeDyn programs (Thompson *et al.*, 1994; Huelsenbeck and Ronquist, 2001; Chevenet *et al.*, 2006; Dereeper *et al.*, 2008; Dereeper *et al.*, 2010). The phylogenetic tree was visualized in FigTree v1.4.4. (Rambaut, 2018). The sequence was deposited in GenBank under access number MW581271. The phylogenetic tree was deposited in TreeBASE (Accession URL: http://purl.org/phylo/treebase/phylows/study/TB2:S28312?x-accesscode=288be19013aeff456bd2b892c2905a0e&format=html).

2.3 Cultivation and production parameters of Pleurotus spp.

2.3.1 Preparation of the primary inoculum

The primary inoculum was obtained in two forms: wheat grains (WG) and pellets (WP). The former were prepared with wheat seeds hydrated for 12 h. Excess water was drained and 250 g of the wheat were placed in polypaper bags. These were sterilized for 15 min and then inoculated with 15, 1-cm^2 squares of the mushroom's mycelium. Incubation was performed at 28ºC under total darkness until the mycelium completely invaded the seeds. The WP were prepared in 250-mL Erlenmeyer flasks containing 100 mL of potato dextrose broth (Sigma - Aldrich, México). They were sterilized and inoculated with 10 mL of the mushroom's mycelium suspended in sterile water, and incubated at 28ºC under stirring at 160 rpm until uniform pellets formed.

2.3.2 *Selection and characterization of the substrate*

The *A. salmiana* bagasse employed consisted of residues from the pulque, inulin, and honey industries. It was provided by the *Corporativo Magueyero San Isidro, S.A. de C.V.* in the municipality of Nanacamilpa, Tlaxcala, Mexico (19°29'00"N 98°32'00"O). Selecting the residue required physicochemical analysis of the bagasse from the shoots, the pineapple, and a 50:50 mixture of shoots:pineapple. The following determinations were made: moisture (M) by the AOAC 930.15 method, ash (C) by the AOAC 942.05 method, organic material (OM) by difference of ash, crude protein (CP) by the adapted A.O.A.C. 955.04 method, total organic carbon (TOC) by calculation (OM/1.74) (Gouleke, 1977), and the C/N ratio. pH was measured in extracts of distilled water in a weight/volume ratio of 1:5.

2.3.3 *Inoculation of the substrate*

Bagasse from the shoots of *A. salmiana* was washed in distilled water and pasteurized at 65ºC for 60 min. Excess water was drained and the material was left to cool at ambient temperature. Once cooled, 27 bags, each containing 150 g (dry weight) of *A. salmiana* bagasse, were inoculated for each experiment (WG, WP) and distributed in 5 treatments for later supplementation with urea (J.T. Baker®). The procedure followed the methodology proposed by López-Rodríguez *et al*. (2008) for inoculation with WG, while for inoculation with WP, we took the total content of the pellets from each flask, filtered the culture broth and deposited the pellets in the center of the polypaper bags that contained the previously sterilized substrate, according to Abdullah *et al*.'s modified protocol (2013).

2.3.4 *Supplementation with urea*

During supplementation of the substrate, the total nitrogen content (TN) of the bagasse of the *A. salmiana* shoots was taken as the initial parameter. The experimental design for WG and WP consisted in 9 treatments (T1: 0.77% TN, T2: 0.95% TN, T3: 1.14% TN, T4: 1.32% TN, T5: 1.5% TN) and 3 repeats in which the minimum, maximum, and central values for total N in the *A. salmiana* bagasse were 0.95, 1.5, and 1.32% TN, respectively. Nitrogen was calculated in percentual amounts equivalent to the weight in grams of the urea. The solutions were prepared with distilled water and sterilized with a 0.2-μm cellulose membrane (Merck millipore, Sigma Aldrich, México). A potentiometer was used to measure pH.

Supplementation was performed 3 days after inoculation using a sterile syringe to cover all the bags. The amount of solution administered was calculated on the basis of the moisture of the inoculated bags, adjusted to 80%. Once all the bags were supplemented, they were kept under darkness until primordia appeared, protocol modified by Monterroso (2009).

2.3.5 *Fructification and harvesting*

After the appearance of the primordia, the bagasse was exposed to light at a temperature of 18-20ºC and a relative humidity of 80-90% until mature fruiting bodies were obtained (López-Rodríguez *et al*., 2008). The mushrooms were gathered manually with a sterile scalpel and measured. This procedure was repeated for each harvest.

2.3.6 *Evaluation of productive parameters*

The following parameters were recorded: running time of the mycelium on the substrate, precociousness (time of appearance of primordia), and fructification. The productive parameters determined were biological efficiency (BE) and production rate (PR).

$$\text{Biological efficiency (BE)} = \frac{\text{Weight of the fresh mushrooms (g)}}{\text{Weight of the dry substrate (g)}} \times 100 \quad (1)$$

$$\text{Production rate (PR)} = \frac{\text{Biological efficiency}}{\text{Days from seeding to the end of production}} \quad (2)$$

2.4 *Morphological and proximal chemical characterization of the fruiting bodies*

The fruiting bodies from each treatment were characterized morphologically by texture, growth, color, form of the laminae, and pileus. For the proximal chemical composition, moisture (M) was determined by method 930.15, crude protein (CP) by the adapted 955.04 method, ethereal extract (EE) by method 920.39, and ash (C) by method 942.05, following the methodologies established by the AOAC (Association of Official Analytical Chemists).

2.5 *Statistical analyses*

All experiments were analyzed by one-way ANOVA analysis of variance with a significance level of

($p \leq 0.05$); to compare the individual differences in biological efficiency, production rate, chemical composition of the substrates and fruiting bodies between the treatments, a comparison of the means was made using a Tukey multiple comparison test with a level of significance ($p \leq 0.05$) at the Minitab® Statistical Software version 19.1.

3 Results and discussion

3.1 Molecular identification

The molecular study of the mushrooms isolated from *A. salmiana* allowed us to obtain one amplification product with a size of 750 pb. It was identified as *Pleurotus djamor*. The sequence was deposited in GenBank under access number MW581271, and a phylogenetic tree was elaborated (Fig. 1 Accession URL: http://purl.org/phylo/treebase/phylows/study/TB2:S28312?x-access-code=288be19013aeff456bd2b892c2905a0e&format=html)usingthesequencesof{\protect\protect\protect\edefT1{T1}\let\enc@update\relax\protect\edeftxr{txr}\protect\edefm{m}\protect\edefn{n}\protect\xdef\T1/txr/m/it/10{\T1/txr/m/n/10}\T1/txr/m/it/10\size@update\enc@update\ignorespaces\relax\protect\relax\edef{it}\edef{sc}\protect\edefn{}\protect\xdef\T1/txr/m/it/10{\T1/txr/m/n/10}\T1/txr/m/it/10\size@update\enc@updatePleurotus} available at GenBank.

Values of 600-800 pb have been reported for *Pleurotus* abalonus, *Pleurotus* cystidiosus, *Pleurotus* cystidiosus var. Formosensis, *Pleurotus* fuscoquamulosus, *Pleurotus* smithii, and *Pleurotus* australis, *P. djamor*, *Pleurotus cornucopiae* and *P. pulmonaris* (Imtiaj *et al*., 2011), but recently fragments of 700 pb were found for *P. djamor* and *P. ostreatus* (Doroteo *et al*., 2018).

The mushrooms that grow on plants of the genera Opuntia, Yucca, Agave, and Phytolacca, among others, are described as *P. opuntiae* , but because worldwide the genus *Pleurotus* includes several taxa and species, controversy exists due to the absence of valid sequencing data and/or phylogenetic reconstructions. This has generated significant ambiguities regarding the exact identity and distribution of *P. opuntiae* (Zervakis *et al*., 2019).

The mushroom *P. djamor* has a cosmopolitan distribution that includes material initially identified as *P. ostreatoroseus*, *P. parsonsiae*, and *P. salmoneostramineus* , *P. djamor* is a pantropical species found in wild form in several countries in the Americas, including Mexico, though its distribution is probably much broader (Zervakis *et al*., 2019).

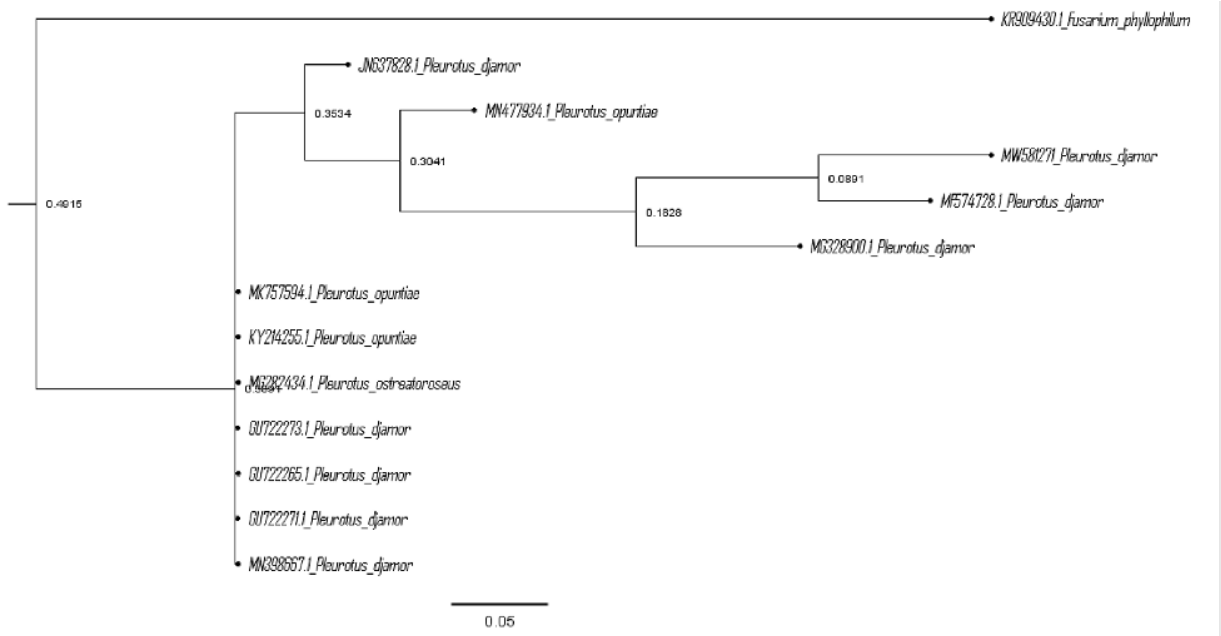

Fig. 1. Phylogenetic tree generated from the sequences of the ITS region of strains of *Pleurotus*.

Table 1. Chemical composition of *Agave salmiana* bagasse.

	Shoots	Pineapples	Pineapples- shoots (50:50)
Moisture (%)	10.61 ± 0.4^b	10.14 ± 0.3^a	11.92 ± 0.3^b
Ash (%)	10.63 ± 0^a	10.91 ± 0.8^a	11.33 ± 0.3^a
Organic matter (%)	89.37 ± 0.8^a	89.09 ± 0.8^a	88.67 ± 0.3^a
Crude protein (%)	4.80 ± 0^c	11.51 ± 0.5^a	8.75 ± 0.8^b
Nitrogen (%)	0.77 ± 0^c	1.84 ± 0.1^a	1.40 ± 0.1^b
TOC (%)	51.4 ± 0^a	51.2 ± 0^a	50.9 ± 0.2^a
Relation C/N	66.7	27.8	36.3
pH	5.14 ± 0.6^a	5.90 ± 0.2^a	5.77 ± 0.03^a

Means not labeled with the letter "a" are significantly different from the mean of the control level in the Tukey Multiple Comparison Test with a significance level ($p \leq 0.05$). ± Standard deviation. TOC: Total organic carbón

Table 2. Precociousness and harvests of *P. djamor* on *A. salmiana* bagasse after the WG and WP treatments.

Treatments	**T1**			**T2**			**T3**			**T4**			**T5**		
	P	1ª	2ª	P	1ª	2ª	P	1ª	2ª	P	1ª	2ª	P	1ª	2ª
WG	14	11	13	11	8	26	11	8	25	9	8	25	10	8	23
WP	14	13	0	14	13	0	18	10	0	18	10	0	18	10	0

T1: Substrate not supplemented; T2: 0.95% of nitrogen; T3: 1.14% of nitrogen; T4: 1.32% of nitrogen; T5: 1.5% of nitrogen P: Precociousness (d), 1ª: First harvests (d), 2ª: Second harvests (d)

Table 3. Production parameters of *P. djamor* on *A. salmiana* bagasse using wheat seeds.

Type of inoculum	**T1**		**T2**		**T3**		**T4**		**T5**	
	BE	PR	BE	PR	BE	PR	BE	PR	BE	PR
WG	58.33 ± 9^a	0.97 ± 0.2^a	61.11 ± 10^a	1.02 ± 0.2^a	63.41 ± 8^a	1.06 ± 0.1^a	70.00 ± 9^a	1.17 ± 0.1^a	70.56 ± 10^a	1.18 ± 0.2^a
WP	20.56 ± 4^a	0.34 ± 0.1^a	23.33 ± 3^a	0.39 ± 0.1^a	44.07 ± 7^b	0.73 ± 0.1^b	42.22 ± 5^b	0.70 ± 0.1^b	38.11 ± 5^b	0.64 ± 0.1^b

T1: Substrate not supplemented; T2: 0.95% of nitrogen; T3: 1.14% of nitrogen; T4: 1.32% of nitrogen; T5: 1.5% of nitrogen; BE: Biological efficiency; PR: Production rate. Means not labeled with the letter "a" are significantly different from the mean of the control level in the Tukey Multiple Comparison Test with a significance level ($P \leq 0.05$). ± Standard deviation

3.2 Culture and productive parameters of P. djamor

Table 1 shows the results of the characterization of various sections of the bagasse of *A. salmiana*. The residue of the pineapples is rich in crude protein (CP) at 10.91%, compared to just 4.8% in bagasse from the shoots. According to Heredia-Solís *et al*., (2014), the content of CP in *A. salmiana* and *Agave weberi* (*A. weberi*) bagasse does not exceed 4%.

The nitrogen content in the bagasse of the shoots, pineapples, and the mixture (50:50) was measured at 0.77, 1.84, and 1.40%, respectively; figures that concur with those reported for bagasse from shoots, which range from 0.1-1% of N (Koutrotsios *et al*., 2014). Mushrooms of the genus *Pleurotus* have the ability to grow on materials with low nitrogen concentrations. The results of our nitrogen analysis indicate that the bagasse from shoots of *A. salmiana* is a good candidate for use as an alternative substrate for cultivating edible mushrooms. Nitrogen is essential for protein synthesis, purines, pyridine, and chitin in mushrooms (0.03 % a 1.0 %), but high levels can shrink yields (>1.5 %). Thus, it is extremely important to ensure that the substrates utilized to cultivate edible mushrooms contain an adequate nitrogen content that will allow correct growth (Monterroso 2009; Bellettini *et al*., 2019).

During cultivation, we analyzed two types of primary inocula: a traditional one that utilizes wheat grains as support, and a second made from pellets of the mushroom itself. Observations showed that the type of primary inoculum used did not affect the development of *P. djamor*, and that the use of the inocula had no impact on the mycelial growth of the mushroom on the substrate. The inoculum in pellet form (WP) colonized the *A. salmiana* bagasse in 13 days, while the inoculum in grain form (WG) required 25 days. This represents a competitive advantage for *P. djamor* compared to other microorganisms that could compete for the substrate and contaminate the crop. In addition, due to its origin, WP does not host spores of

other mushrooms or microorganisms because it exerts greater control (Abdullah *et al*., 2013). The seeds that resulted from the WG treatment were homogeneous, especially in the wheat grain, while the WP pellets varied in size, were semi-uniform, and beige in color. Neither product showed signs of contamination.

Table 2 shows the time of appearance of the primordia (precociousness), and the first and second harvests of the experiments inoculated with WG and WP. Table 3 displays the effect of different concentrations of urea on BE and PR. Because the bags inoculated with WG increased the concentration of nitrogen, precociousness decreased. In treatment T1 (substrate not supplemented with nitrogen), 14 days were required for the first primordia to appear, while in T2 and T3 the time of appearance was 11 days, and for treatment T5 it was 10. In contrast, in the bags with WP precociousness increased with higher concentrations of nitrogen, reaching 14 days in T1 and T2, and 18 days in T3, T4, and T5.

Two harvests were obtained from the bags inoculated with WG. The first (T1) at 25 days from inoculation. For treatments T2 to T5, the first harvest was obtained after an average of 17 ± 1 days. Regarding the second harvest, for T1 it was obtained after 38 days, while for T2 to T5 the time required was 41-45 days. The treatments inoculated with WP only produced one wave of mushrooms at 27 ± 1 days after inoculation. This represents an economic disadvantage for production. It is likely that the WG treatments included nutrients that promoted the growth of the mushroom, while the WP inoculum lacked these compounds that strongly impact the number of harvests achieved. Studies have described that supplementing substrates with nitrogenated sources improves the production of mushrooms of the genus *Pleurotus* spp. (Naim *et al*., 2020), but other observations have shown deficiencies in mycelial growth and yields when nitrogen sources such as ammonium chloride were applied to *P. ostreatus* and *P. cystidiosus* at concentrations above 0.09 and 0.05% (Hoa and Wang, 2015), and with ammonium sulfate and urea at concentrations of 1% and 1.5%, respectively (Naraian *et al*., 2009). The growth, development, quality, and quantitative aspects (biological efficiency, productivity) are closely-related to the type of nutrients present in the substrate, an equilibrium in the C/N ratio, and other conditions of cultivation (Bellettini *et al*., 2019). The absence of a second harvest could be due to the inoculum in pellet form (WP) since it did not contain the characteristic nutrients of wheat grain that provide energy for mycelial growth and development (Royse *et al*., 2004). Other possibilities include the nutritional exhaustion of the substrate during mushroom growth, and/or the accumulation of toxic substances that impeded fructification (Fanadzo *et al*., 2010). The C/N ratio is a determining factor for mushroom production because excess nitrogen can affect the degradation of lignin, thus impeding the development of the mycelium and the formation of fruiting bodies. In the phase of development of the fruiting bodies, a low C/N ratio is favorable. Bellettini *et al*., (2015) recommend and C/N ratio of 28-30 of carbon and 1% nitrogen for growing mushrooms.

The BE and PR of the treatments inoculated with WG increased with respect to the non-supplemented treatment (T1) and as the concentration of TN in the bagasse increased. The maximum BE achieved was 70%, while the maximum PR was 1.18, both in T5. Heredia-Solís *et al*., (2014) reported the same BE with *P. ostreatus* using a substrate made of bagasse of *A. salmiana* and 40% of BE with A. weberi. 33.24% using a substrate of bagasse of *Agave angustifolia* mixed with 30% nogal shavings and 5% wheat bran (Heredia-Solís *et al*., 2016). The BE and PR of the treatments inoculated with WP were lower, with T3 showing the highest values for these two parameters (44% and 0.73). The evidence from this experiment allowed us to determine that the primary inoculum based on pellets (WP) did not generate higher yields in the cultivation of *P. djamor*, as was expected. In contrast, the BE and PR of the bags inoculated with WG and supplemented with urea as a source of nitrogen improved. Valenzuela-Cobos *et al*., (2020), used a reconstituted strain of *Pleurotus djamor* grown on wheat straw showed the highest biological efficiency (125.84%) and production rate (2.79%), also this strain produced on a mixture of oak sawdust, wheat straw, millet seed, seed husk of cotton and CO3 presented the highest productivity parameters: biological efficiency (98.43%) and productivity rate (2.27%).

The fruiting bodies of the WG and WP experiments are shown in Fig. 2. In both cases, they were of beige color with regular growth, a cottony texture, and smooth decurrent laminae. However, the form of the pileus of the mushrooms inoculated with WG was flabelliform, while that of the mushrooms inoculated with WP was infundibuliform. In the WG treatments, 31% of the fruiting bodies from T1 reached a size of 5-8 cm, 39% measured 8-13 cm, but only 4% achieved a size of 13-16 cm. In T2 with WG, 54% of the mushrooms measured 8-13 cm.

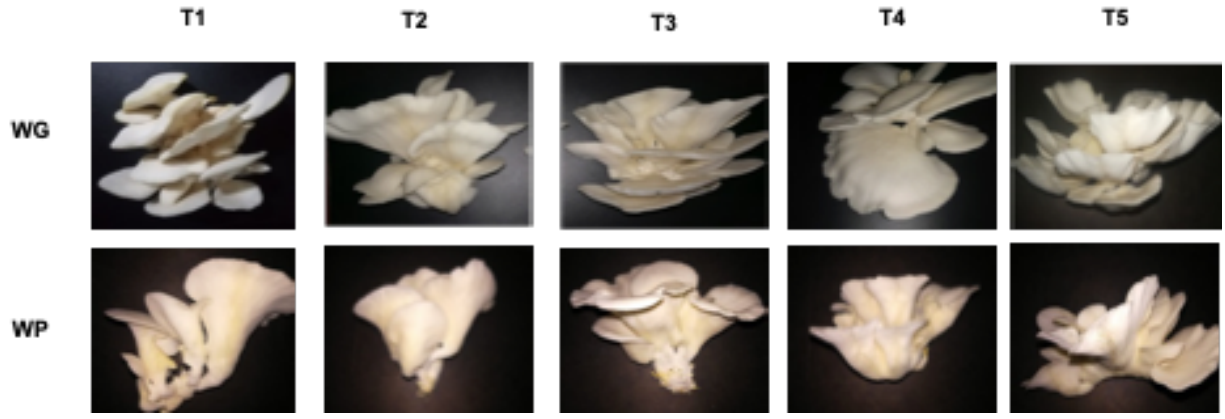

Fig. 2. Fruiting bodies from the different treatments of the experiments with WG and WP. The bodies are of beige color, with regular growth, cottony texture, and decurrent laminae.

For T3 the figures were 62 for 8-13 cm and 24% between 5 and 8 cm. For T4, 39% of the carpophores reached a size of 8-13 cm. For T5, 48% of the fruiting bodies measured 8-13 cm. Although some mushrooms from T4 and T5 achieved average size, some carpophores showed malformations in the clusters and caps. In addition, almost 20% of the fruiting bodies in T4 detained their development after day 14.

Regarding the WP experiment, 45.5% of the fruiting bodies in T1 measured 5-8 cm, but only 6% reached 10-13 cm. In T2, 35% of the carpophores were classified in a size of 5-8 cm. In contrast, observations of T3 showed that 16% of the mushrooms achieved a size ≥16 cm with a predominance of carpophores between 10 and 13 cm. The average size in T4 was 10-13 cm (37%), while in T5, 21% of the carpophores detained their growth.

Deformations of the fruiting bodies -especially elongated stipes and reduced coloration- have been reported due to the effects of cultivating conditions (high luminosity, temperature during fructification, CO_2 levels) (Hoa and Wang, 2015). However, considering that the environmental conditions in the present study were the same for all levels of nitrogen in the substrate, we deduced that higher N concentrations inhibited the growth of the fruiting bodies, and induced deformations in the mushrooms. High N content also produced slow growth of the stems of the fruiting bodies and delays their formation. It is possible, therefore, that the malformations found in this study are related to an imbalance of the C/N ratio. The use of different agricultural residues used in mushroom cultivation provides a direct relationship with productivity and the color of the fruit bodies (Valenzuela-Cobos *et al*., 2019).

3.3 Nutritional composition of the fruiting bodies

The nutritional quality and composition of the fruiting bodies depends on both the cultivation conditions (temperature, moisture, pH, etc.) and the substrate, since this is the source of the nutrients and lignocellulosic material that supports growth, development, and fructification. The use of a waste lignocellulosic material as substrate is key to maximizing yields and enhancing product disposition (Sardar *et al*., 2017). For this reason, we opted to cultivate *P. djamor* on the same source from which it was isolated (i.e., *A. salmiana* bagasse). The effect of the type of inoculum and the substrate supplemented with urea on nutritional composition of the fruiting bodies are shown in Tables 4.

One of the most important parameters for this type of food is the amount of protein content, since it is well-known that species of *Pleurotus* spp. grow on substrates with low nitrogen content yet the fruiting bodies they produce have higher concentrations of N. Protein content, however, is influenced by factors that include the nature and nutritional components of the substrate, the strain, the stage of development, and the timing of post-harvest analysis (Sardar *et al*., 2017). The genus *Pleurotus* sp. is characterized by low fat content, high protein content, and a low concentration of lipids in the fruiting bodies (Bellettini *et al*., 2019).

Table 4. Proximal chemical analysis of the mushrooms from the WG and WP experiments.

	WG				WP			
	M (%)	C (%)	CP (%)	EE (%)	M (%)	C (%)	CP (%)	EE (%)
T1	86.33 ± 2^a	8.09±0.2^a	26.27±0.7^a	6.58±0.1^a	85.22±2^a	7.37±0.2^a	16.03±0.4^a	7.76±0.1^a
T2	89.58±1^b	7.58±0.1^a	23.22±0.7^b	6.02±0^a	89.58±1^b	7.64±0.2^a	15.84±0.9^a	7.88±0.5^a
T3	89.14±2^b	7.67±0^a	22.73±0.6^b	6.50±0.1^a	87.85±2^a	8.17±0.1^b	21.89±0.9^b	8.18±0.1^a
T4	89.83±1^b	7.42 ± 0.6^a	21.91 ±0.5^b	7.13±0.1^a	89.83±1^b	8.21±0.1^b	24.86±0.6^c	7.90±0.1^a
T5	88.86±0.7^b	8.25± 0.3^a	15.20 ± 0^c	6.83±0^a	89.02±1^b	8.29±0^b	22.94±0.2^b	8.36±0^b

T1: Substrate not supplemented; T2: 0.95% of nitrogen; T3: 1.14% of nitrogen; T4: 1.32% of nitrogen; T5: 1.5% of nitrogen; M: Moisture; C: Ash; CP: Crude protein; EE: ethereal extract. Means not labeled with the letter "a" are significantly different from the mean of the control level in the Tukey Multiple Comparison Test with a significance level ($P \leq 0.05$). ± Standard deviation.

The CP content of the fruiting bodies in our WP and WG treatments was found to be within the ranges established for the genus *Pleurotus*; that is, 17-25%, with 20.3% of CP for *P. ostreatus* (España-Rodríguez *et al*., 2021), and 20.7-28% for *P. djamor* (Salmones, 2017). The highest CP content (26.27%) occurred in T1 of the mushrooms inoculated with WG without added nitrogen. We expected that in addition to improving BE and PR, supplementing the substrate with a source of N (urea) would increase CP content, but as the concentration of urea in the substrate increased in the WG treatments, the CP of the mushrooms decreased significantly. Although the concentration of CP in the mushrooms inoculated with WP achieved a value considered typical for this species, it was below the level of the mushrooms inoculated with WG. Treatment T4 (WP) had the highest protein content. Bellettini *et al*., (2019) described wheat seeds can provide nutrients to the inoculum that enrich the substrate and define the development of the mycelium in early stages. Because the inoculum with WP did not contain nutrients from wheat, it may have impeded optimal growth. Therefore, it is probable that the type of substrate and inoculum influence the nutritional composition of the fruiting bodies.

The mushrooms in experiments WG and WP largely avoided suffering statistically-significant changes ($p \leq 0.05$) in fat content, as this ranged from 7.42-8.36%, while species like *Pleurotus eryngii* may contain up to 5.18% of fat (Sardar *et al*., 2017). The percentage of moisture depends on the strain, substrate, and growing environment (temperature, relative humidity) (España-Rodríguez *et al*., 2021). The moisture determined for the fruiting bodies in this study ranged from 85.22-89.83% in the two experiments and showed statistically-significant differences with respect to controls (T1) ($p \leq 0.05$). These values concur with reports in the literature that indicate 85-90.9% of moisture (Sardar *et al*., 2017).

Up to now, this mushroom has not been cultivated on *A. salmiana* bagasse, but its high adaptability to cultivation conditions could well make it attractive in international markets as an exotic mushroom that can be cultivated on maguey bagasse.

The suitability of a substrate for cultivating mushrooms depends on its chemical composition, availability, and cost. In this regard, residual *A. salmiana* bagasse from pulque, inulin, and agave honey production is a promising option that can compete on a small scale with other substrates, since its generation is growing daily, with reports of the generation of 0.5 tons of bagasse from inulin production, and 0.16 tons from producing just 0.4 tons of agave honey (Hoz-Zavala *et al*., 2017). In addition, its availability, though not specific, contains the elements necessary for the developmental and reproductive functions of edible mushrooms (Heredia-Solís *et al*., 2017). Moreover, in comparison to similar substrates, when supplemented with urea it reaches a BE greater than that of bagasse of *A. tequilana*, *A. augustifolia*, and *A. weberi*. In general, then, exploiting *A. salmiana* bagasse represents a sustainable option in ecological, social, and commercial terms (Heredia-Solís *et al*., 2014; Heredia-Solís *et al*., 2017).

Conclusions

This is the first report to address the isolation of *P. djamor* grown on *Agave salmiana* as an atypical substrate. Results represent an invitation for further study and commercialization. During cultivation on *A. salmiana* bagasse, the growth of the fruiting bodies depended on the type of inoculum and the concentration of urea. Supplementing the substrate

with urea (1.32%) to provide nitrogen improved both BE and PR by as much as 70% and 1.17, respectively, in the cultivation of *P. djamor*. At higher concentrations (1.5%), however, this inhibited the growth of the fruiting bodies and produced malformations. *A. salmiana* bagasse is a suitable alternative substrate due to its chemical composition and high availability because it permits the growth of the mushroom without altering the optimal nutritional composition of this genus, thus providing economic and environmental advantages.

Acknowledgment

The authors thank CONACYT for Fellowship No. 732368 granted for the Doctor's Degree studies of Brianda Susana Velázquez De Lucio.

References

Abdullah, N., Ismail, R., Johari, N.M.K., Annuar, M.S.M. (2013). Production of liquid spawn of an edible grey oyster mushroom, *Pleurotus pulmonarius* (Fr.) Quél by submerged fermentation and sporophore yield on rubber wood sawdust. *Scientia Horticulturae 161*, 65-69. https://doi.org/10.1016/j.scienta.2013.06.026

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J. (1990). Basic local alignment search tool. *Journal of Molecular Biology 215*, 403-410. https://doi.org/10.1016/s0022-2836(05)80360-2

AOAC. (1990). *Official Methods of Analysis*, 15th ed. Association of official analytical chemists. USA.

Bellettini, M.B., Fiorda, F.A., Bellettini, S. (2015). Aspectos gerais do cultivo de cogumelo *Pleurotus ostreatus e djamor* pela técnica Jun - Cao. Apprehendere, Guarapuava.

Bellettini, M.B., Fiorda, F.A., Maieves, H.A., Teixeira, G.L., Ávila, S., Hornung, *P.*S. *et al.* (2019). Factors affecting mushroom *Pleurotus* spp. *Saudi Journal of Biological Sciences 26*, 633-646. https://doi.org/10.1016/j.sjbs.2016.12.005

Chevenet, F., Brun, C., Bañuls, A.L., Jacq, B., Christen, R. (2006). TreeDyn: towards dynamic graphics and annotations for analyses of trees. *BMC Bioinformatics 7*, 439. https://doi.org/10.1186/1471-2105-7-439

Dereeper, A., Audic, S., Claverie, J.M., Blanc, G. (2010). BLAST-EXPLORER helps you building datasets for phylogenetic analysis. *BMC Ecology and Evolution 10*, 1-6. https://doi.org/10.1186/1471-2148-10-8

Dereeper, A., Guignon, V., Blanc, G., Audic, S., Buffet, S., Chevenet, F., *et al.* (2008). Phylogeny. fr: robust phylogenetic analysis for the non-specialist. *Nucleic Acids Research 36*, W465-W469. https://doi.org/10.1093/nar/gkn180

Díaz, A.S., Rentería, L.F., Cortez, J.A., Palacios, E.S. (2014). PCR: reacción en cadena de la polimerasa. In: *Herramientas Moleculares Aplicadas en Ecología: Aspectos Teóricos y Prácticos* (R.A. Cornejo, D.A. Serrato, A. Rendón, M.M.G. Rocha, eds.) SEMARNAT, México.

Doroteo, L.A., Segura, *P.*B.Z., Arce, R.V., Fernández, J.Y., Aguilar, M.E.G., Mendoza PCG, del Toro GV. (2018). Utilización de marcadores ITS e ISSR para la caracterización molecular de cepas híbridas de *Pleurotus djamor*. *Revista Iberoamericana de Micología 35*, 49-55. https://doi.org/10.1016/j.riam.2017.06.003

España-Rodríguez, M., Hernández-Domínguez, E.M., Velázquez-De Lucio, B.S., Villa-García, M., Álvarez-Cervantes, J. (2021). Productivity and proximate chemical analysis of *Pleurotus* spp. grown on *Agave salmiana* bagasse as an alternative substrate. *Agrociencia. 55*, 569-581. https://doi.org/10.47163/agrociencia.v55i7.2604

Fanadzo, M., Zireva, D.T., Dube, E., Mashingaidze, A.B. (2010). Evaluation of various substrates and supplements for biological efficiency of *Pleurotus ostreatus sajor-caju* and *Pleurotus ostreatus*. *African Journal of Biotechnology 9*, 2756-2761. https://doi.org/10.5897/AJB2010.000-3100

Gouleke, C.G. (1977). Biological processing: Composting and hydrolysis. In: *Handbook of*

Solid Waste Management (D.G. Wilson eds). Pp. 197-225. Van Norstrand Reinhold, Nueva York.

Heredia-Solís, A., Bañuelos-Valenzuela, R., Esparza-Ibarra, E. (2017). Producción de *Pleurotus ostreatus* sobre sustratos de *Agave salmiana* y *Agave weberi*. *Biotecnología y Sustentabilidad 2*, 31-36.

Heredia-Solís, A., Esparza-Ibarra, E., Romero-Bautista, L., Cabral-Arellano, F., Bañuelos-Valenzuela, R. (2014). Bagazos de *Agave salmiana* y *Agave weberi* utilizados como sustrato para producir *Pleurotus ostreatus*. *Revista Iberoamericana de Ciencias 1*, 103-110.

Hoa, H.T., Wang, C.L. (2015). The effects of temperature and nutritional conditions on mycelium growth of two oyster mushrooms (*Pleurotus ostreatus* and *Pleurotus cystidiosus*). *Mycrobiology 43*, 14-23. https://doi.org/10.5941/myco.2015.43.1.14

Hoz-Zavala, M., Esther, E., Nava-Diguero, *P.* (2017). Situación del agave y sus residuos en Tamaulipas. *Revista de Energías Renovables 1*, 19-31.

Huanca-Mamani, W., Martínez, R.S., Sepúlveda-Chavera, G. (2014). A fast and efficient method for total DNA extraction from soil filamentous fungi. *Idesia 32*, 75-78. http://dx.doi.org/10.4067/S0718-34292014000200010.

Huelsenbeck, J.*P.*, Ronquist, F. (2001). MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics 17*, 754-755. https://doi.org/10.1093/bioinformatics/17.8.754

Imtiaj, A., Lee, T.S., Ohga, S. (2011). Sequence variation of *Pleurotus* species collected from Eastern Asia. *Micología Aplicada International 23*, 1-10.

Koutrotsios, G., Mountzouris, K.C., Chatzipavlidis, I., Zervakis, G.I. (2014). Bioconversion of lignocellulosic residues by *Agrocybe cylindracea* and *Pleurotus ostreatus* mushroom fungi - Assessment of their effect on the final product and spent substrate properties. *Food Chemistry 161*, 127-135. https://doi.org/10.1016/j.foodchem.2014.03.121

López-Rodríguez, C., Hernández-Corredor, R., Suárez-Franco, C., Borrero, M. (2008). Evaluación del crecimiento y producción de *Pleurotus ostreatus* sobre diferentes residuos agroindustriales del departamento de Cundinamarca. *Universitas Scientiarum 13*, 128-137.

Mkhize, S.S., Cloete, J., Basson, A.K., Zharare, G.E. (2016). Performance of *Pleurotus ostreatus* mushroom grown on maize stalk residues supplemented with various levels of maize flour and wheat bran. *Food Science and Technology 36*, 598-605. https://doi.org/10.1590/1678-457X.08516

Monterroso, F.O.G. (2009). Efecto de la suplementación de la caña de maíz (*Zea mays* L.) con nitrato de amonio, nitrato de potasio y urea en el cultivo del hongo *Pleurotus ostreatus* (Cepa ECS-152). Doctoral thesis. Universidad de San Carlos de Guatemala. http://www.repositorio.usac.edu.gt/9488/

Naim, L., Alsanad, M.A., El Sebaaly, Z., Shaban, N., Abou, F.S., Sassine, Y.N. (2020). Variation of *Pleurotus ostreatus* (Jacq. Ex Fr.) *P.* Kumm (1871) performance subjected to different doses and timings of nano-urea. *Saudi Journal of Biological Sciences 27*, 1573-1579. https://doi.org/10.1016/j.sjbs.2020.03.019

Naraian, R., Sahu, R.K., Kumar, S., Garg, S.K., Singh, C.S., Kanaujia, R.S. (2009). Influence of different nitrogen rich supplements during cultivation of *Pleurotus florida* on corn cob substrate. *Environmentalist 29*, 1-7. https://doi.org/10.1007/s10669-008-9174-4

Nava-Cruz, N.Y., Medina-Morales, M.A., Martinez, J.L., Rodríguez, R., Aguilar, C.N. (2015). Agave biotechnology: an overview. *Critical Reviews in Biotechnology 35*, 546-559. https://doi.org/10.3109/07388551.2014.923813

Nylander, J.A.A. (2004). MrModeltest Version 2. Uppsala University, Uppsala http://www.abc.se/?nylander/. program distributed by the author. Accessed 15 Jan 2021.

Ogundele, G.F., Abdulazeez, R.O., Bamidele, O.*P.* (2014). Effect of pure and mixed substrate on oyster mushroom (*Pleurotus ostreatus*) cultivation. *Journal of Experimental Biology and Agricultural Science 2*(2S), 215-219.

Rambaut, A. (2018). FigTree v1. 4. 4. Institute of Evolutionary Biology, University of Edinburgh, Edinburgh. http://tree.bio.ed.ac.uk/software/figtree/. Accessed: 20 Jan 2021.

Royse, D.J., Rhodes, T.W., Ohga, S., Sanchez, J.E. (2004). Yield, mushroom size and time to production of *Pleurotus cornucopiae* (oyster mushroom) grown on switch grass substrate spawned and supplemented at various rates. *Bioresource Technology 91*, 85-91. https://doi.org/10.1016/s0960-8524(03)00151-2

Royse, D.J., Sánchez, J.E. (2017). Producción mundial de setas *Pleurotus* spp. con énfasis en países iberoamericanos. In: *La biología, el Cultivo y las Propiedades Nutricionales y Medicinales de las Setas Pleurotus spp*. (D.J. Royse, J.E. Sánchez eds.) Pp. 17-25. El Colegio de la Frontera Sur. Tapachula.

Salmones, D. (2017). *Pleurotus djamor*, un hongo con potencial aplicación biotecnológica para el neotrópico. *Revista Mexicana de Micología 46*, 73-85.

Sardar, H., Ali, M.A., Anjum, M.A., Nawaz, F., Hussain, S., Naz, S., Karimi, S.M. (2017). Agro-industrial residues influence mineral elements accumulation and nutritional composition of king oyster mushroom (*Pleurotus eryngii*). *Scientia Horticulturae 225*, 327-334. http://dx.doi.org/10.1016/j.scienta.2017.07.010

Thompson, J.D., Higgins, D.G., Gibson, T.J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research 22*, 4673-4680. https://doi.org/10.1093/nar/22.22.4673

Valenzuela-Cobos, J. D., Vásquez-Véliz, G.K., Zied, D.C., Franco-Hernández, O.M., Sánchez-Hernández, A., Garín-Aguilar, M.E., & del Toro, G.V. (2019). Bioconversion of agricultural wastes using parental, hybrid and reconstituted strains of *Pleurotus* and *Lentinula. Revista Mexicana de Ingeniería Química 18*, 647-657. https://doi.org/10.24275/uam/izt/dcbi/revmexingquim/2019v18n2/Valenzuela

Valenzuela-Cobos, J.D., Grijalva-Endara, A., Marcillo-Vallejo, R., & Garcés-Moncayo, M. F. (2020). Production and characterization of reconstituted strains of *Pleurotus* spp. cultivated on different agricultural wastes. *Revista Mexicana de Ingeniería Química 19*, 1493-1504. https://doi.org/10.24275/rmiq/Bio1126

Zervakis, G.I., Venturella, G., Fryssouli, V., Inglese, *P.*, Polemis, E., Gargano, M.L. (2019). *Pleurotus opuntiae* revisited-An insight to the phylogeny of dimitic *Pleurotus* species with emphasis on the *P. djamor* complex. *Fungal Biology 123*, 188-199. https://doi.org/10.1016/j.funbio.2018.12.005