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CULTIVATION OF *PLEUROTUS* MUSHROOMS ON BRAZILIAN COFFEE HUSK AND EFFECTS OF CAFFEINE AND TANNIC ACID

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ABSTRACT

The possibility of using the total coffee residue (husks) obtained from the Brazilian coffee industry as substrate for cultivation of *Pleurotus* mushroom and degradation of caffeine and phenolic compounds present in this residue were studied. Eight strains of *P. ostreatus* and two strains of *P. sajor-caju* were tested on a medium prepared from aqueous extract of coffee husk and agar. Based on the best mycelial growth and biomass production in plate, the strain *P. ostreatus* LPB 09 was selected for further studies. The first fructification occurred 20 days after inoculation, and the biological efficiency reached about 96% after 60 days. It was found that fruiting bodies accumulated 0.157-mg/g caffeine on a dry weight basis. After cultivation, the content of caffeine and phenolic compounds in the husk was reduced at 60.6% and 79.1%, respectively. The results indicated the feasibility of using coffee husk without any pre-treatment for the cultivation of *Pleurotus*. We propose that after cultivation, the residue might be useful for feeding of ruminant as several toxic compounds decreased, while the protein concentration increased (9.62%).

Key words: Cultivation, coffee residue, *Pleurotus*, caffeine, phenolic compounds, detoxification.

INTRODUCTION

Coffee is one of the most important beverages in the world and its yearly production is about one million tons (green bean) in more than 50 countries. However, the main by-products, pulp and husks amounts to one million tons, which remains a serious problem due to the presence of toxic materials such as caffeine, free phenols (monomers) and polyphenols (tannins) [3]. The outer skin and a part of mucilage form the coffee pulp (i.e., the exocarp and part of the mesocarp of the coffee cherry), produced during the wet processing; whereas all parts except the bean form the coffee husk, produced by dry processing in Brazil.

Many have studied, proposed uses for the wastes such as silage, aerobic composting, biogas production, vermiculture, animal feed (cattle, pork, chickens, fishes), and production of ethanol, vinegar, single-cell protein, enzymes, biopesticides, and probiotics [3, 13]. However, successful transfer of technologies into industrial scale use have been limited [13].

Martinez-Carrera [13] succeeded in producing mushrooms on a large scale with fresh coffee pulp (wet process). It was allowed to drain for 4-8 h and then piled up into long pyramidal heaps for a short aerobic fermentation. For mushroom cultivation, the fermented coffee pulp was pasteurised by immersion in hot water at 70–90 C for 1-2 h or in an appropriate room (tunnel) with steam at 60-100 C for 6-24 h. The caffeine content in the pulp after pasteurisation reached 0.20-0.25% [11], and the biological efficiency (B.E.) reached 159.6%. The author confirmed that the caffeine content of that range did not inhibit the mycelial growth [12].

In Brazil, the coffee cherries (*Coffea arabica* L.) are processed principally by the dry process [14], resulting in two parts, one is the coffee husk (residue), the other is the green bean (traded coffee). Brand *et al.* [2] reported the content of caffeine and phenolic compounds in coffee pulp as 0.75% caffeine and 3.7% phenolic compounds, while in coffee husks it was 1.2% caffeine and 9.3% phenolic compounds. It is noted that the toxic compounds (caffeine and phenolic compounds) in Brazilian coffee husk are much higher than in coffee pulp, so it is more difficult to treat coffee husk than pulp. Fan *et al.* [5] reported that raw coffee husk can be treated with hot water (100 C, 1 h) for cultivation of *Lentinus edodes*. Previous results [11] confirmed that the toxic materials can be minimised by hot water treatment, but it also was noted that the other residue (waste water) would lead to environmental pollution. We evaluated the feasibility of using untreated Brazilian coffee husk for the cultivation of *Pleurotus* mushrooms in order to avoid the process of water treatment. The degradation of toxic materials, particularly caffeine, was also studied. To confirm the results, the effect of tannic acid and caffeine on the mycelial growth of *Pleurotus* was also tested.

MATERIALS AND METHODS

Micro-organisms and screening. Eight strains of *P. ostreatus* (Jacq. ex Fr.)Kumm. and two of *P. sajor-caju* (Fr.)Sing. from the culture collection of the Laboratory of Biotechnological Processes (LPB), were used for screening purposes. The majority of the strains have been introduced, and their codes are *P. ostreatus* LPB 01, 03, 08, 09, 22, 23, 24, 31; and *P. sajor-caju* LPB 19 and 20. Strains were maintained on potato-dextrose-agar (PDA) at 4 C. All strains were screened in a medium containing the extract of coffee husk, which was prepared by cooking the coffee husks (40g/l) in distilled

water for 1 h, filtered, and made up to 1 l with distilled water. The pH was adjusted to 7 and after mixing with agar (2%), the medium was autoclaved at 121 C for 15 min. Then 15 ml per dish was poured into Petri dishes (9-cm diameter), and allowed to cool. Plates were inoculated with fungal cultures, and the radial mycelial growth and biomass productions were measured. Biomass was determined by dissolving the agar and separation of mycelia on filter paper [1, 5].

Spawn preparation. The sawdust of *Eucalyptus* spp. (80%) and rice bran (20%) was used for spawn preparation. The mixture was adjusted to 60% moisture, and then placed in glass jars of 500 ml capacity. The spawn in the jars became totally colonised and ready for inoculating after 20 days of mycelial growth [5].

Cultivation. The coffee husks were adjusted to 55-60% moisture content, placed in plastic bags (20 x 35 cm), and autoclaved at 121 C for 1.5 h. The inoculation method depended on the spawn rate. The spawn was inoculated only one place or mixed thoroughly with the substrate. Inoculated bags were incubated at 24 C in the dark. After 15 days, bags were transferred to an environmental chamber (90% relative humidity), and the plastic was removed to promote fruiting-body development [1, 5]. The dry weight of the spawn was included as substrate when B.E. was calculated.

Effects of caffeine and tannic acid. The caffeine and tannic acid were from Sigma. Experiments were conducted in PDA plates with the addition of caffeine (0, 30, 50, 100, 500, 1,000, 2,500 mg/l) and tannic acid (0, 100, 500, 1000, 5,000 and 10,000 mg/l). After one week, the radial growth of mycelia was measured and aerial mycelium was harvested using a scraper. The agar medium without mycelia was also collected for analysis of caffeine and tannic acid. The total biomass was measured as described above, plus the dry weight of aerial mycelia [5].

Analyses of caffeine and phenolic compounds. To analyse caffeine, the spectrophotometric method (276.5 nm) was used according to manual edited by Lutz Adolfo Institute [8], modified according to references [7, 10]. Samples (2 g) were mixed with 15 ml concentrated H_2SO_4 in a 100-ml glass beaker, and heated in a boiling water bath for 15 min. The mixture was added to 50 ml-distilled water, and heated again for 15 min as described above. After using Whatman filter paper, the filtrate was neutralised using NaOH (1 N). Caffeine was extracted from the filtrate with chloroform. Caffeine was determined by extracting with chloroform and drying at 104 C. The concentration of phenolic compounds, expressed as tannic acid, was determined according to Folin-Denis method [6]. Samples (5 g) were mixed with distilled water (200 ml) and heated for 2 h. After filtering, a 5 ml sample

Table 1. Mycelial growth of *Pleurotus* strains on coffee-husk extract medium*.

Strains	Radial growth (mm/day)	Biomass (mg/ dish)
P. ostreatus LPB 09	9.7 ±1.67^{a}	43.40 ±0.14^{a}
P. ostreatus LPB 01	9.5 ±1.38^{a}	40.10 ±1.84^{b}
P. ostreatus LPB 22	9.3 ±1.53^{ab}	35.70 ±0.00^{c}
P. ostreatus LPB 08	9.0 ±2.85^{c}	31.90 ±4.80^{c}
P. sajor-caju LPB 20	9.0 ±2.07^{c}	24.50 ±2.30^{de}
P. ostreatus LPB 23	9.0 ±1.80^{c}	25.60 ±6.00^{e}
P. ostreatus LPB 24	8.8 ±1.25^{d}	18.80 ±0.56^{f}
P. ostreatus LPB 31	8.8 ±1.22^{d}	15.80 ±0.56^{f}
P. sajor-caju LPB 19	8.1 ±1.81^{e}	22.30 ±2.00^{e}
P. ostreatus LPB 03	6.6 ±1.73^{f}	14.20 ±0.35^{f}

*Temperature of incubation: 24 C for 9 days; initial pH: 7.0. The data represent the average of three measures. Values with different superscript letters in the same column differ significantly among the groups at $p<0.05$.

Table 2. The effect of caffeine on the mycelial growth of *Pleurotus ostreatus* LPB 09 on potato-dextrose agar (PDA), after 6 days of incubation, as well as its accumulation in the mycelium and the culture medium.

Caffeine			Growth	Biomass
Initial (mg/l)	Final (mg/l)	Mycelium (mg/g)	(mm/day)	(mg/dish)
0	0	0	13.12 ±0.21[a]	55.3 ±1.43[a]
30	0	0.066 ±0.0121[a]	12.56 ±0.43[a]	53.4 ±2.36[a]
50	4.02 ±0.243[a]	0.1342 ±0.0013[b]	12.23 ±0.26[a]	52.8 ±1.28[a]
100	58.0 ±8.321[b]	3.79 ±0.432[c]	11.02 ±0.32[a]	47.3 ±1.55[b]
500	399 ±21.566[c]	9.2 ±2.387[d]	8.13 ±0.42[b]	32.6 ±2.35[c]
1000	860 ±58.098[d]	13.9 ±3.143[e]	4.52 ±0.41[c]	12.4 ±2.44[d]
2500	na	na	0	0

na: not analysed. Values with different superscript letters in the same line differ significantly among the groups at $p<0.05$.

was mixed with an equal amount of Folin-Denis reagent and saturated Na_2CO_3 (10 ml). The volume was made up to 100 ml with distilled water. The concentration of phenolic compounds was determined by reading the absorbance at 760 nm in a spectrophotometer. Mycelial samples were reduced to 10 mg (fresh) for analyses of caffeine and phenolic compounds (same as above).

Statistical analysis. Experiments were carried out in triplicate and means results are presented. Comparison between means was carried out using a t-test or analysis of variance (ANOVA) as appropriate.

RESULTS AND DISCUSSION

Table 1 shows the behaviour of ten strains of *Pleurotus* spp. on the coffee-husk extract medium. Although all strains grew well on this medium, the strains of *P. ostreatus* showed better growth, in general, than *P. sajor-caju*. After nine days of incubation, *P. ostreatus* LPB 09 showed the best growth with high-density mycelial growth, having the highest radial growth rate of 9.7 mm/day and a biomass of 43.4 mg/plate. Further investigations were carried out using this strain.

Table 2 shows the effect of different concentrations of caffeine on the mycelial growth and biomass production of *P. ostreatus* LPB 09, grown on PDA after six days of incubation. As the caffeine concentration increased, the mycelial growth and the biomass production decreased. It was noticed that the concentration of caffeine above 100 mg/l showed a significantly negative effect, and no mycelial growth was observed when the concentration reached 2500 mg/l. The mycelium absorbed the caffeine, as its concentration varied from 0.066-13.9 mg/g, increasing proportionally to the concentration in the medium. *Pleurotus* may not be capable of degrading the caffeine, but the mushroom can absorb it. This is in agreement with previous research work by Martinez-Carrera *et al*.[12], which demonstrated that mycelial growth on agar plates was gradually inhibited at caffeine concentrations ranging from 0.25–2.0 mg/ml.

Table 3 shows the effect of different concentrations of tannic acid on the mycelial growth of *P. ostreatus* LPB 09, grown on PDA

Table 3. The effect of tannic acid on the mycelial growth of *Pleurotus ostreatus* LPB 09 grown on potato-dextrose agar (PDA), after 7 days of incubation, as well as its degradation.

Tannic acid				
Initial (mg/l)	Final (mg/l)	Mycelium	Growth (mm/day)	Biomass (mg/dish)
0	0	0	11.29 ±0.11[a]	50.1 ±1.55[a]
100	29.6 ±5.35[a]	0	11.76 ±0.23[a]	52.8 ±1.76[a]
500	277.1 ±23.47[b]	0	8.71 ±0.00[b]	43.1 ±1.21[b]
1000	429.5 ±42.13[c]	0	2.72 ±0.24[b]	10.2 ±1.12[c]
5000	na	na	1.58 ±0.34[d]	3.3 ±1.56[d]
10000	na	na	0.82 ±0.11[e]	1.33 ±0.12[e]

na: not analysed. Values with different superscript letters in the same line differ significantly among the groups at $p<0.05$.

after seven days of incubation. It was observed that the tannic acid, at a concentration of 100 mg/l in the medium, stimulated the mycelial growth; while above a concentration of 500 mg/l, it began to have a negative effect. At concentrations higher than 1000 mg/l, fungal growth decreased. No tannic acid was found in the mycelium at any concentration studied. Its concentration in the culture medium decreased, showing that *Pleurotus* had the capacity to degrade tannic acid.

The first fructification started 20 days after inoculation (5 days after the opening of plastic bags), while four flushes were harvested during 60 days of mushroom cultivation (**Fig. 1**). The total biological efficiency was 96.5%. Martinez-Carrera *et al.* [11, 13] obtained a higher biological efficiency of 175.8% on pasteurised coffee pulp in Mexico, however, the methodology used produced waste water causing environmental pollution. This water, which can be recycled in crop soils after appropriate treatment, is to be added to that generated by the wet processing method of

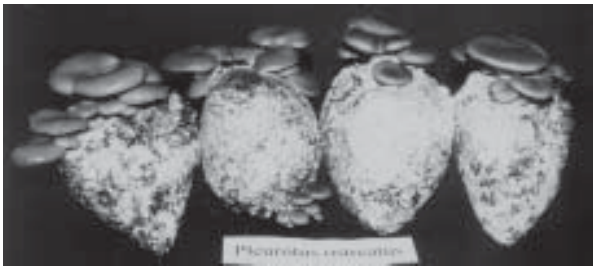

Fig. 1. Fruiting bodies of *Pleurotus ostreatus* cultivated on the Brazilian coffee husk.

coffee cherries. Barbosa [1] used coffee husk for the production of *Pleurotus*, but had no success. They reported that mycelial growth was initially strong and vigorous, but after a few days was inhibited; apparently due to the greater concentration of toxic compounds in the coffee husk, than in the coffee pulp. Initially, we had the same problem when using a 2% spawning rate. After many attempts, satisfactory results were obtained at a spawning rate of 20%, homogeneously mixed with the coffee husk. Our results on coffee husk under Brazilian conditions, although lower than that of Martinez-Carrera *et al.* [13], seem promising in terms of future technological applications.

Table 4 shows that the concentration of caffeine in the coffee husk was reduced by 60.6% after mycelial growth and fruiting of *Pleurotus*. Analysis of fruiting bodies showed the presence of a caffeine concentration

Table 4. Change of the content of caffeine and tannins in the coffee husk, after growing *Pleurotus ostreatus* LPB 09.

	Initial (%)	Final (%)	Reduction (%)
Caffeine	0.65 ± 0.12[a]	0.197 ± 0.093[b]	-60.69± 4.32
Tannins	3.65 ± 0.21[a]	0.76 ± 0.14[b]	-79.17± 8.12

*Values with different superscript letters in the same line differ significantly among the groups at $p<0.05$.

of 0.157 mg/g dry fruiting body. This confirmed that the *Pleurotus* did not completely degrade the caffeine, but the caffeine accumulated in the fruiting bodies. Martinez-Carrera *et al*. [11, 13] reported that the caffeine reduction during *Pleurotus* cultivation is not significant, suggesting that the initial caffeine content was low (0.20-0.25%). The coffee pulp was pasteurised by immersion in hot water at 70 C for 15 min. By contrast, the concentration of phenolic compounds was reduced significantly in the coffee husk, reaching values of 79.1%. However, no phenolic compounds were found in the fruiting bodies showing that *Pleurotus* was capable of degrading phenolic compounds present in the coffee husk.

Ribereau-Gayon [15] reported that plant phenolic compounds include a large range of compounds, such as simple phenols, phenolic acids, polyphenols including various brown oxidised products (widely known as tannins). According to Bressani [3], there are two groups of tannins (polyphenols) in the coffee husk: hydrolysable tannins and condensed tannins. Wong and Wang [16] reported that *P. sajor-caju* was capable of degrading tannins in the spent coffee grounds. Our results are in agreement with this previous research work.

Bressani [3] demonstrated that a ration supplemented with above 30% coffee pulp caused high mortality in young rats and chickens. Cabezas *et al*.[4] reported that the ration supplemented with more than 20% coffee pulp had a negative effect on cows, and similar results on swine were reported by Jarquin [9]. We propose that spent substrate, after oyster mushroom cultivation, could be used as animal feed because the caffeine content is reduced and the protein content increased by 9.62%. However, further studies are needed in this respect.

There are some limitations for large-scale application of the process: elevated cost for sterilisation and inoculation with aseptic conditions. Rural cultivator could have financial problem using sterile substrate and 20% spawn. It is believed that this technique has promise, because, today, people are concentrating on sustainable systems and ambient living.

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