Maintenance culture medium and inoculum based on peach palm leaves for *Pleurotus* spp. production

Meio de manutenção e inóculo a partir de folhas de pupunheira para a produção de **Pleurotus** spp.

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ABSTRACT: This study evaluated the use of immersion water from peach palm leaves (PPLDA) as a component of the culture medium for the maintenance of *Pleurotus* spp. and the use of agricultural waste, peach palm leaves, as inoculum support for the fungi. The performance of the inoculum based on peach palm leaves (PPL) for the production of Pleurotus spp. fruiting bodies was compared with that using wheat grains (WG) as inoculum support. PPLDA culture medium (immersion water of peach palm leaves, dextrose, and agar) showed a lower radial velocity of mycelial growth for both fungi than that obtained with the culture medium WDA (wheat extract, dextrose and agar), commonly used as maintenance medium for Pleurotus spp. However, the type of inoculum support does not significantly influence the linear velocity of P. ostreatus mycelial growth, reaching 6.71 mm/day on wheat grains and 6.18 mm/day on peach palm leaves. Thus, when the inoculum based on peach palm leaves is utilized, the immersion water used for preparing this support can be used for preparing the PPLDA maintenance culture medium, diminishing the production costs of Pleurotus mushrooms. Data also showed that when Pleurotus sajor-caju was cultivated on peach palm leaves, using PPL as inoculum support, the fruiting bodies production parameters (Y = 47%, BE = 3% and Pr = 0.2 g/day) did not differ fromthat obtained using WG.

KEYWORDS: Pleurotus sajor-caju; Pleurotus ostreatus; agricultural waste.

RESUMO: Este trabalho avaliou a utilização da água de imersão das folhas de pupunheira como componente do meio de cultivo para manutenção da cepa fúngica e a produção de inóculo de Pleurotus spp. utilizando como suporte folhas de pupunheira, um resíduo agrícola. Ainda, para verificar a capacidade do inóculo à base de folhas de pupunheira (FP) de produzir corpos frutíferos, foi realizado um experimento comparando FP com o inóculo à base de grãos de trigo (GT). Para tanto, realizou-se a comparação das velocidades de crescimento radial entre o meio de cultivo TDA (extrato de trigo, dextrose e ágar) utilizado para a manutenção de fungos do gênero Pleurotus e o meio FPDA (água de imersão de folhas de pupunheira, dextrose e ágar), e a comparação das velocidades de crescimento linear entre os grãos de trigo, utilizados como suporte para o inóculo de fungos do gênero Pleurotus e as folhas de pupunheira. O meio de cultivo TDA apresentou velocidade radial superior ao meio FPDA para as duas espécies, no entanto, o tipo de suporte não influenciou significativamente a velocidade de crescimento linear de P. ostreatus, que apresentou 6,71 mm/dia em grãos de trigo e 6,18 mm/dia em folhas de pupunheira. Assim, quando o inóculo à base de folhas de pupunheira é utilizado, a água de imersão deste suporte pode ser aproveitada para a confecção do meio de cultivo de manutenção (FPDA), diminuindo os custos produção. Observou-se que Pleurotus sajor-caju, quando cultivado em folhas de pupunheira utilizando inóculo FP, apresentou cerca de 47% de rendimento, 3% de eficiência biológica e 0,2 g/dia de produtividade, sem diferença significativa com o inóculo GT.

PALAVRAS-CHAVE: Pleurotus sajor-caju, Pleurotus ostreatus, resíduos agrícolas

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INTRODUCTION

Brazil is the largest producer, consumer, and exporter of hearts of palm in the world. In 2014, the production was 146,279 tons, with 22,954 tons produced in the south of Brazil (IBGE, 2016). Due to the depletion of natural reserves of Juçara palms (*Euterpe edulis*) generated by severe devastation, the cultivation of alternative palm trees, such as peach palms (*Bactris gasipaes*) for hearts of palm production has increased in the Southern and Central areas of the country (Chaimsohn; Durigan, 2004). Factors that encourage peach palm cultivation are that the cultivation is high density (5000–6600 plants/ha), occupying a smaller geographical area (Bovi, 2000); it can be grown in several agro-bioclimatic regions (Santos et al., 2008); and it can be harvested earlier (around 2 years) than the Juçara species (7 years) (Chaimsohn, 2000).

The cultivation and extraction of hearts of palm generate large amounts of waste and only a small part of the biomass is commercialized in the form of pickled and bottled hearts of palm. Most of the residual biomass remains in the soil after extraction (Soto et al., 2005). According to Israel (2005), in order to obtain the heart of palm, it is necessary to cut down the palm tree and remove the fibrous leaves that cover its sheath. Only 1 m of a palm tree with an approximate height of 15 m is commercially exploited. In the industrial processing of preserved hearts of palm, the palm tree arrives with coated sheaths with an average weight of 5 kg, of which approximately 95% is considered waste (Seben et al., 2012). The leaves, the stem, and the outer sheaths are discarded in the farming area, and the middle sheaths are discarded in the industrial processing (ISRAEL, 2005). The permanence of this residue in the soil encourages the humidification process, mobilizing intense microbial activity and temporarily causing a nitrogen deficiency, which is consumed by microorganisms at the expense of plants (REICHER, 2009). One way to reduce this environmental impact is reusing the waste generated from this process as a substrate for the production of edible mushrooms, providing the opportunity of another source of income for farmers (Philippoussis, 2009). The use of such waste in mushroom production can utilized due to the fact that some fungi are able to decompose lignocellulosic residues. This is the case of fungi of the genus *Pleurotus*, from the Basidiomycetes class, which, along with other fungi, form a group called "white rot fungi", because they form a white mycelium and have a single lignocellulolitic enzyme complex, with enzymes such as cellulase, ligninase, cellubiase, hemicellulases, and laccase, that degrade lignin as much as cellulose (Carvalho et al., 2010). According to Kakon et al. (2012), mushrooms of the genus Pleurotus have high gastronomic value, are rich in protein and vitamins, contain fiber, carbohydrates and several minerals, and are low in fat.

The cultivation of *Pleurotus* in its solid state involves two steps. The first step is the introduction of inoculum in

the substrate in order to enable mycelial growth through cell division. In the second step, the formation of fruiting bodies occurs (CHANG; MILES, 2004). The use of agricultural waste as solid substrate for mushroom cultivation requires the preparation of the substrate. First, the waste must be ground into particles of 2 cm to 5 cm, immersed in water for about 12 h, placed in polypropylene bags, and sterilized at 121°C for 1 h (Bonatti et al., 2004). The purpose of the trituration of the substrate is the formation of a system of pores in order to facilitate gas exchange and temperature control. The immersion of the substrate in water is important for solubilizing many compounds that can inhibit the mycelial growth (CHANG; MILES, 2004). This process generates the immersion water as more waste. The solid inoculum, or "spawn", comprises a solid support such as grains from rye, rice, wheat, millet, and sorghum (CHANG; MILES, 2004), inoculated under aseptic conditions with an agar disk containing the mycelium (Bonatti et al., 2004).

Given the above, this study evaluated the use of the immersion water of the substrate as a component of the culture medium for the maintenance of fungal strain in Petri dishes, and the production of inoculum of *Pleurotus* spp. using peach palm leaves as growing support. In order to check the capacity of the inoculum based on peach palm leaves (PPL) to produce fruiting bodies, an experiment was conducted comparing this inoculum with that based on wheat grains (WG), generally used in solid state cultivation of *Pleurotus*.

MATERIAL AND METHODS

Microorganisms

Pleurotus sajor-caju was purchased from the Culture Collection of Basidiomycetes of the Instituto de Botânica de São Paulo, under the code CCB 019. Pleurotus ostreatus was purchased from "Deutsche Sammlung von Mikroorganismen und Zellkuturen Gmbh" under the code DSM 1833. The strains were maintained in WDA (Wheat extract, Dextrose, and Agar) (Furlan et al., 1997) and under refrigeration (4°C).

WDA culture medium was composed of 20 g/L of dextrose and 15 g/L of agar dissolved in wheat extract (wheat grains boiled in water, 1:2 (w/v), for 10 min). The culture medium was disposed in Duran flasks and sterilized in autoclave at 121°C (1 atm), for 20 minutes. After the temperature cooled to around 40°C, 20 mL of the culture medium were poured into previously sterilized Petri dishes measuring 90 mm ϕ x 15 mm high. After medium solidification, the center of each Petri dish was inoculated with a 12 mm agar disk containing fungal mycelium. The fungus was incubated at 28 \pm 2°C until the Petri dish was completed covered by the mycelium.

Utilization of the immersion water in the composition of the maintenance culture medium

Peach palm leaves were dried at 60°C for 24 h, ground in a feed crusher, packed in a raffia bag, and immersed in water for 12 h (1:6 w/v). The leaves were removed and the residual immersion water was added to 20 g/L of dextrose and 15 g/L of agar, generating the PPLDA (immersion water of peach palm leaves, dextrose, and agar). The culture medium was autoclaved at 121°C (1 atm), for 15 min. Petri dishes prepared with WDA culture medium, widely reported as a maintenance culture medium for *Pleurotus* strains (Furlan et al., 1997; Bonatti et al., 2004; Rampinelli et al., 2010; Borges et al., 2013) were used as a control.

Petri dishes containing WDA and PPLDA were divided into four quadrants and inoculated, at their center, with an agar disk of 12 mm diameter containing seven-day-old mycelium of *Pleurotus sajor-caju* CCB 019 or of *Pleurotus ostreatus* DSM 1833. The Petri dishes were incubated at $28 \pm 2^{\circ}$ C. The growth advance of the mycelium (in millimeters) was measured daily in each quadrant and the radial growth velocity was calculated by the relation between fungal mycelium growth (mm) and time (days). The experiment was conducted in five replicates.

Utilization of peach palm leaves for inoculum production

Peach palm leaves were dried at 60°C for 24 h, ground in a feed crusher, packed in a raffia bag, and immersed in water for 12 h (1:6 w/v). The leaves were removed, drained until reaching about 70% humidity (MADAN et al., 1987), and disposed in glass tubes (1.5 cm diameter and 15 cm length) sealed with cotton wool covered with gauze. The tubes were autoclaved at 121°C (1 atm) for 1 h and inoculated in a laminar flow cabinet pressing the edge of the glass tube on the agar of a Petri dish containing fungal mycelium. This procedure allowed the insertion of a 15 mm diameter agar disk at the end of the tube. The tubes were incubated at 28 ± 2 °C and the linear growth of the mycelium was monitored through the measurement of the daily advance (in millimeters) of the mycelium. The radial growth velocity was calculated by the relation between fungal growth (mm) and time (days). The experiment was conducted in five replicates. In addition, an assay using wheat grains instead of peach palm leaves as growth support was used as a control. The wheat grains were washed and boiled in water (1:2, w/v) for 10 min. The water was drained and the cooked grains were added to CaCO₃ and CaSO₄ at ratios of 0.35% and 1.3%, respectively, relative to the mass of grains before boiling. This procedure aimed to keep the pH slightly alkaline and the grains unpacked (Bonatti et al., 2004). The addition of these components to the peach palm leaves was not necessary.

Performance of the peach palm leaves inoculum on production of *Pleurotus* spp. fruiting bodies

The cooked grains and the peach palm leaves after immersion were packed in polypropylene bags (250 g dry mass/bag). CaCO $_3$ and CaSO $_4$ were added to the wheat grains in ratios of 0.35% and 1.3%, respectively, related to the mass of grains after cooking. The bags were sealed and sterilized in autoclave at 121°C (1 atm) for 1 h. After cooling, each bag was inoculated with 3 agar disks of 12 mm in diameter containing fungal mycelium grown in WDA medium (for wheat grains support) or PPLDA (for peach palm leaves support). All bags were incubated at 28 \pm 2°C, in the absence of light, until complete colonization of the support.

For substrate preparation, the peach palm leaves were ground in a feed crusher, dried at 60° for 1 h, and packed in raffia bags. The bags were immersed in water for 12 h. Afterwards, the leaves were removed and drained for about 2 h until reaching about 70% moisture (MADAN et al., 1987). The drained leaves were added to 5% (dry weight) rice bran and packed in polypropylene bags in an amount corresponding to 150 g of dry weight. The bags were sterilized in autoclave at 121°C (1 atm) for 2 hours and the inoculation was done in laminar flow cabinet using 10% of inoculum, considering the dry weight of the substrate. The inoculum consisted of palm peach leaves (PPL) or wheat grains (WG) colonized with the mycelium of P. ostreatus or P. sajor-caju. The incubation occurred in the absence of light, at 28°C, until complete substrate colonization. Fruiting bodies induction was achieved by plastic bag perforation and incubation under controlled temperature (28 ± 2°C), relative air humidity (90 \pm 2%), and light (12 hours/day). The harvest moment was determined visually, when the edge of the pileus was planar, in a stage that precedes the sporulation (Fig. 1). The fruiting bodies were harvested with a scalpel, weighted, dried at 40°C for 24 hours in a forced air circulating oven and weighted again for moisture content determination (Bonatti et al., 2004).

The experiments were implemented in seven replicates and were evaluated in terms of yield (Y%: ratio between fresh fruiting bodies and initial substrate weights), biological efficiency (BE%: ratio between dried fruiting bodies and initial substrate weights), and productivity (Pr (g/day): ratio between dried fruiting bodies weight and total time of cultivation) (Holtz et al., 2009).





Figure 1. Harvest moment of fruiting bodies of *Pleurotus sajor-caju* (A) and *Pleurotus ostreatus* (B).

Statistical Analysis

All the data were analyzed throughout the test to reject deviant values (Dixon's Q Test) (RORABACHER, 1991) and were submitted to the variance analysis through the Tukey Test, with a 95% confidence level (ANOVA).

RESULTS AND DISCUSSION

Evaluation of immersion water in the composition of the maintenance culture medium

Full colonization of the Petri dishes containing WDA maintenance culture medium occurred in 5 days for *P. sajor caju* and *P. ostreatus*, with a significantly higher radial velocity of growth (mm/day) than that obtained with PPLDA (Fig. 2). When PPLDA was used, full colonization of the Petri dishes occurred in 7 and 6 days for *P. sajor-caju* and *P. ostreatus*, respectively. Figure 3 shows the visual aspect of the Petri dishes colonized by the mycelium.

Visual analysis shows that mycelium growth of *P. ostreatus* is more vigorous when cultivated in PPLDA than in WDA (Fig. 3B), suggesting that PPLDA is more nutritious for this species. *P. sajor-caju* has grown better in WDA (Fig. 3A). PEDRA; MARINO (2006) found that the vigorous growth of the mycelium can be influenced by the substrate composition. However, in our research, the vigor of the mycelium was not reflected in the radial growth velocity since the growth was slower in PPLDA than in WDA (Fig. 2), regardless of the

mycelium vigor. Bernardi et al. (2007) also observed that *P. ostreatoroseus* growing in rice husks showed bigger mycelium growth regardless of the lesser vigor visually shown by the lesser density of the hyphae.

FAN et al. (2001) reported values for mycelial velocity of 8.98 mm/day and 8.12 mm/day for *P. sajor-caju* LPB 20 and *P. sajor-caju* LPB 19, respectively, both grown in medium containing coffee husks extract, dextrose, and agar. These velocities are similar to those found in our research using wheat grains.

Donini et al. (2005) found values of radial growth of *P. ostreatus* BF24 of 24.1, 17.3, 17.8 mm/day, and 10.7 mm/day when cultivated in an infusion of elephant grass, soybean straw, sugar cane, or corn cob in boiling water for 30 min added to 10 g/L of dextrose, respectively. Sales-Campos et al. (2011) using culture media composed of the infusion of marupá straw and açaí seeds in boiling water for 30 min added to bran cereals, CaCO₃, and 12 g/L of dextrose, reported radial growth of *P. ostreatus* of 81 mm in 7 days, reaching 11.6 mm/day of radial growth velocity. Carvalho et al. (2013) evaluated the mycelial growth of *P. ostreatus* in culture media containing 80% leaves from different banana cultivars supplemented with 20% wheat bran, and boiled for 15 min. The authors reported velocity values ranging from 12.00 to 13.75 mm/day.

The values of radial velocity reported in other scientific sources are higher than those found in this body of work using PPLDA. It should be noted that during the immersion of the leaves and the cooking of the residues and the grains, several compounds are solubilized (Chang; Miles, 2004), becoming available for the maintenance of the microorganisms. The results reported in this study and in other sources show that the type

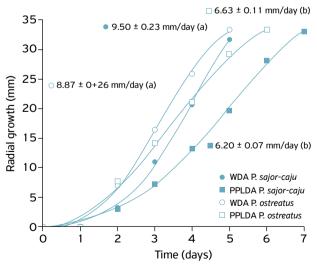


Figure 2. Rate of radial growth (mm) with time (days) and radial velocity of growth (V_R^{\pm} standard deviation) of *Pleurotus sajor-caju* and *Pleurotus ostreatus* in WDA (wheat extract, dextrose and agar) and PPLDA (immersion water of peach palm leaves, dextrose and agar). Equal letters mean values without significant differences based on Tukey Test, with 95% confidence level (ANOVA).

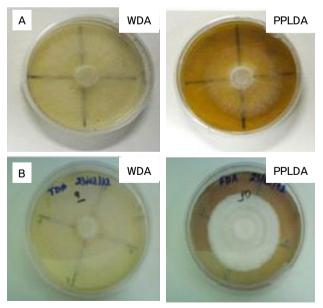


Figure 3. Radial growth of the mycelium of *Pleurotus sajor-caju* (A) and *Pleurotus ostreatus* (B) in WDA (wheat extract, dextrose, and agar) and PPLDA (immersion water of peach palm leaves, dextrose, and agar).

of substrate utilized for preparing the culture medium, the kind of treatment of the substrate and the fungal strain, have direct influence on the mycelial growth velocity. Despite taking a few more days to complete the colonization of the Petri dish, the PPLDA culture medium composed of peach palm immersion water, instead of the wheat extract (more expensive) utilized for preparing WDA, (Fig. 2), is still viable as a maintenance culture medium for *P. sajor-caju* and *P. ostreatus*.

Evaluation of the inoculum production

The values of mycelial linear growth (mm/day) on the analyzed substrates (wheat grains and peach palm leaves) for *P. sajor-caju* and *P. ostreatus* are shown in Figure 4. *P. sajor-caju* colonized the wheat grains (WG) faster (20 days) than *P. ostreatus* (27 days). However, there were no significant differences in the linear growth of both species when peach palm leaves (PPL) were used as substrate. Additionally, the type of inoculum support did not significantly influence the linear growth velocity of *P. ostreatus*, which reached 6.71 mm/day and 6.18 mm/day in WG and in PPL, respectively.

Donini et al. (2006) reported higher values for linear growth velocity of *P. ostreatus* BF24 when they evaluated elephant grass (*Pennisetum* sp.) as the substrate, supplemented with soy, wheat, rice, and corn bran, at concentrations of 0, 10 and 20%. The linear growth velocity was 20.9 mm/day for the experiment executed without supplementation, and this value decreased when the supplementation increased. Palheta et al. (2011) evaluated the mycelial linear growth of the strains *Pleurotus ostreatus* and *Pleurotus florida* in cupuaçu husks (CC) supplemented with 10% rice bran (FA) reaching values of 4.0 mm/day and 3.1 mm/day for *Pleurotus florida* and *Pleurotus ostreatus*,

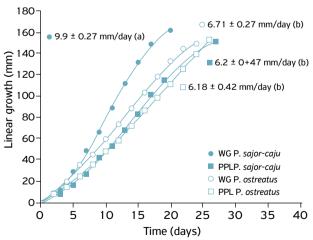


Figure 4. Rate of linear growth (mm) with time (days), and linear velocity of growth ($V_L\pm$ standard deviation) of *Pleurotus sajor-caju* and *Pleurotus ostreatus* in wheat grains (WG) and in peach palm leaves (PPL). Equal letters mean values without significant differences based on the Tukey Test, with 95% confidence level (ANOVA).

respectively. These values were lower than those achieved in our research, using wheat grains and peach palm leaves.

Figure 5 shows visual aspects of the linear mycelial growth of *P. sajor-caju* (Fig. 5A) and *P. ostreatus* (Fig. 5B), in glass tubes containing wheat grains and peach palm leaves.

Greater mycelium vigor, revealed by the greater density of the hyphae (Bernardi et al., 2007) can be observed for both species grown on wheat grains. However, the greater vigor of the mycelium was not reflected in the linear growth velocity, since no significant differences in velocities achieved in wheat grains and peach palm leaves were observed (Fig. 4).

Performance of the peach palm leaves inoculum on production of *Pleurotus* spp. fruiting bodies

Results in terms of yield (Y%), biological efficiency (BE%), and productivity (Pr - g/day) for cultivation of *P. sajor-caju* and *P. ostreatus* using inoculum based on wheat grains (WG) or in peach palm leaves (PPL) are shown in Table 1.

Table 1 shows that the production parameters did not differ significantly for both fungi when WG or PPL were utilized. According to these results, the inoculum based on peach palm

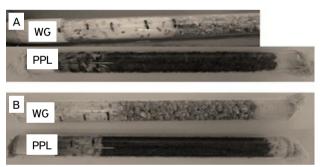


Figure 5. Linear mycelial growth in wheat grains (WG) and in peach palm leaves (PPL) disposed in glass tubes inoculated with *Pleurotus sajor-caju* (A) and *Pleurotus ostreatus* (B).

Table 1. Mean values \pm standard deviation of yield (Y%), biological efficiency (BE%), and productivity (Pr - g/day) achieved with the cultivation of *P. sajor-caju* and *P. ostreatus* using inoculum based on wheat grains (WG) or peach palm leaves (PPL)

Inoculum	Y (%)	BE (%)	Pr (g/day)
Pleurotus sajor-caju			
WG	$47.7 \pm 7.2 \ a$	$3.79 \pm 0.24 c$	$0.25 \pm 0.02 d$
PPL	$46.3 \pm 6.5 \ a$	3.03 ± 0.33 <i>c</i>	$0.20 \pm 0.02 \ d$
Pleurotus ostreatus			
WG	38.6 ± 14.6 a	3.88 ± 1.11 <i>c</i>	0.11 ± 0.03 e
PPL	23.3 ± 10.7 b	2.64 ± 1.16 c	0.11 ± 0.05 e

Equal letters mean values without significant differences based on the Tukey Test, with 95% confidence level (ANOVA).

leaves can be suggested as a substitution for the inoculum based on wheat grains. This proposal is based on the fact that peach palm leaves are considered waste and have a low cost of acquisition. Additionally, comparing the two species, *P. sajor-caju* showed higher productivity than *P. ostreatus* (0.11 g/day) in both inoculum supports (0.25 g/day in WG and 0.20 g/day in PPL), showing a higher preference for peach palm leaves than *P. ostreatus*. Holtz et al. (2009) found higher productivity for *P. ostreatus*, about 0.37 g/day, using cotton waste from the textile industry as substrate. However, the authors used 20% of inoculum based on wheat grains, instead of 10% as used in our body of work.

Table 1 also shows that *P. sajor-caju* reached a higher yield than *P. ostreatus* using PPL inoculum. However, the conversion of this value to BE (considered a more accurate parameter as the ratio between the weight of the fruiting bodies and the weight of the substrate, both without moisture interference) makes that difference disappear.

CONCLUSION

The maintenance culture medium based on wheat extract (WDA) promoted radial growth velocities greater than that

obtained with the culture medium based on immersion water of peach palm leaves (PPLDA) for both fungal species, *P. sajorcaju* and *P. ostreatus*.

The inoculum based on wheat grains (WG) favors the linear growth velocity for the *P. sajor-caju* strain.

The immersion water of peach palm leaves can be utilized for the preparation of the fungi maintenance culture medium and also lowers production costs since peach palm leaves are considered agro-industrial waste.

In the evaluation for both fungal species (*P sajor-caju* and *P. ostreatus*), there is no significant difference in biological efficiency (BE%) and productivity (Pr g/day) when inoculum based on peach palm leaves or wheat grains are used.

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