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Molecular characterization of *Pleurotus ostreatus* commercial strains by random amplified polymorphic DNA (RAPD)

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The genetic variability of strains of *Pleurotus ostreatus* expresses basidiomata with distinct morphological characteristics and responses of different production. Therefore, choosing the correct strain to be used is one of the factors limiting the cultivation of *P. ostreatus*. Thus, we evaluated the genetic variability (Random Amplified Polymorphic DNA (RAPD)), coloring pileus and yield, four commercial strains of *Pleurotus ostreatus* grown in the state of São Paulo (POS 98-38, POS 09-100, POS 09-101 and POS 09-102). Molecular characterization showed two different groups, with 69% similarity between them. Within the group that contains the three samples collected from mushroom growers, strains POS 09-100, POS 09-101, and POS 09-102 showed similarities of 93%, which showed pileus light gray color and yield statistically different (Tukey 5%) relative to the second group. The second group was only formed by strain POS 98-38, which showed staining of the pileus dark gray and lower yield in the first group. Therefore, the genetic variability between strains of *P. ostreatus* analyzed showed genetic differences, morphological and yield.

Key words: Random amplified polymorphic DNA (RAPD), strain, morphology, yield, number of clusters.

INTRODUCTION

The *Pleurotus* genus belonging to the order *Agaricales* is considered one of the most important genus of edible mushrooms currently known for the great variety in the morphology of individuals (Lopes, 1999). Species of edible mushrooms with medicinal properties and important biotechnology and environmental applications are included in this genus (Cohen et al., 2002).

Among the main cultivated species are: *Pleurotus ostreatus*, *Pleurotus sajor-caju*, *Pleurotus citrinopileatus, Pleurotus pulmonarius, Pleurotus cystidiosus, Pleurotus sapidus, Pleurotus abalones, Pleurotus salmoneo-stramineus, Pleurotus ferulae* and *Pleurotus ostreatus* var. "Florida" (Maziero, 1990; Wang et al., 2001). However, the correct description of the species and varieties of commercial *Pleurotus* morphological point of view is influenced by different climates in countries that cultivate these species as well as methods of cultivation in substrate materials that result in different forms of growth (Marino et al., 2003). The color of the pileus is related to the light intensity (Durand, 1976) and the

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Table 1. Origin of the *Pleurotus ostreatus* strains used in the experiments.

Strains	Isolation date	Origin
POS 98/38	06/1998	Database of the Mycology Collection of the Mushroom Research Center
POS 09/100	01/2009	Mushroom collected from a commercial grower cultivated in pasteurized substrate in the region of Sorocaba, São Paulo State, Brazil
POS 09/101	01/2009	Mushroom collected from a commercial grower cultivated in pasteurized substrate in the region of São Pedro, São Paulo State, Brazil
POS 09/102	02/2009	Mushroom collected from a commercial grower cultivated in pasteurized compost in the region of Tatuí, São Paulo State, Brazil

interaction of temperature and light intensity changes the color of the pileus *Lentinula edodes* (Przybylowicz and Donoghe, 1990).

The mushrooms of genus *Pleurotus* have wide geographical distribution, being *P. ostreatus* the mushroom of greatest commercial importance in this genus, distributed in temperate areas and tropical regions (Yang, 1986; Delmas, 1989).

Marino et al. (2003) reported that the gene expression of each isolate could be influenced, between other factors temperature and light intensity. In Brazil, there are some native species of *Pleurotus*, most of them not studied yet, which would have potential to be used for cultivation, with the advantage of being adapted to regional Brazilian climates (Maziero, 1990). About that, Sales-Campos et al. (2010) cultivated a *P. ostreatus* strain occurring in the Brazilian Amazon in wood and agroindustrial wastes and obtained satisfactory results.

Multiple companies or cooperatives have traded the inoculum (spawn) of *P. ostreatus* or the substrate itself colonized. However, very little is known about the origin and genetic variability of the individual used for the commercial production of mushrooms of that species.

The methods for identifying genotypes are important to understand the evolutionary history of fungi. Additionally, the success of the growing trade of mushrooms requires an ability to identify the species correctly and distinguish between lines of a single species (Bunyard et al., 1996).

The taxonomy of species of *Pleurotus*, in spite of all the efforts for clarification, is still confusing (Menolli Jr. et al., 2010). Due to the variety of mushrooms, the groups and distribution of most species are not known (Vilgalys and Sun, 1994). Additionally, the morphology diversity of the mushrooms of multiples species, especially those distributed in different parts of the world, as well as the ones erroneously identified as trading isolates, has led to multiple names for the same species (Menolli Jr. et al., 2010).

The random amplified polymorphic DNA (RAPD) is a convenient method to detect genetic diversity (Marino et al., 2003; Sunagawa and Magae, 2005; Larraya et al., 1999). This method has been particularly successful when applied to check the strains of mushrooms with different origins (Lopandic et al., 2005).

This study evaluated the variability between four commercial strains of *P. ostreatus* (POS-98/38, POS-09/100, POS-09/101 and POS-09/102), collected in the state of São Paulo, and characterized by the method RAPD (Williams et al., 1990).

MATERIALS AND METHODS

The strains of *P. ostreatus* used in the experiment were obtained from mushroom growers of São Paulo State and from the Database of the Mycology Collection of the Mushroom Research Center, College Agronomical Science, Department Plant Production, Botucatu-SP (Table 1).

Isolation and primary array

This technique was used for isolation and getting primary array of commercial strains based on the collection of a healthy mushroom of *P. ostreatus* on cultivation places, and in aseptic environment, in a laminar flow chamber. First, the mushroom was manually sectioned in the middle and, with the help of a cutting clip; fragments were transferred from 3 to 5 mm of the internal pseudotissue to Petri dishes containing 20 ml of CA (Compost Agar) culture medium.

The CA medium was prepared based on the extract of a mixture of sugar cane bagasse (30%) plus braquiaria grass (30%) plus brizantha grass (30%) plus wheat bran (10%). For such, 40 g (dry basis) of the mixture was boiled for 10 min in 500 ml of distilled water. The extract was filtered using a 60-mesh sieve. Later 500 ml of distilled water was added to complete the volume for 1 L. Then the mixture was stored inside bottles of Duran, sterilized at 121°C during 30 min. and after 24 h, added with 7.5 g of Agar and autoclaved again so that the Tindal's process was accomplished. After cooling and in a laminar flow chamber, 20 ml of medium were transferred to sterile Petri dishes.

It is important to emphasize that the fragments were cut away from the gills, to prevent the transfer of spores for the culture medium and, consequently, the risk of genetic recombination and morphological changes of the strains. The colonization of the culture medium occurred under the temperature of 25 ± 1°C in biologic oxygen demand (BOD) for 10 days in the darkness.

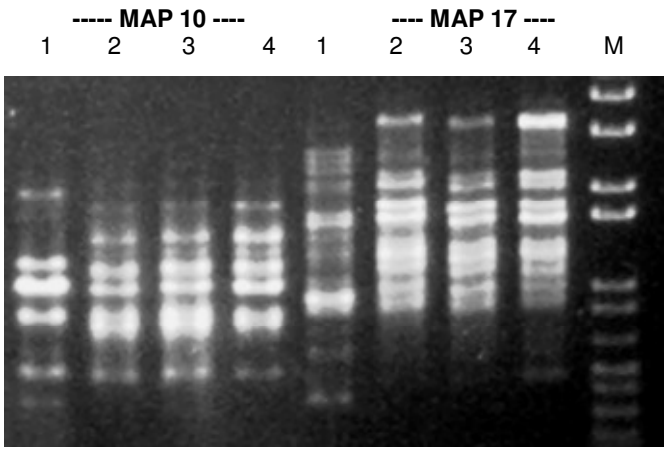

Figure 1. Amplification results of the *P. ostreatus* strains within oligonucleotides MAP 10 and MAP 17. Bands: 1. POS 98-38; 2. POS 09-100; 3. POS 09-101; 4. POS 09-102; M = Molecular marker 1 Kb plus ladder.

The certification and viability of the obtained strains, as well as the strain of the Database of Germplasm of the Mushroom Center Research, were conferred by means of their precultivation in pasteurized substrate, based on sugar cane bagasse (45%), sugar cane straw (45%) and wheat bran (10%) (ingredients dry base). Next, the harvest of healthy mushrooms was carried out and the isolation process was repeated.

Molecular characterization

The strains have been characterized by the RAPD method (Williams et al., 1990). The genetic material was obtained from mycelium cultivated on the plates with solid media, potato-dextrose-agar (BDA). The method described by Doyle and Doyle (1988), modified for use in fungi, was used for the isolation of DNA. Oligonucleotide starter sets of K, O, and M (K-12, K-15, OPA-01, MAP-08, MAP-10, and MAP-17) were used for the amplification of DNA. The reactions occurred in a final volume of 10 µl, including: 50 ng of DNA, 0, 1 mM DNTPs 2, 5, $MgCl_2$ mM, 1 µM of the oligonucleotide starter, buffer reaction (10 mM Tris-HCl, 50 mM KCl), 1 U Taq polymerase. The amplifications were developed with an initial cycle of 94°C, 4 min, 35 cycles of 94°C, 1 min, 40°C, 1 min, 72°C, 1 min, with a final extension period of 72°C, 10 min. The products of the amplification were analyzed in agar gel 0.8%, stained with bluegreen and viewed under ultraviolet light. The data generated were analyzed by the software GEL-PRO ANALYZER version 3.1, by means of which they were calculated the coefficients of similarity by Jaccard's coefficient and building the dendograms for the genetic distances (Figure 1).

Yield, number of clusters, and coloring of pileus

Cultures were grown in substrate from sugar cane bagasse (47.5%, dry basis) + sugarcane straw (47.5%, dry basis) + wheat bran (5%, dry basis), with the C/N adjusted to 60:1. The substrate was prepared in covered shed with concrete floor in a pile (2 m long, 1.5 m wide and 1 m high) with overturned every 48 h and correction of moisture (72%), lasting 9 days. Subsequently the substrate was pasteurized Dalsem climate chamber with a temperature of 59.5 °C for 8 h and conditioning at 46.5°C for 96 h with subsequent cooling to 25°C. Inoculation was performed with 1.5% inoculum (inoculum. Kg^{-1} substrate fresh weight). The inoculum was prepared with wheat grains boiled in water for 30 min and adjusted the pH to 7 and autoclaved in the plastic bags of high-density polypropylene for 4 h. Incubation occurred at 25°C in the darkness with relative humidity of 80% and later harvested manually in three flushes with the production cycle of 60 days. Yield was measured by the production of fresh mushrooms divided by the fresh weight of substrate (fresh weight of mushrooms/fresh weight of substrate*100). The coloration of the pileus was assessed visually demonstrating through pictures.

The experiment was completely randomized (4 strains x 1 substrate) with 15 repetitions. Statistical analysis was performed by the analysis of variance of the average treatment (yield and number of clusters) (Tukey, 5%) by software ASSISTAT Version 7.6 beta (2013) (Silva and Azevedo, 2009).

RESULTS AND DISCUSSION

Primers MAP-10 and MAP-17 were selected according to the amplification of the fragments of DNA in all isolates and because they present clear polymorphic bands, easily detected by the human eye (Figure 1).

The patterns of RAPD revealed variability among the four mushroom strains analyzed. This confirms the high discriminatory ability of the RAPD technique in the studies of genetic diversity of fungi, being useful in the differentiation of isolates from the various species of mushroom commercial strains (Chiu et al., 1996; Colauto et al., 2002; Khush et al., 1992; Ramirez et al., 2001; Zhang and Molina, 1995). Bunyard et al. (1996) analyzing the ribosomal DNA classes for the assessment of genotypic classes of *Pleurotus* spp. among 21 individuals tested, verified a gap of 4 to 25% was seen among the group and between isolates W84 (*P. dryinus*) and 609 (*P. cystidiosus*). Thus, the phylogenetic relations between different populations also provide a tool to understand the biogeographical history of *Pleurotus* species (Vilgalys and Sun, 1994).

The formation of two different groups appears in the dendograms shown in Figure 2. A group with the three strains collected from producers of *P. ostreatus* in the State of Sao Paulo, that is, POS 09-100, POS 09-101 and POS 09-102. The other group with the strain of the database of the Mycology Collection of the Mushroom Research Center, POS 98-38.

The four strains of *P. ostreatus* presented genetic similarity of 38%. According to Ravash et al. (2010), the high genetic diversity detected within groups is probably due to an efficient flow of gene between compatible strains within each group. These authors report even the adaptation of certain groups of fungi from different places due to the accumulation of genetic differences within the same species due to the process of isolation. Similar reports were described by Bunyard et al. (1996), who verified that the species *P. ostreatus* is too heterogeneous, especially when obtained from distant geographical locations and more variation might occur, in relation to the phylogenetic tree, between populations geographically isolated than between different kinds of

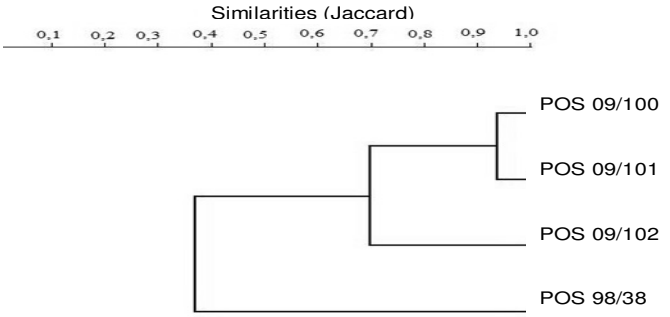

Figure 2. Dendogram based on the UPGMA method, according to the amplification patterns obtained within oligonucleotides MAP 10 and MAP 17 for all *Pleurotus ostreatus* strains.

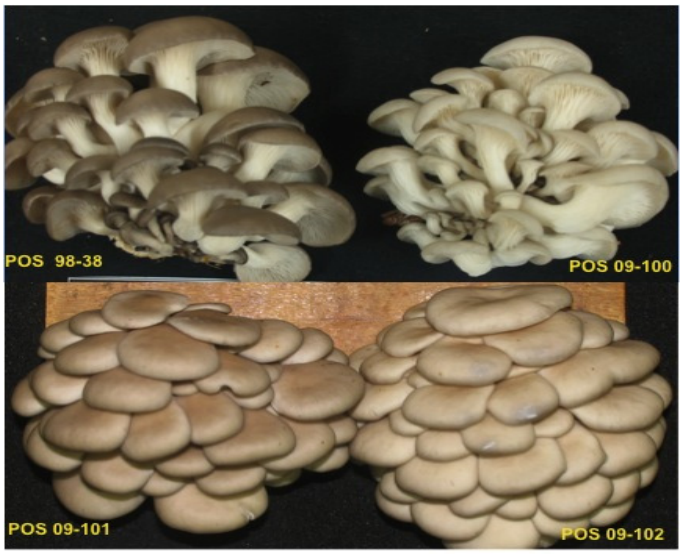

Figure 3. Coloration of clusters of the strains POS 98-38, POS 09-100, POS 09-101 and POS 09-102 of *P. ostreatus*.

Table 2. Yield and number of clusters of strains POS 98-38, POS 09-100, POS 09-101 and POS 09-102 of *P. ostreatus* grown in pasteurized substrate.

Strains	Yield	Number of clusters
POS 98-38	2.52^{c}	1.86^{c}
POS 09-100	17.05^{b}	18.33^{b}
POS 09-101	20.00^{ab}	22.86^{b}
POS 09-102	23.50^{a}	29.46^{a}
CV %	40.03	36.66
Test F	31.97**	47.01**

Means followed by the same letter do not differ statistically among themselves. The test was applied Tukey test at 5% probability.

Pleurotus. The group containing the three strains collected from growers has similarity of 69%. Still within this group, the strains POS 09-100 and POS 09-101 showed similarity of 93%, indicating low genetic variability. Menolli Jr. et al. (2010), also obtained a genetic similarity of similar 98% between 12 isolates from *Pleurotus* spp. Bunyard et al. (1996), reported a similarity between 21 isolates from *Pleurotus* Spp., where almost all of them were grouped together and many species have been considered similar to the *P. ostreatus* within this group, that is, 536 (*P. ostreatus* var. florida - 598), 529 (one wild isolate of *P. sapidus*), and 397 (a commercial strain of *P. cornucopiae*) were reported as being similar to the *P. ostreatus*. Marino et al. (2006), evaluating the molecular characterization of seven isolates from *P. ostreatus*, verified a similarity of 85% among them, in spite of having different origins.

Among the main morphological factors, the change of color of mushrooms is very common in species and depends on the environmental conditions. In some cases, a single strain of *P. ostreatus* grown in different regions may present different morphological characteristics, what might lead the grower to erroneously consider them as distinct strains of a same species. About that, Marino et al. (2003), observed that the activity of *P. ostreatus* submitted to a luminous intensity of 120 lux and 28°C did not present pileus colouring, but the luminous intensity of 900 lux at 15°C caused the reduction of staining with the increase of luminosity in isolates. In this experiment, there was a difference in color of the pileus, where the strain POS 98-38 showed staining pileus darker compared with other strains. This can be explained by the greater genetic variability among the other strains. Strains POS 09-101 and POS 09-102 showed no differences in relation to coloration of the pileus being regarded by the analysis by RAPD similar 98% (Figure 3).

Bunyard et al. (1996) verified that the morphology of the mushroom of isolate 397 (commercial strain of *P. cornucopiae*) was almost identical to *P. ostreatus*, that is, although originally identified as *P. cornucopiae*, the results suggest that the isolate was not handled correctly and might, in fact, be an isolate from *P. ostreatus*. Menolli Jr. et al. (2010) reported that the combination of morphological studies and molecular phylogenetic analysis provide a useful tool to understand the systematic and species limits of the genus *Pleurotus*.

Another factor observed in this experiment was the yield and the number of clusters of *P. ostreatus* strains. The Yield of POS 09-101 and POS 09-102 strains were statistically equal unlike POS 98-38 and POS 09-100 strains suggesting that genetic similarities revealed by RAPD test. Thus, the genetic variability of the strains is correlated with yield, number of clusters, and coloring pileus (Table 2).

Conclusion

The analysis of the molecular characterization in this work detected the existence of genetic variability among the strains evaluated, being strain POS 98-38 the most

distinct from the others in all evaluations. This variability makes possible future studies in relation to their agronomic characteristics.

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