MATEUS DIAS NUNES

TRATAMENTO DE SUBSTRATO COM CAL HIDRATADA PARA CULTIVO DE *Pleurotus spp.*: VISÃO MICROBIOLÓGICA, QUÍMICA E ECONÔMICA

Tese apresentada a Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, para obtenção do título Doctor Scientiae.

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SUMÁRIO

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RESUMO

NUNES, Mateus Dias, D. Sc. Universidade Federal de Viçosa, Fevereiro de 2016. **Tratamento de substrato com cal hidratada para cultivo de Pleurotus spp.: visão microbiológica, química e econômica**. Orientadora: Maria Catarina Megumi Kasuya. Coorientadores: Wendel Batista da Silveira e Marliane de Cássia Soares da Silva.

Cogumelos são considerados alimentos saudáveis devido ao baixo teor calórico, além de possuir alto teor de proteínas e minerais, podendo, assim, serem utilizados como fontes de diversos nutrientes. As principais técnicas utilizadas para o preparo do substrato para o cultivo de cogumelos são a pasteurização e a esterilização. Entretanto, estas técnicas são onerosas. Neste estudo, visando criar alternativas para cultivo de cogumelos de seis espécies do gênero Pleurotus, avaliou-se a utilização de técnicas alcalinas, utilizando bagaço de cana, capim elefante, casca de café Conilon e Arabica e serragem com farelo de arroz. Esta técnica já vem sendo utilizada com sucesso no México, entretanto sem qualquer padronização. As técnicas alcalinas testadas permitiram boa produção de cogumelos, inclusive com composição nutricional similar aos substratos esterilizados, mostrando o potencial da utilização destas técnicas. Além disso, demonstramos que estas técnicas apresentam custo de implementação e manutenção bem menor que a esterilização. Os substratos mais adequados para o cultivo foram o capim elefante, bagaço de cana e serragem com farelo de arroz. A casca de café foi o substrato em que mais se observou o aparecimento dos contaminantes, mostrando a importância da escolha do substrato para a utilização destas técnicas. Em relação às técnicas alcalinas, observamos que a imersão em solução de cal hidratada 2% por 4 h é efetiva para evitar o aparecimento de contaminantes, com redução de até 99% da microbiota residente e a efetiva alcalinização do substrato. Por fim, algumas espécies de cogumelos não foram capazes de serem cultivadas utilizando técnicas alcalinas por inibição da formação dos corpos de frutificação ou do crescimento micelial. Dessa forma, as técnicas alcalinas, principalmente imersão em 2% da solução de cal hidratada, demonstram apresentar potencial para serem utilizadas para produção de diferentes espécies de cogumelos. Assim, os cogumelos podem se tornar fonte de renda e alimentar para as populações, melhorando a qualidade de vida, principalmente das regiões carentes, pois utilizando esta técnica pode-se produzir cogumelos em diferentes resíduos agrícolas com baixo investimento. Entretanto, deve ser salientado que a

escolha do substrato e espécies de fungos adaptadas às condições alcalinas é fundamental para cultivo de cogumelos utilizando as técnicas alcalinas.

ABSTRACT

NUNES, Mateus Dias, D. Sc. Universidade Federal de Viçosa, February, 2016. **Treatment of substrate using lime for Pleurotus mushrooms cultivation: microbiological, chemical and economic vision.** Adviser: Maria Catarina Megumi Kasuya. Co-advisers: Wendel Batista da Silveira and Marliane de Cássia Soares da Silva.

Mushrooms are health food due to low calories content and high level of minerals and proteins, thus it can be used as source of nutrients. The main techniques used to treat substrate for mushrooms growth are pasteurization and sterilization, however they are expensive. In this study, we evaluated the technical viability of alkaline techniques, which has been successfully used in Mexico, to cultivate mushrooms of six Pleurotus species using as substrates: sugar cane bagasse, elephant grass, sawdust/rice bran and Conilon and Arabica coffee husk. The alkaline techniques had productivity and produced mushroom with nutritional composition as well as sterilization method, showing the potential of these techniques. We also showed that the cost of implementation and maintenance were lower than sterilization method. The most suitable substrates were sugar cane bagasse, elephant grass and sawdust/rice bran. Coffee husk substrate was that present higher contamination, showing the importance of choosing the substrates for use alkaline method. Comparing the alkaline techniques, we observed that soaking in 2% lime solution for 4 h was efficient, avoiding avoid contaminants growth by reduction until 99% of resident microbiota found in the substrate and was also effective alkalization. Finally, some mushrooms species were not able to be cultivated using alkaline techniques, due to inhibition of fruiting bodies formation or mycelial growth. Therefore, alkaline techniques, mainly soaking in 2% lime solution, show potential to be used to mushrooms cultivation of different species of Pleurotus in deprived regions. So, mushrooms can be a good source of funds and food for the population, increasing their quality of life, mainly in deprived region, since these alkaline methods can use different agricultural residue with low investment. However, it should be pointed that the choosing of suitable substrate and mushrooms species adapted to alkaline conditions are essential to cultivate mushrooms using alkaline techniques.

INTRODUÇÃO GERAL

1. Cogumelos

Corpos de frutificação, ou cogumelos, de algumas espécies de basidiomicetos são apreciados na culinária de diversos países. Entre as principais espécies produzidas comercialmente estão Agaricus bisphorus, Pleurotus ostreatus e Lentinula edodes. Espécies de Pleurotus são valorizadas na culinária pelo seu sabor, textura, aroma e valor nutricional. O seu alto valor nutricional é devido ao alto conteúdo de proteínas, fibras, vitaminas, minerais e baixo teor de lipídeos (Barros et al,. 2008, Manzi et al,. 1999). Além disso, a composição de proteínas dos cogumelos, que é comparável à encontrada em carnes, apresenta elevada biodisponibilidade proteica (Flegg e Maw 1997, Longvah e Deosthale 1998). Isso é muito importante, do ponto de vista nutricional, para indivíduos vegetarianos, seja devido a questões religiosas, culturais ou financeiras. Devido a todos esses fatores, é desejável e interessante à inserção de cogumelos na alimentação diária humana, principalmente em regiões carentes, onde é observada uma alimentação diária deficiente e inadequada.

Além das questões nutricionais, o consumo dos cogumelos também pode trazer benefícios para saúde humana. Cogumelos da espécie Agrocybe aegerita possuem propriedades anti-inflamatória e antitumoral (Diyabalanage et al,. 2008). Outras espécies dos gêneros Agrocybe, Lentinula, Volvariella e Pleurotus também contêm em sua composição metabólitos com propriedades antioxidantes, antimicrobianos e compostos com atividade hipoglicemiante (Cheung et al,. 2003, Jose et al,. 2004, Ngai et al,. 2005, Wong e Chye 2009). Estudos também sugerem que espécies de Pleurotus ostreatus possuem compostos com propriedade hipocolesterolêmica, uma característica muito importante devido à elevada mortalidade causada por doenças cardiovasculares

em diversos países (Yusuf e Anand 2010, Bobek et al,. 1994, Bobek et al,. 1993). Portanto, além de ser um alimento considerado saudável, o consumo de cogumelos pode trazer benefícios para saúde humana, justificando ainda mais a realização de trabalhos que visam à inserção do mesmo na alimentação diária.

2. Cogumelos: Métodos de preparo de substrato para produção de cogumelos

Atualmente, os métodos de preparo de substrato mais utilizados para produção de espécies de cogumelos comestíveis são a esterilização e a pasteurização. Esses métodos têm sido estudados e aplicados com sucesso na produção comercial de diversas espécies de cogumelos. Entretanto, esses métodos apresentam custo e investimentos iniciais elevados, além de necessitar de mão de obra especializada, tornando a sua implementação em regiões carentes inviável.

Uma técnica alternativa de baixo custo e simples de ser executada têm sido utilizadas com sucesso no México, obtendo-se uma eficiência biológica (produtividade) similar aos métodos convencionais (Léon-Monzón et al,. 2004) A técnica consiste em submergir o substrato que será utilizado para crescimento fúngico em solução de 2% de cal hidratada durante 12 a 48 h (Contreras et al,. 2004). Além disso, o método compostagem com ventilação passiva e a imersão em água a 100 ºC durante 90 min também tem demonstrado ser eficiente e de baixo custo para produção de cogumelos (Avendaño-Hernandez e Sánchez 2013, Jaramillo Mejía and Albertó 2013). Porém, esses métodos são demorados, favorecendo a remoção de nutrientes do substrato e uma distribuição de umidade não homogênea no substrato, o que pode favorecer o desenvolvimento de contaminantes (Contreras et al,. 2004, Jaramillo Mejía e Albertó 2013). Além disso, contaminantes podem produzir substâncias indesejadas, como

micotoxinas, que são prejudiciais à saúde humana (Bennett e Klich 2003). De fato, contaminação bacteriana e fúngica já foram relatadas (Contreras et al,. 2004, Léon-Monzón et al,. 2004), justificando o desenvolvimento de métodos mais rápidos e a padronização dos mesmos, a fim de reduzir o aparecimento de contaminantes.

Outro fator importante que deve ser considerado é quais espécies de cogumelos comestíveis são capazes de serem cultivadas utilizando as técnicas alcalinas de produção. Estudos prévios utilizaram apenas a espécies de Pleurotus ostreatus e Pleurotus pulmonarius para avaliação das técnicas alcalinas (Avendaño-Hernandez e Sánchez 2013, Contreras et al,. 2004, Jaramillo Mejía e Albertó 2013, Léon-Monzón et al,. 2004), restringindo o potencial de possíveis consumidores. Dessa forma, o conhecimento de quais espécies são capazes de serem produzidas pelas técnicas alcalinas é fundamental para disseminação dessas técnicas, além de permitir a elaboração de estratégias de comercialização mais adequadas.

Também deve ser considerado no processo de produção, o efeito dos métodos alcalinos na composição mineral dos cogumelos, uma vez que os minerais são essenciais para o balanço e funções do corpo humano, o que já vem sendo objeto de diversos estudos em diferentes alimentos (McDowell 2003, Kohlmeier 2015, Tsuji et al,. 2016). Cogumelos apresentam concentrações consideráveis de alguns importantes minerais, como por exemplo, potássio e fósforo, o que os torna fonte alimentar de minerais (Kalač 2009). Entretanto, a composição mineral dos cogumelos é afetada diretamente pela concentração dos mesmos no solo/substrato (Borovička et al,. 2010, Brzostowski A et al,. 2011, Drewnowska M et al,. 2012). Dessa forma, é plausível o questionamento em relação ao efeito da alcalinização e da submersão do substrato em solução de cal sobre a composição mineral dos cogumelos. Esse efeito deve ser

esclarecido, tendo em vista o potencial dos cogumelos serem uma importante fonte alimentar de minerais em regiões carentes.

Além disso, é também importante uma comparação de custos entre os métodos alternativos e os métodos padrões para melhor compreensão da viabilidade econômica proporcionada por estas técnicas. Essa informação permitirá um planejamento mais exato dos custos necessários para a implementação destas metodologias.

Diante do exposto, o objetivo geral deste estudo foi avaliar a viabilidade técnica e financeira, da produção de cogumelos de diferentes espécies de fungos do gênero Pleurotus, cultivadas em diversos substratos tratados utilizando diferentes métodos.

Para atender o objetivo geral o trabalho foi desenvolvido para atingir as seguintes metas:

1. Determinar a viabilidade técnica e financeira para cultivo de Pleurotus ostreatus utilizando métodos alcalinos e comparando-los com o método convencional de esterilização.
2. Determinar o efeito de diferentes substratos na viabilidade técnica para cultivo de Pleurotus ostreatus utilizando três métodos alcalinos.
3. Avaliar a relação entre o pH da solução e o pH do substrato no controle de qualidade durante o processo de cultivo dos cogumelos.
4. Avaliar os parâmetros técnicos para cultivo utilizando métodos alcalinos e avaliar o efeito do tempo de submersão na alcalinização e na redução da microbiota residente.
5. Determinar a viabilidade técnica para cultivo de diferentes espécies de Pleurotus utilizando os melhores métodos alcalinos testados.

6. Avaliar o efeito dos métodos alcalinos sobre a composição mineral dos cogumelos.

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CAPÍTULO 1: Alkaline methods are rapid, easy and economical methods to grow Pleurotus ostreatus mushrooms

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Alkaline methods are rapid, easy and economical methods to grow Pleurotus ostreatus mushrooms

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ABSTRACT

Simple and economical methods have been successfully used to produce Pleurotus spp. mushrooms in many countries and regions, including Mexico, India, China, Indonesia and Africa. Such simple methods include immersion in hot water or in alkaline solutions without pasteurization or sterilization treatments. However, these methods are time consuming. Here, we evaluated the viability of rapid alkaline immersion methods (15' = boil for 15 min in 0.5% lime solution; 30' = boil for 30 min in 0.5% lime solution; 4 h = immersion for 4 h in 2.0% lime solution) compared to the sterilizing method, and we found that mushroom production of rapid methods worked as well as the sterilizing method. However, we observed effects on mushroom composition and the time required to finish production. Though, cost estimates showed that the alkaline methods are much more economical than the sterilizing method (USD = 15': 17066.47, 30': 17074.32, 4 h: 16975.94 and sterilizing method: 46043.54). Additionally, selection of substrates with low resident microbial load and with suitable features for mushroom production seemed important for avoiding the growth of antagonistic fungi. Therefore, alkalization can be a money- and time-saving substrate treatment method for P. ostreatus mushroom cultivation.

Keywords: money-saving method; time-saving method; non-sterile method; mushroom cultivation.

1. Introduction

Protein-energy malnutrition affects millions of persons each year, mainly in developing countries. (Grover and Ee 2009). To avoid ill health, daily dietary protein consumption is essential for humans. P. ostreatus mushroom cultivation could be an interesting alternative to alleviate this problem, as it provides a food with high protein

content while using agricultural wastes for its cultivation (Nunes et al,. 2012, Bonatti et al,. 2004). Thus, its growth substrate may be acquired for little or even no money while contributing to environmental conservation. Also, P. ostreatus has a shorter time growth in comparison to others edible mushrooms and demands few environments control, making this specie an excellent alternative for mushroom production (Sánchez 2010).

Sterilization and pasteurization have typically been used to prepare the substrate for commercial P. ostreatus cultivation. However, these methods are expensive and require large investments, making them difficult to implement in poorer areas and countries. An alternative method that is simple and economical has been successfully used in the state of Chiapas, Mexico (Léon-Monzón et al,. 2004). Pleurotus growers commonly prepare the substrate simply by soaking it in a lime solution for 12 to 48 h. Avendaño-Hernandez and Sánchez (2013) also showed that an alternative, simple and inexpensive method of self-pasteurization was suitable, at the farm level, for substrate preparation and P. ostreatus production. Simple and low-cost methods are essential for allowing mushroom cultivation in depressed regions.

Previous studies showed that soaking the substrate in a solution with 0.5% or 2.0% lime for 12 to 36 h or immersing it in hot water (100 °C) for 90 min is a sufficient treatment for P. ostreatus mushroom production (Contreras et al,. 2004, Avendaño-Hernandez and Sánchez 2013, Léon-Monzón et al,. 2004, Jaramillo Mejía and Albertó 2013). However, these methods are very time consuming, and they remove nutrients from the substrate (Jaramillo Mejía and Albertó 2013, Houdeau et al,. 1991), possibly affecting mushroom composition (Ashraf et al,. 2013, Sales-Campos et al,. 2009).

It is clear that the cost of soaking methods is lower than that of the typical thermal methods (pasteurization and sterilization), mainly because soaking methods do

not require energy input and the acquisition of expensive machines. However, as the aim of immersion methods is to decrease the cost of P. ostreatus mushroom cultivation, a cost comparison between alkaline immersion methods (AIM) and the standard thermal treatments is fundamental for a better understanding of the true cost savings achieved using AIM.

In this study, we evaluated the technical of AIM techniques. We tested the effects of these methods on P. ostreatus mushroom yield, flush cycles and composition. The emergence of antagonistic fungi during the mycelial run and the estimated cost of these methods were also evaluated and compared.

2. Materials and methods

2.1. Microorganism and inoculum production

The fungus used was P. ostreatus isolate PLO 06 (GenBank access KC782771). This fungus belongs to the collection of the Laboratório de Associações Micorrízicas / Departamento de Microbiologia / BIOAGRO / UFV. The fungus isolate was grown in a Petri dish containing potato dextrose agar culture medium (PDA; Fluka) at pH 5.8 and was incubated at 25°C. After seven days, the mycelium was used for inoculum production in a substrate based on sorghum grains that was previously boiled and autoclaved at 121 °C for 90 min (de Assunção et al,. 2012).

2.2. Experimental design

The basic design consisted of using one out of four kinds of mushroom cultivation methods and quantifying the effect on mushroom yield, flush cycle and chemical composition.

For the test, it was chosen sugar cane bagasse and elephant grass (Pennisetum purpureum). The bagasse was chosen because it is a byproduct of the sugar/alcohol industry in Brazil and can be easily acquired at a low cost or even for free. Elephant grass was used because it is a plant species that is very cheap to produce, has very fast gowth, and that is generally discarded by animal farmers when it becomes old. The substrates were air dried, crushed and passed through a 0.5-mm mesh. As prior research reported that supplementation with an N source can increase mushroom production (Nunes et al,. 2012), these substrates were mixed with wheat bran at a 4:1 (w/w) prior to the alkaline treatments.

The substrates were subjected to disinfestation methods, as shown in the item 2.3, packed, inoculated with spawn and randomly incubated in dark room at 25 °C for 15 to 35 d (n = 6 each; total = 48). During mycelial run the growth of antagonistic fungi was performed according to Fig A1. After incubation time the packages were randomly transferred to a fruiting room with controlled temperature and humidity of 20 °C and 80%, respectively. The packages were transferred as soon as mycelial run end for each substrate/disinfestation method group (n = 6). After 3 to 5 d mushroom harvest was performed and flush cycle was determined. The fresh weight of the mushrooms was immediately recorded to determine the biological efficiency {BE = (fresh weight of mushrooms/dry weight of substrate) x 100}. Then, packages were randomly transferred to incubation room (dark room at 25 °C) for 15 d. This procedure allows fungi to recovery for a new flush. After new incubation period, mushrooms were harvest and flush cycle were performed as shown before. This process was repeated twice allowing three harvests to be done.

Subsequently, the mushrooms were dried at 60 °C, ground and passed through a 2-mm mesh before their mineral content and nutritional value were determined.

2.3. Disinfestation methods

2.3.1. Alkaline treatments

In this study, we treated the substrate by (i) boiling it in water containing 0.5% $Ca(OH)_2$ for 15 min (15'), (ii) boiling it in water containing 0.5% $Ca(OH)_2$ for 30 min (30'), or (iii) submerging it in water containing 2% $Ca(OH)_2$ for 4 h (4 h). The purpose of increasing the pH and boiling is to inhibit or hamper the growth of contaminants and to reduce the number of resident microorganisms, respectively. We opted to perform treatments with boiling because they are intermediate between sterilization and alkaline soaking treatments. Soaking for 4 h was successfully used previously in our laboratory (unpublished data). After treatment, the substrates were centrifuged at 1.800 rpm for 5 min to remove excess water. Next, 300 g of each sample was packaged in a plastic bag and inoculated with 15 g of spawn.

2.3.2. Sterilization treatment

We choose sterilization as standard treatment because this method is more suitable to cultivation of some Pleurotus species than pasteurization. The substrates were moistened with water to 70% of the retention capacity, and 300 g of each substrate were placed in a polypropylene bag. The bags containing the substrates were then autoclaved at 121 °C for 2 h. After sterilization, the substrates were inoculated with 15 g of spawn.

2.4. Chemical composition

2.4.1. Proximate analysis

To obtain their moisture content, samples of the mushrooms were dried in an oven at 105 °C overnight before being analyzed for chemical composition, with respect to moisture, protein, fat, carbohydrates and ash, using the AOAC procedures (2005)

with minor changes. The crude protein content (N × 4.38) of the samples was estimated using the macro Kjeldahl method; the crude lipid content was determined by extracting a known weight of powdered mushroom sample with petroleum ether, using a Soxhlet apparatus; the ash content was determined via incineration at 600 ± 15 °C. Total carbohydrates were calculated as follows: Total carbohydrates (g) = 100 - (g crude protein + g crude fat + g ash). Total energy was calculated according to the following equation: Energy (kcal) = 4 x (g protein + g carbohydrate) + 9 x (g lipid).

2.4.2. Mineral content

Samples (200 mg) of dry mushrooms were subjected to digestion with a mixture of nitric acid and perchloric acid (3:1, v:v) and 500 μL hydrogen peroxide at 200 °C. (Tedesco et al,. 1995). Complete oxidation of the organic matter occurred when no further darkening of the solution occurred upon continued heating and when a clear yellow or white solution was finally obtained. Finally, the mixture was cooled, quantitatively transferred to a volumetric flask and diluted with ultra-pure water (Milli-Q system, Millipore, USA) to a final volume of 25 mL. The solutions were then transferred to suitable plastic containers after filtration through Whatman No. 42 filter paper (Kent, U.K.) to remove any insoluble silicates or other solid materials. The resulting solutions were used for direct spectrophotometric analysis. Three blank digests were processed in the same way.

The concentrations of iron (Fe), zinc (Zn), potassium (K), sodium (Na), calcium (Ca) and magnesium (Mg) were determined using an atomic absorption spectrometer (Optima 3300 DV; Perkin Elmer, Waltham, MA), using specific standards and procedures for each mineral, as recommended by the manufacturer. It was used

wavelength with higher sensitivity for each mineral and integration time of 15 s, in duplicate for each analysis.

2.5. Statistical analyses

The experiment used a randomized/factorial design. The results were expressed as the mean values with standard deviation. Test for differences in the biological efficiency, flush cycle, nutritional value and mineral content of mushroom between substrates (sugar cane bagasse and elephant grass) and disinfestation methods (15', 30', 4 h, auto) were carried out using two-way ANOVA. The post-hoc HDS-Tukey tests were applied when differences were significant ($p < 0.05$).

2.6. Cost estimates of mushroom cultivation

The data used to estimate the cost of mushroom cultivation were based on our experience during one year at small mushroom company in Viçosa-Brazil and considered the daily production of 25 kg of substrate for fungal growth. The cost analysis included the initial capital investment required for building and equipment acquisition, fixed costs and variable costs (Singh et al,. 2010).

To estimate the investment required for equipment acquisition, the average expenditure on various products in Brazil, such as coats, trays, buckets, gloves, freezers, baskets, industrial ovens, centrifuges, weight balances, etc. were calculated. To estimate the cost of laminar-flow biosafety cabinets, we considered quotes from two different companies in Brazil. To estimate the cost of an autoclave, we considered a quote from a Chinese company well known for making sterilizers for mushroom production. It should be noted that autoclave import duties were not considered because of their variation among different countries.

The depreciation of the buildings and equipment were calculated according to the straight-line method. Maintenance costs were based on the maintenance cost of similar equipment housed at the Universidade Federal de Viçosa (Federal University of Viçosa), Brazil.

For variable cost estimates, the average expenditures on various services in Brazil were considered. These costs were also based in our experience. Water and energy costs were based on their prevailing price in the State of Minas Gerais, Brazil. Note that all cost estimates were calculated in April 2014.

The financial results of the study were calculated on the basis of the national monetary unit, and the results were then reported by converting the values to the US dollar (USD) at the average exchange rate of the Banco do Brasil (Bank of Brazil) over the research period (1 USD = 3.60 Brazilian Real).

3. Results

Both of the studied substrate compositions showed similar trends of BE ($P > 0.05$; Table 1). Additionally, the BE of alkaline treatments was similar to sterilization treatment ($P > 0.05$; Table 1). For both substrates, the highest differences in the BE among the substrate treatments were observed at the first flush; however, in the other flushes these differences were compensated, resulting in a similar total BE.

The substrate treatments had effects on the flush cycle ($P < 0.05$; Table 2). Sterilized substrate was able to produce mushrooms faster than non-sterilized substrate in the first and second flush when elephant grass was used for fungal cultivation (Table 2), but we found no differences in the third flush ($F_{(3;21)} = 2.96$; $p = 0.056$). The same trend was observed with sugar cane bagasse, with boiled substrate presenting a smaller

total flush cycle than sterile substrate in the first flush (Table 2; $P < 0.05$) but with small observed differences in the second flush ($F_{(3;20)} = 2.86$; $p = 0.063$).

The growth of antagonistic fungi was greater in the elephant grass substrate (Fig 1). With this substrate, 15' and 4 h treatments resulted in colonization by antagonistic fungi in all packages, with a higher prevalence in the 4 h substrate treatment (Fig 1).

Table 3 and 4 show variations ($P < 0.05$) among the substrate treatments in the nutritional value and mineral content of the mushrooms. The levels of fat, ash and Zn decreased when the substrates were boiled or immersed in water for 4 h ($P < 0.05$). The same trend was observed for the level of K with sugar cane bagasse. Likewise, the level of protein decreased when the substrates were boiled (Table 3; $P < 0.05$). On the other hand, the Ca level showed a clear increase in the boiled-substrate mushrooms, most likely due to the presence of Ca in the solutions used for the alkaline methods (Table 4).

Cost estimates for mushroom cultivation using different substrate treatments showed that the initial investment and monthly costs of the quick AIM were similar (Table 5). However, the investment and monthly cost of the autoclave method was almost double that of other methods (Table 5).

4. Discussion

Studies concerning alkaline soaking methods for mushroom production have attracted the interest of Central and South American mushroom growers due to their easy implementation, good production and, particularly, their low cost (Contreras et al,. 2004, Léon-Monzón et al,. 2004, Hernández et al,. 2003). These features explain the fast dissemination of these methods in Mexico (Léon-Monzón et al,. 2004). However,

prior studies used methods that are more time consuming than the sterilization process, thus justifying the evaluation of faster methods.

In this context, we showed that the AIM can be used for mushroom production with yields similar or equal to those with sterilized substrates (Table 1). We obtained BE values for P. ostreatus comparable to those described using other substrates prepared via pasteurization, sterilization or alternative preparation methods (Contreras et al,. 2004, Hernández et al,. 2003, Avendaño-Hernandez and Sánchez 2013, Hasan et al,. 2010, Siqueira et al,. 2012), showing that these techniques have good potential for use in mushroom cultivation. Moreover, the use of faster techniques decreases the risk of bacterial contamination (Contreras et al,. 2004) and causes less nutrient removal from the substrate, both of which have been shown to negatively affect the mushroom yield (Jaramillo Mejía and Albertó 2013).

Evaluation of the flush cycle is important for mushroom growers, as the flush cycle affects production costs. The flush cycle was influenced by AIM (Table 2), but it showed different trends among the substrates and flushes. However, in later flush cycles on both substrates, little to no effect on the flush cycle was observed (Table 2). Therefore, as most oyster mushroom growers harvest more than one flush cycle, the quick AIM is suitable for oyster mushroom cultivation, reinforcing the potential of these techniques.

Adjusting the pH of the substrate to alkaline levels without further thermal treatment has been reported as an approach that can prevent or decrease the growth of competitor fungi without substantially affecting the mycelial run of P. ostreatus (Hernández et al,. 2003, Contreras et al,. 2004). In the present study, the substrate composition influenced the growth of antagonistic fungi (Fig 1). The main

contaminating fungi we observed were Trichoderma sp., Penicillium sp. and Aspergillus sp., all of which are common contaminants in P. ostreatus mushroom cultivation (Avendaño-Hernandez and Sánchez 2013, López-Averalo et al,. 1996). In contrast, studies using similar techniques but different substrates neither observed nor mentioned the growth of fungal contaminants (Jaramillo Mejía and Albertó 2013, Contreras et al,. 2004). Thus, the substrate seems to be more relevant to the appearance of contamination than the method used to prepare the substrate. Indeed, substrate age and quality have been noted to affect the emergence of antagonistic microorganisms (Houdeau et al,. 1991, Jaramillo Mejía and Albertó 2013). Such considerations are very important in the case of alkaline methods, as they aim not to eliminate the resident microbiota but to create conditions that promote the growth of P. ostreatus rather than the growth of antagonistic microorganisms. Beyond that, it is well known that mushroom yield is affected by substrate composition. Prior studies, using methods similar to the techniques used in this study, reported the influence of substrate composition on mushroom yield (Jaramillo Mejía and Albertó 2013, Contreras et al,. 2004). Thus, the selection of an adequate substrate seems to be an essential step before employing alkaline methods to produce P. ostreatus mushrooms.

Mushrooms have great nutritional value as they are quite rich in protein, contain important essential amino acids and fiber, and are poor in fat. This combination of nutritional features is one of the reasons for the currently observed rise in mushroom consumption and production (FAOSTAT 2010). Thus, evaluation of the effects that quick AIM has on mushroom nutritional value and mineral content is warranted. In this study, we observed that, irrespective of the observed decreases in the content of protein and some minerals in the mushrooms (Table 3 and 4), their nutritional profiles showed similar or higher values than those reported in other studies (Reis et al,. 2012,

Çağlarırmak 2007). Due to the high levels of calcium in the substrate, we observed the bioaccumulation of this element in the mushroom (Table 4). This finding is interesting as calcium in mushroom has high absorption than green vegetables and is beneficial for the prevention of osteoporosis and related fractures (Reid 2002, Chapuy et al,. 1992, Dawson-Hughes et al,. 1997, Reid et al,. 1993, Heaney et al,. 1988). Moreover, the decrease in fat content (Table 3) increases the attractiveness of mushrooms to consumers because, as noted by Carels et al,. (2006), people tend to base their judgments about the healthiness of food on factors such as the food's perceived fat content.

Here, we show for the first time an estimate of the cost difference between soaking treatments and the sterilizing method (Table 5). It is clear that the quick AIM are much more economical than the sterilizing method in both initial investment and monthly cost, showing the high cost-benefit ratio of these methods. Should be pointed out that mushroom grower will invest only one time in infrastructure; therefore, after this step the maintenance of AIM production is cheap and believable to be made in deprived regions. The overall costs of AIM were, on average, one third those of the autoclave method. However, we estimated the cost using Brazilian parameters, which may be substantially different from the parameters of other countries where Pleurotus growers use soaking alkaline methods, such as Mexico. We also note that the cost of labor was not considered in our study as labor costs vary greatly among different regions and countries. Therefore, further studies of the costs of alkaline immersion methods should consider other regions and countries to enable a more realistic estimate of the overall cost savings of these methods.

5. Conclusion

The present study clearly shows that quick AIM are simple, economical, effective and fast methods for P. ostreatus mushroom production. However, an appropriate substrate should be carefully selected.

6. Competing of interest

The author(s) declare that they have no competing interests.

7. Acknowledgements

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Tables and Figures

Table 1: Biological efficiency (%) of Pleurotus ostreatus cultivated in elephant grass and sugar cane bagasse using different substrate treatments (mean ± SD) and results of two-way ANOVA evaluating the factors substrate and disinfestation method. MS = mean square; F = F statistic; P = significance level.

Flush/Substrate	Disinfestation method *				Factor	MS	F	P
	15 min	30 min	4 h	Autoclave				
1º Flush								
Elephant grass	93.77 ± 01.09 ^{aA}	87.09 ± 02.86 ^{abA}	59.92 ± 12.62 ^{cA}	68.87 ± 15.62 ^{bcB}	Substrate	4.5	0.03	0.871
Bagasse	76.98 ± 15.25 ^{abB}	74.11 ± 12.74 ^{abA}	60.42 ± 14.08 ^{bA}	95.00 ± 09.08 ^{aA}	Disinfestation method	1599.8	9.52	< 0.000
					Interaction	1343.3	8.00	< 0.000
2º Flush								
Elephant grass	17.08 ± 03.84 ^{bB}	27.74 ± 12.76 ^{abA}	30.03 ± 14.87 ^{abA}	34.99 ± 11.39 ^{aA}	Substrate	203.6	1.40	0.243
Bagasse	37.36 ± 21.61 ^{aA}	34.34 ± 08.55 ^{aA}	31.44 ± 08.74 ^{aA}	23.05 ± 03.31 ^{aB}	Disinfestation method	36.6	0.25	0.859
					Interaction	580.4	4.00	0.014
3º Flush								
Elephant grass	11.89 ± 01.63 ^{bA}	18.15 ± 04.79 ^{abA}	22.48 ± 09.85 ^{abA}	28.50 ± 13.32 ^{aA}	Substrate	650.9	10.69	0.002
Bagasse	15.33 ± 05.71 ^{aA}	17.44 ± 05.76 ^{aA}	18.74 ± 07.26 ^{aA}	00.00 ± 00.00 ^{bB}	Disinfestation method	132.4	2.17	0.105
					Interaction	736.1	12.08	< 0.000
Total								
Elephant grass	122.75 ± 11.75	132.98 ± 16.91	112.43 ± 23.85	132.37 ± 24.22	Substrate	323.7	1.18	0.284
Bagasse	129.67 ± 12.22	124.00 ± 03.50	110.60 ± 07.67	115.45 ± 11.60	Disinfestation method	662.7	2.41	0.080
					Interaction	348.0	1.27	0.298

* 15 min: boil for 15 min in 0.5% lime solution; 30 min: boil for 30 min in 0.5% lime solution; 4h: immersion for 4 h in 2.0% lime solution; Autoclave: Sterilization for 60 min in 121 ºC; Means with different lowercase letters within a line and uppercase letters within a column, for each flush/factor , are significantly different (P < 0.05).

Table 2: Flush cycle (days) of Pleurotus ostreatus cultivated in elephant grass and sugar cane bagasse using different substrate treatments (mean ± SD) and results of two-way ANOVA evaluating the factors substrate and disinfestation method. MS = mean square; F = F statistic; P = significance level.

Flush/Substrate	Disinfestation method *				Factor	MS	F	P
	15 min	30 min	4 h	Autoclave				
1º Flush								
Elephant grass	20.00 ± 1.09 ^{bcB}	23.17 ± 2.86 ^{bA}	27.83 ± 3.46 ^{aA}	17.00 ± 0.00 ^{cB}	Substrate	645.15	90.00	< 0.000
Bagasse	26.83 ± 1.72 ^{bA}	26.20 ± 2.59 ^{bA}	32.33 ± 4.23 ^{aA}	31.71 ± 2.98 ^{aA}	Disinfestation method	109.73	15.40	< 0.000
					Interaction	88.19	12.38	< 0.000
2º Flush								
Elephant grass	40.00 ± 2.53 ^{aA}	40.33 ± 4.08 ^{aA}	42.17 ± 3.66 ^{aA}	35.00 ± 0.00 ^{bB}	Substrate	46.15	6.28	0.016
Bagasse	39.16 ± 1.72 ^{aA}	41.00 ± 2.74 ^{aA}	42.17 ± 3.92 ^{aA}	43.00 ± 0.00 ^{aA}	Disinfestation method	24.36	3.29	0.030
					Interaction	55.42	7.48	< 0.000
3º Flush **								
Elephant grass	54.00 ± 5.55 ^{B}	52.67 ± 6.65 ^{B}	57.83 ± 5.42 ^{A}	60.00 ± 1.00	Substrate	563.63	25.82	< 0.000
Bagasse	61.16 ± 2.40 ^{A}	65.80 ± 3.42 ^{A}	61.67 ± 2.66 ^{A}	not observed	Disinfestation method	15.29	0.70	0.505
					Interaction	62.96	2.88	0.072

* 15 min: boil for 15 min in 0.5% lime solution; 30 min: boil for 30 min in 0.5% lime solution; 4h: immersion for 4 h in 2.0% lime solution; Autoclave:
** Autoclave treatment was not considerate for two-way ANOVA.
Sterilization for 60 min in 121 ºC.
Means with different lowercase letters within a line and uppercase letters within a column, for each flush/factor , are significantly different (P < 0.05).

Table 3: Nutritional value of Pleurotus ostreatus cultivated in elephant grass and sugar cane bagasse in a fresh weight basis using different substrate treatments (mean ± SD) and results of two-way ANOVA evaluating the factors substrate and disinfestation method. MS = mean square; F = F statistic; P = significance level.

Nutritional value/ Substrate	Disinfestation method *				Factor	MS	F	P
	15 min	30 min	4 h	Autoclave				
Moisture (g/100)								
Elephant grass	93.03 ± 0.30	93.17 ± 0.47	93.02 ± 0.25	92.68 ± 0.14	Substrate	1.75	12.13	0.003
Bagasse	93.27 ± 0.51	93.57 ± 0.25	93.61 ± 0.61	93.81 ± 0.21	Disinfestation method	0.07	0.55	0.655
					Interaction	0.23	1.64	0.220
Proteins (g/100)								
Elephant grass	1.50 ± 0.08 ^{bA}	1.79 ± 0.04 ^{bA}	1.39 ± 0.14 ^{bA}	2.49 ± 0.19 ^{aA}	Substrate	0.95	119.68	< 0.000
Bagasse	1.27 ± 0.03 ^{bB}	1.06 ± 0.07 ^{cB}	1.67 ± 0.06 ^{aA}	1.69 ± 0.02 ^{aB}	Disinfestation method	0.48	60.89	< 0.000
					Interaction	0.27	34.37	< 0.000
Fat (g/100)								
Elephant grass	0.12 ± 0.01 ^{bA}	0.11 ± 0.01 ^{bA}	0.08 ± 0.01 ^{cA}	0.19 ± 0.02 ^{aA}	Substrate	0.020	158.73	< 0.000
Bagasse	0.06 ± 0.01 ^{bB}	0.05 ± 0.01 ^{bB}	0.05 ± 0.01 ^{bB}	0.12 ± 0.02 ^{aB}	Disinfestation method	0.010	79.99	< 0.000
					Interaction	0.0005	4.43	0.019
Ash (g/100)								
Elephant grass	0.50 ± 0.03 ^{bA}	0.51 ± 0.03 ^{abA}	0.49 ± 0.01 ^{bA}	0.59 ± 0.05 ^{aA}	Substrate	0.030	55.70	< 0.000
Bagasse	0.51 ± 0.04 ^{aA}	0.39 ± 0.02 ^{bB}	0.39 ± 0.01 ^{bB}	0.52 ± 0.01 ^{aA}	Disinfestation method	0.017	32.85	< 0.000
					Interaction	0.004	9.05	0.001
Carbohydrates (g/100)								
Elephant grass	4.73 ± 0.22	4.42 ± 0.40	4.66 ± 0.14	3.96 ± 0.03	Substrate	0.001	0.01	0.927
Bagasse	4.88 ± 0.48	4.88 ± 0.19	4.21 ± 0.55	3.85 ± 0.23	Disinfestation method	0.798	6.91	0.004
					Interaction	0.218	1.89	0.174

Energy (Kcal)								
Elephant grass	25.28 ± 1.14	24.73 ± 1.60	24.45 ± 1.04	26.78 ± 0.63	Substrate	26.17	12.79	0.003
Bagasse	23.93 ± 1.88	23.18 ± 0.90	23.21 ± 2.25	22.31 ± 0.83	Disinfestation method	0.91	0.45	0.724
					Interaction	3.04	1.49	0.258

* 15 min: boil for 15 min in 0.5% lime solution; 30 min: boil for 30 min in 0.5% lime solution; 4h: immersion for 4 h in 2.0% lime solution; Autoclave: Sterilization for 60 min in 121 ºC.

Means with different lowercase letters within a line and uppercase letters within a column, for each nutritional value/factor , are significantly different ($P < 0.05$).

Table 4: Mineral content (dry mass) of Pleurotus ostreatus cultivated in elephant grass and sugar cane bagasse using different substrate treatments (mean ± SD) and results of two-way ANOVA evaluating the factors substrate and disinfestation method. MS = mean square; F = F statistic; P = significance level.

Mineral/ Substrate	Disinfestation method *				Factor	MS	F	P
	15 min	30 min	4 h	Autoclave				
Fe (mg Kg^{-1})								
Elephant grass	88.1 ± 11.6	67.3 ± 12.4	95.6 ± 13.2	69.8 ± 03.0	Substrate	5922.3	12.51	0.002
Bagasse	99.2 ± 40.7	97.3 ± 12.0	118.0 ± 17.7	115.3 ± 34.5	Disinfestation method	807.7	1.71	0.192
					Interaction	417.4	0.88	0.465
K (g Kg^{-1})								
Elephant grass	17.80 ± 1.55 ^{abA}	17.07 ± 2.58 ^{abA}	14.17 ± 2.59 ^{bA}	21.51 ± 1.80 ^{aA}	Substrate	> 9843	25.92	< 0.000
Bagasse	13.80 ± 1.63 ^{bB}	11.88 ± 1.26 ^{bB}	12.39 ± 1.58 ^{bA}	18.47 ± 0.47 ^{aB}	Disinfestation method	> 6841	18.02	< 0.000
					Interaction	> 4196	1.10	0.366
Na (mg Kg^{-1})								
Elephant grass	463.0 ± 497.0	506.0 ± 224.0	462.5 ± 198.4	363.0 ± 209.0	Substrate	117701	1.96	0.177
Bagasse	350.0 ± 35.4	275.0 ± 125.0	393.8 ± 55.4	241.7 ± 94.6	Disinfestation method	21486	0.36	0.784
					Interaction	8536	0.14	0.933
Mg (g Kg^{-1})								
Elephant grass	1.58 ± 0.05 ^{bA}	1.78 ± 0.12 ^{aA}	1.83 ± 0.07 ^{aA}	1.79 ± 0.08 ^{aA}	Substrate	710134	22.70	< 0.000
Bagasse	1.45 ± 0.39 ^{abA}	1.21 ± 0.15 ^{bB}	1.36 ± 0.18 ^{abB}	1.78 ± 0.08 ^{aA}	Disinfestation method	139395	4.46	0.013
					Interaction	144915	4.63	0.011
Ca (g Kg^{-1})								
Elephant grass	2.02 ± 0.70 ^{a}	1.14 ± 0.78 ^{ab}	0.76 ± 0.23 ^{b}	0.23 ± 0.11 ^{b}	Substrate	22155	0.05	0.829
Bagasse	1.81 ± 1.35 ^{a}	1.34 ± 0.83 ^{a}	0.54 ± 0.04 ^{ab}	0.26 ± 0.05 ^{b}	Disinfestation method	> 42213	9.08	< 0.000
					Interaction	83411	0.18	0.909

Zn (mg Kg^{-1})								
Elephant grass	46.5 ± 3.5 ^{cA}	62.9 ± 2.6 ^{bB}	65.3 ± 2.7 ^{bA}	76.6 ± 6.9 ^{aB}	Substrate	> 23935	2355.7	< 0.000
Bagasse	72.3 ± 21.1 ^{bA}	75.1 ± 7.3 ^{bA}	71.8 ± 17.0 ^{bA}	106.0 ± 8.5 ^{aA}	Disinfestation method	37832	37.24	< 0.000
					Interaction	25504	25.10	< 0.000

* 15 min: boil for 15 min in 0.5% lime solution; 30 min: boil for 30 min in 0.5% lime solution; 4h: immersion for 4 h in 2.0% lime solution; Autoclave: Sterilization for 60 min in 121 ºC.

Means with different lowercase letters within a line and uppercase letters within a column, for each mineral/factor , are significantly different ($P < 0.05$).

Table 5: Estimate of mushroom cost cultivation using different substrate treatments.

Particulars	Disinfestation method (USD)			
	15 min	30 min	4h	Autoclaved
1. Investment on building				
Green house	2793.64	2793.64	2793.64	2793.64
Small building for mushroom processing	5913.72	5913.72	5913.72	5913.72
2. Investment on equipments				
Industrial oven	66.55	66.55	-	-
Centrifuged	113.55	113.55	113.55	-
Autoclave	-	-	-	25000.00*
Laminar flow biosafety cabinets	-	-	-	2146.29
Coat, caps, mask and gloves	23.22	23.22	23.22	23.22
Trays and buckets	65.48	65.48	65.48	65.48
Spray pump, nozzle and water pipes	378.88	378.88	378.88	378.88
Exhaust fan and freezer	392.88	392.88	392.88	392.88
Thermometer, basket, petis, knife, etc.	47.30	47.30	47.30	47.30
Weighing balance	58.42	58.42	58.42	58.42
Electrical fittings	257.36	257.36	257.36	257.36
Others	19.18	19.18	19.18	19.18
Total investment cost	**10130.23**	**10130.23**	**10016.68**	**37096.42**
3. Fixed cost				
Depreciation on building	127.32	127.32	127.32	127.32
Depreciation and maintenance cost on equipment	12.50	12.50	11.18	340.02
4. Variable cost				
Substrate cost				
Bagasse (transport)**	11.82	11.82	11.82	19.35
Elephant grass	0.70	0.70	0.70	1.15
Wheat rice	13.05	13.05	13.05	21.36
Bags				
Bags	5.95	5.95	5.95	-
Filter paper	5.80	5.80	5.80	-
Bags for autoclaving	-	-	-	113.42
Spawn				
Spawn (1%)	-	-	-	10.59
Spawn (5%)	52.98	52.98	52.98	-
Water				
Industry water charges	10.82	10.82	10.82	10.82
Water for $Ca(OH)_2$ solution	0.53	0.53	0.53	-
Chemical reagent				
$Ca(OH)_2$(solution 2%)	-	-	3.94	-
$Ca(OH)_2$(solution 0.5%)	0.97	0.97	-	-
Energy				
Industry energy charges	56.77	56.77	56.77	56.77
Autoclave energy charges	-	-	-	28.96
Gas for boil water 15 min	4.79	-	-	-
Gas for boil water 30 min	-	9.59	-	-
Gas (unit Laminar flow biosafety cabinets)	-	-	-	2.13
Total (3+4)	**304.07**	**308.87**	**300.93**	**583.41**
Total	**10434.30**	**10439.10**	**10317.61**	**37679.83**

*It was not considerate import duties; * *Bagasse can be acquired for free from sugarcane industry.

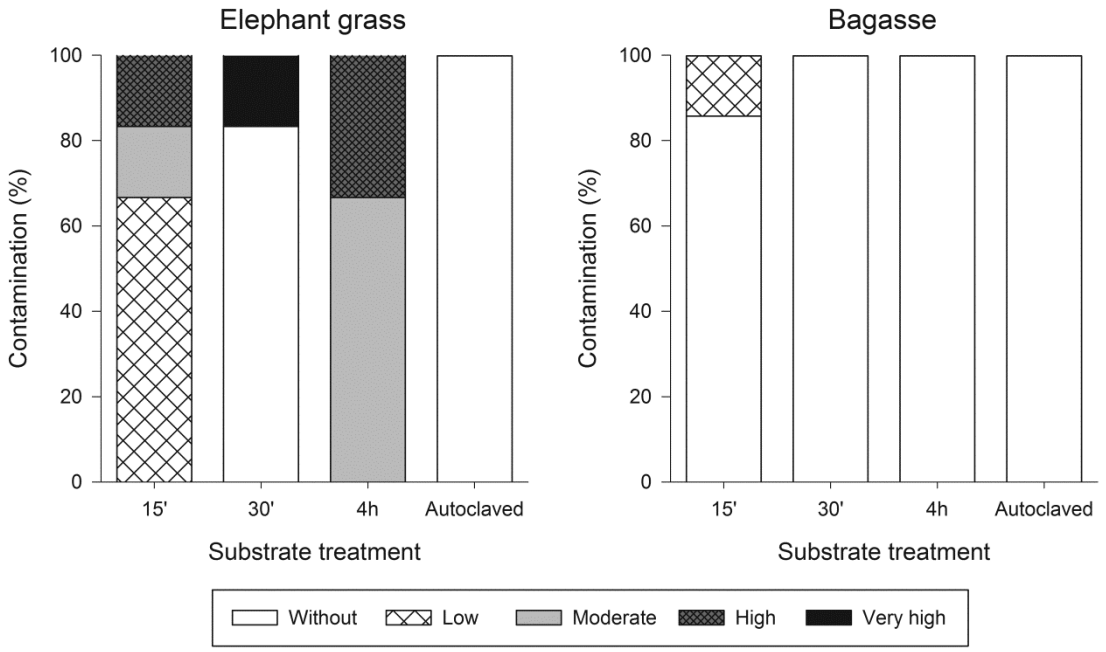

Fig 1: Growth of antagonistic fungi in substrates after disinfestation methods.

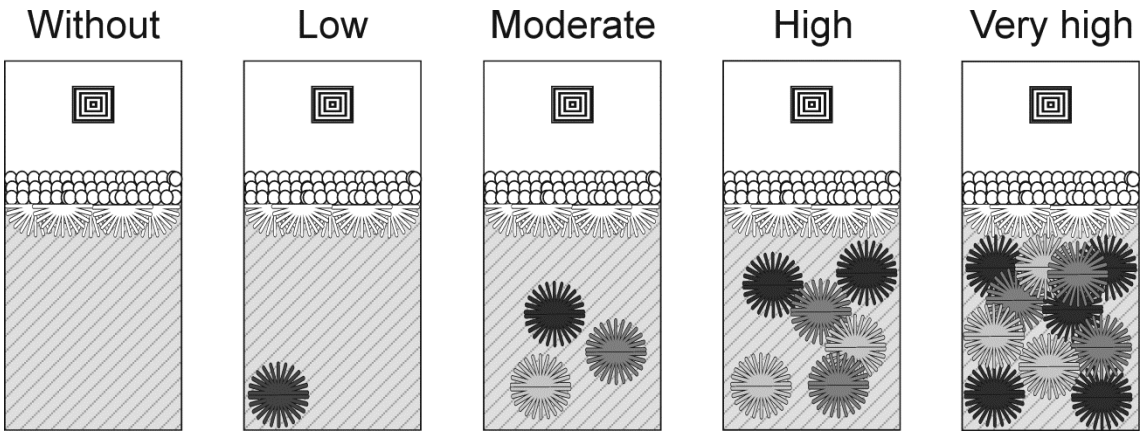

Fig A1: Qualitative parameters used to evaluate the growth of antagonistic fungi in substrate after disinfestation methods.

CAPÍTULO 2: Pleurotus ostreatus mushrooms production in coffee husk using quick and cheap methods and the challenges to use this residue

Formatação de acordo com as normas da revista

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P. ostreatus mushrooms production in coffee husk using quick and cheap methods and the challenges to use this residue

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ABSTRACT

Coffee husk is a residue generated at large amount in Brazil that contains caffeine and tannins, which makes it toxic, resulting in disposal problem. Recently, studies have shown that coffee husk can be use as substrate for mushroom cultivation. However, the main problem seems to be a low mushroom yield. Moreover, sterilization was always performed, making difficult the mushroom cultivation dissemination in deprived regions. Here, we evaluated the viability of alternative methods (without sterilization) to produce P. ostreatus mushrooms in two varieties of coffee husk. We observed that the alternative methods can be used to produce mushroom with higher or similar yield than sterilized coffee husk, even though, the mushroom yield observed here was low. Therefore, immersion of the substrate in water for removal of toxic compounds, careful control of moisture in the substrate and selection of strains adapt to growth in coffee husk seems to be efficient approaches to improve mushroom yield.

1. Introduction

Brazil is the largest producer of coffee in the world (FAOSTAT 2010). The industrial processing of coffee cherries generated a residue called coffee husk. The coffee husk is rich in nutrients (Yesuf 2010, Rodriguez and Gordillo 2011). However, it also contains toxic compounds such as caffeine, tannins, and polyphenols (Fan et al,. 2000b, Fan et al,. 2003), which limit the uses of this residue (Pandey et al,. 2000). Therefore, most coffee husk is discarded or burned leading to environmental pollution.

Aiming to give a destination to this agro-industrial residue, several studies have been made to use coffee husk as substrate for mushroom production (Fan et al,. 2000a, Fan et al,. 2000b, Fan et al,. 2001, Fan et al,. 2003, Nunes et al,. 2012, Dias et al,. 2003, da Silva et al,. 2012, de Assunção et al,. 2012). Furthermore, coffee husk can be easily found in Brazil throughout the year, a very important characteristic to use any residue as substrate for mushroom production. The production of mushroom is an interesting strategy, because it provides food that is a good source of protein, fiber, vitamins and minerals for humans (de Assunção et al,. 2012, Barros et al,. 2008). However, many studies, up to date, had a low mushroom yield or were not able to produce mushrooms in coffee husks (Nunes et al,. 2012, da Silva et al,. 2012, de Assunção et al,. 2012, Dias et al,. 2003, Beaux and Soccol 1996).

Moreover, all previous studies used sterilized coffee husk for mushroom production. Sterilization is an expensive method that requires great investment, which makes difficult its implementation in deprived regions and countries. Utilization of alternative simple and cheap methods is essential to disseminate the mushroom production, as has been shown in Mexico (Léon-Monzón et al,. 2004).

Herein, we evaluated the technical viability of quick and cheap methods (Chapter 1) for mushroom production in coffee husk. We tested the effect of these methods on the P. ostreatus mushroom yield, flush cycle and composition using two varieties of coffee husk. Appearance of antagonistic fungi during mycelial run was also evaluated. Moreover, we assessed the main factors that influence the mushroom yield. This will offer useful information to produce mushroom in coffee husk, independently of the method used to treat the coffee husk.

2. Materials and methods

2.1. Microorganism and inoculum production

The fungus used was P. ostreatus, isolate PLO 06. This fungus belongs to the collection of the Laboratório de Associações Micorrízicas / Departamento de Microbiologia / BIOAGRO / UFV. This isolate grew in a Petri dish containing potato dextrose agar culture medium (Fluka) at pH 5.8 and incubated at 25 ºC. After seven days, the mycelium was used for inoculum production in a substrate based on rice grains that was previously boiled and autoclaved at 121 ºC for 90 min (de Assunção et al,. 2012).

2.2. Substrate, methods of treatment and inoculation

In this experiment, the substrate used for mushroom cultivation were Arabica (from region of Minas Gerais, Brazil) coffee husk/wheat bran at a 4:1 (w/w) and Conilon (from region of Espírito Santo, Brazil) coffee husk /wheat bran at 4:1 (w/w). Addition of nitrogen source was previously showed to increase the biological efficiency of coffee husk (Nunes et al,. 2012).

2.2.1. Alkaline treatments

In this study, we treat the substrate by (i) boiling it in water containing 0.5% of Ca $(OH)_2$ during 15 min, (ii) boiling it in water containing 0.5% of Ca $(OH)_2$ during 30 min, (iii) submerging it in water containing 2% of Ca $(OH)_2$ during 4h. The purpose of increasing the pH and boil is to inhibit or hamper the growth of contaminants and reduce the resident microorganism, respectively. We opted to perform treatments with boiling because they are intermediate between sterilization and alkaline soaking treatments. Soaking for 4 h was successfully used previously in our laboratory

(unpublished data). After the treatments, the substrates were centrifuged at 1800 rpm for 5 min to remove excess of water. Then, 300 g of each sample were placed in plastic bags and inoculated with 15 g of spawn. The bags were closed and incubated at 25 ºC during 20 to 30 d. After the incubation period, the packages were transferred to a fruiting room with controlled temperature and humidity of 20 ºC and 80%, respectively. There were six packages for each substrate.

2.2.2. Sterile treatment

The substrates were moistened with water at 70% of the retention capacity and 300 g of each substrate was placed in polypropylene bags. Next, the bags containing the substrates were autoclaved at 121 ºC for 2 h. After sterilization, the substrates were inoculated with 15 g of spawn and incubated at 25 ºC during 15 to 35 d. After incubation period, the packages were transferred to a fruiting room, with controlled temperature and humidity of 20ºC and 80%, respectively. There were six packages for each substrate.

2.3. Mushroom production

Three times of mushroom harvest were performed at intervals from the 15th to the 65th days after inoculation. The fresh weight of mushrooms was recorded to determine the biological efficiency (BE):

BE = (fresh weight of mushrooms/dry weight of substrate) x100.

Subsequently, the mushrooms were dried in an oven at 60 ºC. To determine the content of minerals and the nutritional value, the dried mushrooms were ground using a knife mill and passed through a 2-mm sieve.

2.4. Proximate analysis

2.4.1. Chemical composition

To obtain moisture contents, samples of the mushrooms were dried in an oven at 105 °C overnight. Samples of mushrooms were analyzed for chemical composition (moisture, protein, fat, carbohydrates and ash) using the AOAC procedures (2005) with minor changes. The crude protein content (N × 4.38) of the samples was estimated by the macroKjeldahl method; the crude fat was determined by extracting a known weight of powdered mushroom sample with petroleum ether, using a Soxhlet apparatus; the ash content was determined by incineration at 600 ± 15°C. Total carbohydrates were calculated by the difference. Total energy was calculated according to the following equation (AOAC 2005).

Energy (kcal) = 4 x (g protein + g carbohydrate) + 9 x (g lipid).

2.4.2. Mineral content

Two hundred miligrams of dried mushrooms were digested with a mixture of nitric acid and perchloric acid (3:1, v:v) and 500 μL H_2O_2 at 200 ºC. (Tedesco et al,. 1995). At last, the mixture was cooled, and dilute with ultra-pure water (Milli-Q system, Millipore, USA) to a final volume of 25 mL. The solutions were filtered through Whatman No. 42 filter paper (Kent, U.K.), in order to remove any insoluble silicates and other solid materials. Resulting solutions were used for direct spectrophotometric analysis. Three blank digest was carried out in the same way.

The concentrations of iron (Fe), zinc (Zn), potassium (K), sodium (Na), calcium (Ca) and magnesium (Mg) were determined in an atomic absorption spectrometry (Optima 3300 DV; Perkin Elmer, Waltham, MA), using specific standards and

procedures for each mineral as recommended by the manufacturer. It was used wavelength with higher sensitivity for each mineral and integration time of 15 s in duplicate for each analysis.

2.4.3 Caffeine analysis

After disinfestation of the substrate, 40 g of each substrate were separated and dried in oven at 75 ºC over night. Then, 2 g of each substrate were boiled in 50 mL of water during 15 min. The coffee extract was filtered through 0.45 um membrane and used for analysis. The extracts were prepared in triplicate.

For HPLC analysis, a mobile phase consisting of 50% methanol and 50% water (v/v) was run isocratically at 1mL/min through a 150 x 4.6 mm C-18 column having a 5 pm particle size (Ultracarb 5 ODS20, Phenomenex, Torrance, CA). Caffeine eluted around 7 min and was detected at 273 nm (Casal et al,. 2000).

Caffeine (Sigma, St. Louis, MO) was dissolved in purified water to form standard solutions. These solutions were prepared in duplicate and serially diluted to prepare the standard curve. The standard curve had a linear correlation of 0.999. Caffeine reference material (Sigma) was used to determine analytical precision. The certified caffeine was 105.87% recovered, showing an acceptable analytical precision.

2.5. Statistical analyses

The experiment used a randomized design. The results were expressed as mean values and standard deviation. The data, except the qualitative data, were subjected to analysis of variance (ANOVA), and the averages were compared by Tukey's test ($p < 0.05$). Should be pointed that we do not have interest to compare which coffee husk is

better for mushroom production. Here, we focus only in disinfestation methods, justifying the one-way ANOVA analysis in table 1.

Pearson's correlation coefficients were used to analyze the relationship between relative contamination growth (RCG) and mushroom yield ($p < 0.05$) using Sigma Plot software (Version 11.0). The degree of antagonistic (DA) growth was numerical classified as follow: 0 = without; 1 = low; 2 = moderate; 3 = high; 4 = very high. Then, DA values and the percentage of contaminated packages (Figure 1 A, Chapter 1) were used to calculate the RCG ($RGC = 0 * without_{\%} + 1 * low_{\%} + 2 * moderate_{\%} + 3 * high_{\%} + 4 * very\ high_{\%}$).

3. Results

3.1. Mushroom yield

One of the most important features to evaluate the viability of a new method to produce mushroom is the mushroom yield. Here, we observed that the mushroom yield obtained by the alkaline immersion methods (AIM) was higher than sterilization process, for both coffee husk varieties (Table 1), showing the potential of the AIM methods. Mycelial growth was inhibit or reduced in the autoclaved substrates, justifying the low biological efficiency observed (Table 1). The biological efficiency was different among the substrates and treatments (Table 1; $P < 0.05$). Table 1 shows that the highest mushroom yield for Arabica substrate was observed when immersion in lime solution was used, on the other hand, the boil treatments were more efficient for Conilon substrate. Furthermore, it was possible to observe a higher prevalence of unviable packages when the substrate was boiled and immersed in lime, for Arabica and Conilon, respectively (Table 1).

3.2. Harvest time and time required for mushroom formation

In order to measure the total time required to produce the mushrooms, a feature also relevant to mushroom growers, the harvest time spent to finish the mushroom production and the time required for mushroom formation were observed. It was not observed differences in the harvest time and time required for mushroom formation among the treatments for both coffee husks varieties (Table 2; $P > 0.05$). The immersion in lime solution was able to produce mushrooms faster ($P < 0.05$) than the others treatments in the first flush for Arabica substrate, however, this difference was compensated in the next flush, resulting in similar harvest time in the second flush ($P > 0.05$).

3.3. Contamination

Contamination is the main problem when no-sterilized methods are used for mushroom production. With the purpose of evaluate the contamination growth effect on the mushroom yield, visual observations were daily made to rank the degree of contamination and then correlated with mushroom yield. It was observed a higher prevalence of antagonistic growth in the boiled substrates for both coffee husk varieties (Figure 1). However, we found no correlation between relative contamination growth and mushroom yield for Arabica ($r = - 0.272$, $p = 0.728$) and Conilon ($r = 0.264$, $p = 0.736$).

3.4. Chemical composition and mineral content

Table 3 shows the effect of substrate treatments on nutritional value of the mushrooms. Protein and lipid content decreased when coffee husk substrate was boiled (Table 3; $P < 0.05$), most likely reflecting nutrients removal from the substrate during the boil. The same trend was observed for ash content in the Conilon substrate (Table 3;

$P < 0.05$). On the other hand, the ash content of the mushrooms produced in the sterile Arabica substrate was lower (Table 3; $P < 0.05$). Regarding the mineral content of the mushrooms, no influence of substrate treatments on mineral value of mushrooms produced in Conilon coffee husk was observed (Table 4; $P > 0.05$), but should be pointed out that the comparison with autoclaved substrate was not realized due to inhibition of the mycelial growth. On the contrary, we found an increase ($P < 0.05$) in the mineral content of the mushrooms produced in the no-sterile Arabica substrate, besides the bioaccumulation of Ca (Table 4).

3.5. Caffeine

In order to determine if the disinfestation methods remove caffeine from the coffee husks, a compound toxic for fungi, the caffeine was measure in the substrate after the treatments. Table 5 shows that disinfestation methods were efficient in decrease the caffeine content in the coffee husks ($P > 0.05$).

4. Discussion

Nowadays, one problem of coffee industry is the management of the coffee husk generated during the coffee processing. Usually, this residue is discarded without any processing step leading to environmental problems. Use of this residue as substrate for mushroom cultivation seems to be an interesting strategy to add economic value to this residue and preventing environmental pollution. Many studies successfully reported production of mushrooms in sterile coffee husk (Fan et al,. 2000a, Fan et al,. 2000b, Fan et al,. 2001, Fan et al,. 2003, Nunes et al,. 2012, Dias et al,. 2003, da Silva et al,. 2012, de Assunção et al,. 2012). However, in many South America countries, mushrooms are an expensive food, especially when compared with meat, making its

commercialization difficult, thus justifying investigations of cheap methods to cultivate mushrooms in coffee husk. This will improve the competitiveness of this commodity; besides making cheaper, the infrastructure implementation required for mushroom cultivation.

In this study, we showed that alkaline immersion methods (AIM) can be successful used to produce mushroom in coffee husk (Table 1) The mushroom yield obtained was similar to others studies that used sterilized coffee husk for mushroom cultivation (da Silva et al,. 2012, de Assunção et al,. 2012, Nunes et al,. 2012, Dias et al,. 2003) and the time required to produce the mushrooms, in overall, was similar between treatments (Table 2). The AIM had slight effect on the nutritional value and the mineral content of the mushrooms, with protein and lipid level decreasing when the substrate was boiled (Table 3) and K, Mg, Zn and Ca levels increasing when AIM were used, which a clear bioaccumulation of Ca in the mushrooms (Table 4). However, the protein, lipid and minerals levels observed here were similar or higher than those reported in others studies (Chapter 1,(Çağlarırmak 2007, Vetter 1994, Gupta et al,. 2013)), showing an adequate profile of macronutrients and micronutrients in the mushrooms produced by AIM.

Appearance of contamination should be avoided, even already been observed that there is no influence in mushrooms production. Growth of antagonistic microorganism should be avoided because some common contaminants, in mushroom production, produce harmful substances for human health, as mycotoxins (Bennett and Klich 2003, Waśkiewicz 2014). Due to the capacity to accumulate substances found in the substrate in the fruiting bodies, it is possible that, in the presence of contaminants occur accumulation of harmful substance in mushrooms, which is not desirable and

need to be avoided. Therefore, future studies should seek to evaluate if mushrooms produced in contaminate substrates accumulates harmful substances for humans.

Relevant differences between coffee husks varieties were observed in the mushroom yield (Table 1), suggesting that, beyond the disinfestation methods, characteristics of these substrates is influencing the mushroom production. Indeed, the fact that both sterile coffee husks were not suitable for mushroom production (Table 1) reinforces this hypothesis, because previous studies reported that sterile coffee husk are appropriate for mushroom cultivation (Fan et al,. 2003, da Silva et al,. 2012, Pandey et al,. 2000, de Assunção et al,. 2012, Nunes et al,. 2012, Fan and Soccol 2001, Fan et al,. 2000b).

One of these characteristics seems to be presence of tannins and caffeine in the coffee husk, compounds that exercise toxic effect on P. ostreatus and Lentinula edodes (Pandey et al,. 2000, Fan et al,. 2003, Beaux and Soccol 1996) and can significantly affect the fungal growth. Immersion in water or boil remove those compounds from the substrate (Table 5), leading to levels more suitable for mycelial growth and consequently, to an increase in mushroom yield, as observed in this study (Table 1). Indeed, prior studies reported to boil the coffee husk before use this residue for mushroom production (da Silva et al,. 2012, de Assunção et al,. 2012). Therefore, immersion in water seems to be a simple and efficiency alternative to improve the mushroom cultivation in coffee husk.

Other characteristic is the low moisture holding capacity of the coffee husk. The moisture content of dry coffee husks is naturally low, being found values lower than 20% (Brand et al,. 2000, Duku et al,. 2011). Its low moisture holding capacity led to no homogeneous water distribution, allowing some sites of the substrate be with high

moisture level, which decrease the substrate porosity and prevents oxygen transfer (Pandey 1992). On the other hand, low moisture level leads to poor accessibility of nutrients, resulting in poor growth (Pandey 1992). In fact, prior researches showed inhibition or decrease of fungal growth due to high moisture level in coffee husk (Dias et al,. 2003, Fan et al,. 2000b). To avoid or minimize this problem, previous studies reported to adjust the moisture of the substrate 4-5 h before the autoclaving (Fan et al,. 2001, Fan et al,. 2003, Fan and Soccol 2001). Moreover, supplementation with agroindustrial residues with high moisture holding capacity (e.g. sawdust, sugar cane bagasse) and decrease of coffee husk granulometry can be interesting strategies to improve the moisture holding capacity of the substrate.

Finally, use of approaches to select fungi strains more adapted to growth in coffee husk is essential to achieve high mushroom production. The studies of mushroom cultivation in coffee husk carried out so far with high mushroom yields performed a prior screening of fungi strains in PDA medium containing extract of coffee husk (Fan et al,. 2001, Fan et al,. 2003, Fan and Soccol 2001). This leads to selection of fungi strains more adapt to degrade the tannins and caffeine as well as to metabolize carbon resource found in the coffee husk, which will result in a higher energy availability to support the mycelial growth and fructification process.

Therefore, we showed that AIM, a simple and cheap method, can be used to produce mushroom in coffee husk. However, immersion of the substrate in water for removal of toxic compounds, careful control of moisture in the substrate and selection of strains adapt to growth in coffee husk seems to be efficient approaches to improve mushroom yield.

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Tables and Figures

Table 1: Biological efficiency, number of flushes and unviable packages of Arabica and Conilon coffee husk treated by four different methods (mean ± SD).

Substrate	Disinfestation method *	Biological efficiency (%)				Number of flushes	Unviable packages (%)		
		1º	2º	3º	Total		1º	2º	3º
Arabica	15 min	09.25 ± 6.84 [b]	03.86 ± 1.77 [b]	-	12.46 ± 08.62 [b]	2	0.00	0.00	100.00
	30 min	11.58 ± 5.73 [b]	06.79 ± 7.67 [b]	-	15.65 ± 08.37 [b]	2	0.00	50.00	100.00
	4 h	24.01 ± 8.37 [a]	16.29 ± 5.93 [a]	-	40.30 ± 13.15 [a]	2	0.00	0.00	100.00
	Autoclaved	05.00 ± 1.94 [b]	0.00 ± 0.00 [c]	-	05.50 ± 02.91 [c]	1	0.00	100.00	100.00
Conilon	15 min	15.28 ± 5.70 [a]	16.18 ± 4.85 [a]	7.40 ± 5.03 [a]	37.37 ± 07.35 [a]	3	0.00	16.66	33.33
	30 min	20.03 ± 2.59 [a]	15.94 ± 4.32 [a]	8.04 ± 4.49 [a]	42.68 ± 04.06 [a]	3	0.00	0.00	16.66
	4 h	13.07 ± 7.65 [a]	06.66 ± 3.07 [b]	0.00 ± 0.00 [b]	17.51 ± 10.57 [b]	2	0.00	16.66	100.00
	Autoclaved	0.00 ± 0.00 [b]	0.00 ± 0.00 [c]	0.00 ± 0.00 [b]	0.00 ± 0.00 [c]	0	100.00	100.00	100.00

* 15 min: boil for 15 min in 0.5% lime solution; 30 min: boil for 30 min in 0.5% lime solution; 4h: immersion for 4 h in 2.0% lime solution; Autoclave: Sterilization for 60 min in 121 ºC.

Means with different letters within a column are significantly different ($P < 0.05$) according to Tukey's test.

Table 2: Harvest time and time required for mushroom formation of Arabica and Conilon coffee husk treated by four different methods (mean ± SD).

Substrate	Disinfestation method *	Harvest time (d)		
		1º Flush	2º Flush	3º Flush
Arabica	15 min	40.43 ± 6.60 [a]	57.40 ± 6.50 [a]	-
	30 min	43.00 ± 7.77 [a]	56.67 ± 8.74 [a]	-
	4 h	31.50 ± 2.51 [b]	51.17 ± 6.01 [a]	-
	Autoclaved	46.60 ± 0.54 [a]	-	-
Conilon	15 min	34.00 ± 1.22 [a]	46.33 ± 1.63 [a]	57.17 ± 2.86 [a]
	30 min	31.67 ± 4.41 [a]	46.50 ± 6.09 [a]	57.00 ± 1.41 [a]
	4 h	34.80 ± 3.90 [a]	53.00 ± 5.57 [a]	-
	Autoclaved	Inhibited	Inhibited	Inhibited

* 15 min: boil for 15 min in 0.5% lime solution; 30 min: boil for 30 min in 0.5% lime solution; 4h: immersion for 4 h in 2.0% lime solution; Autoclave: Sterilization for 60 min in 121 ºC.

Means with different letters within a column, for each substrate, are significantly different ($P < 0.05$) according to Tukey's test.

Table 3: Nutritional value of mushrooms in a fresh weight basis (mean ± SD).

Substrate	Disinfestation method *	Moisture(g/100)	Proteins(g/100)	Fat(g/100)	Ash(g/100)	Carbohydrates(g/100)	Energy (Kcal)
Arabica	15 min	92.40 ± 0.07 [a]	2.41 ± 0.16 [ab]	0.10 ± 0.02 [b]	0.69 ± 0.01 [a]	4.37 ± 0.25 [a]	27.03 ± 0.14 [a]
	30 min	92.70 ± 0.38 [a]	2.30 ± 0.12 [b]	0.10 ± 0.02 [b]	0.71 ± 0.01 [a]	4.17 ± 0.31 [a]	25.84 ± 1.42 [a]
	4 h	92.38 ± 0.49 [a]	2.66 ± 0.03 [a]	0.15 ± 0.03 [ab]	0.68 ± 0.02 [a]	4.12 ± 0.45 [a]	27.48 ± 1.93 [a]
	Autoclaved	93.08 ± 0.09 [a]	2.68 ± 0.05 [a]	0.19 ± 0.01 [a]	0.63 ± 0.01 [b]	3.49 ± 0.15 [a]	25.27 ± 0.34 [a]
Conilon	15 min	92.56 ± 0.49 [a]	2.32 ± 0.11 [b]	0.10 ± 0.01 [b]	0.58 ± 0.01 [b]	4.49 ± 0.45 [a]	26.80 ± 1.91 [a]
	30 min	92.55 ± 0.11 [a]	2.01 ± 0.12 [c]	0.08 ± 0.01 [b]	0.59 ± 0.02 [b]	4.88 ± 0.23 [a]	26.59 ± 0.33 [a]
	4 h	92.16 ± 0.44 [a]	2.69 ± 0.16 [a]	0.13 ± 0.01 [a]	0.66 ± 0.01 [a]	4.26 ± 0.25 [a]	27.80 ± 0.71 [a]

* 15 min: boil for 15 min in 0.5% lime solution; 30 min: boil for 30 min in 0.5% lime solution; 4h: immersion for 4 h in 2.0% lime solution; Autoclave: Sterilization for 60 min in 121 ºC.

Means with different letters within a column, for each substrate, are significantly different ($P < 0.05$) according to Tukey's test.

Table 4: Mineral content in mushrooms (mean ± SD; mg Kg^{-1} of dry mass).

Substrate	Disinfestation method *	Fe	K	Na	Mg	Ca	Zn
Arabica	15 min	119.4 ± 03.8 [b]	24754.0 ± 866.0 [a]	75.0 ± 25.0 [a]	1229.2 ± 17.7 [ab]	738.0 ± 236.0 [a]	83.5 ± 01.7 [a]
	30 min	105.2 ± 04.0 [b]	25492.0 ± 070.7 [a]	145.7 ± 31.7 [a]	1166.7 ± 35.4 [b]	825.0 ± 035.4 [a]	73.2 ± 01.8 [a]
	4 h	170.2 ± 23.8 [a]	23679.0 ± 778.0 [a]	150.0 ± 43.3 [a]	1291.7 ± 28.9 [a]	1150.0 ± 238.0 [a]	56.1 ± 15.5 [ab]
	Autoclaved	83.3 ± 04.2 [b]	21183.0 ± 437,0 [b]	151.8 ± 66.6 [a]	1000.0 ± 14.4 [c]	225.0 ± 110.9 [b]	47.4 ± 08.6 [b]
Conilon	15 min	237.5 ± 75.0 [a]	24006.0 ± 907.0 [a]	253.3 ± 112.5 [a]	2050.0 ± 158.1 [a]	1531.0 ± 518.0 [a]	105.2 ± 14.6 [a]
	30 min	237.5 ± 59.5 [a]	25019.0 ± 646.0 [a]	185.1 ± 066.6 [a]	1912.5 ± 116.4 [a]	2650.0 ± 926.0 [a]	98.0 ± 23.6 [a]
	4 h	162.5 ± 17.7 [a]	24625.0 ± 106.0 [a]	331.3 ± 027.6 [a]	2012.5 ± 088.4 [a]	1175.0 ± 177.0 [a]	102.2 ± 23.9 [a]

* 15 min: boil for 15 min in 0.5% lime solution; 30 min: boil for 30 min in 0.5% lime solution; 4h: immersion for 4 h in 2.0% lime solution; Autoclave: Sterilization for 60 min in 121 ºC.

Means with different letters within a column, for each substrate, are significantly different ($P< 0.05$) according to Tukey's test.

Table 5: Caffeine in coffee husk after disinfestation treatments.

Substrate	Disinfestation method *	Caffeine (g kg^{-1})
Arabica	15 min	1.19 ± 0.06 [b]
	30 min	0.92 ± 0.04 [c]
	4 h	1.11 ± 0.07 [b]
	Autoclaved	3.85 ± 0.09 [a]
Conilon	15 min	5.63 ± 0.67 [b]
	30 min	3.98 ± 0.45 [c]
	4 h	4.86 ± 0.22 [bc]
	Autoclaved	7.68 ± 0.93 [a]

* 15 min: boil for 15 min in 0.5% lime solution; 30 min: boil for 30 min in 0.5% lime solution; 4h: immersion for 4 h in 2.0% lime solution; Autoclave: Sterilization for 60 min in 121 ºC.
Means with different letters within a column, for each substrate, are significantly different ($P < 0.05$).

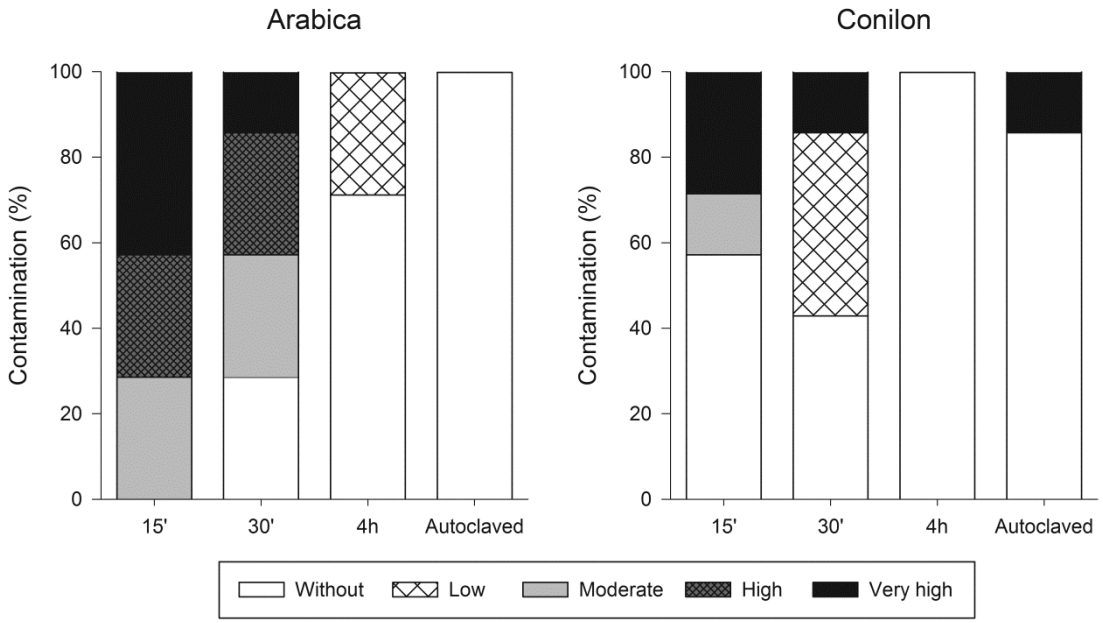

Figure 1: Percentage of Pleurotus ostreatus packages contaminated by fungi antagonism using different substrate treatments.

CAPÍTULO 3: Improving the cultivation of mushrooms on substrates treated by immersion on alkaline solution

Formatação de acordo com as normas da revista

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Improving the mushrooms cultivation on substrates treated by immersion on alkaline solution

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ABSTRACT

Usually, thermal treatments have been used to prepare substrates to cultivate mushrooms. However, these methods are expensive and difficult to be used in deprived regions. Immersion in alkaline solution, due to its simplicity and low cost, seems to be a good alternative. Nevertheless, different soaking time and lime concentration have been used to prepare the substrates. So, we evaluated the effect of concentrations of lime and time of immersion on pH and microbiota in the treated substrate. Then, we evaluated the efficiency of disinfection method based on sawdust and rice bran for six species of Pleurotus, comparing them to sterilization method. Also, the effect of disinfestation methods on mineral content of mushrooms was evaluated. We found that immersion of substrate in lime 2% for 4 h reduces approximately 99% of microbiota and increases the pH of substrate to around 10, showing that this combination is efficient to prepare the substrates. In addition, we obtained mushroom yield as well as sterilized substrates, reinforcing the efficiency of these method of cultivation. The differences in the mineral content of mushrooms were significant for all metal analyzed ($P < 0.05$) and wide variations were observed, showing a species-specific sensitivity to uptake metals ions. These values are comparable with others studies, showing that this mushrooms can be used as dietary source of minerals. So, the use of lime 2% for 4 h is a cheap method with good potential for mushroom production.

Keywords: Pleurotus mushroom production; cheap methods; easy methods

Introduction

Nowadays, the main methods used by mushrooms growers to prepare the substrate for Pleurotus species cultivation are sterilization or pasteurization. Advantages of these conventional methods are the wide applicability, in several species of mushrooms, with low probability of contamination and pests problems (Oei 2003). However, these process are expensive and technically demanding in terms of professional training, which limit its utilization in deprived regions.

An alternative method has been successfully used in Mexico to prepare the substrate for Pleurotus ostreatus and Pleurotus pulmonarius cultivation (Léon-Monzón et al,. 2004, Bernabé-González and Cayetano-Catarino 2009). Substrate is soaked by 12 to 48 h in lime solution. Beyond the low cost, this method uses a chemical compound that is cheap and can be easily found in different countries, is simple in terms of professional training, can be used with different substrates (Léon-Monzón et al,. 2004, Contreras et al,. 2004).

However, some restrictions of application of this method are: (i) prolonged time of immersion, which may lead to no homogeneous substrate wetting and favor growth of antagonistic microorganism (Léon-Monzón et al,. 2004, Contreras et al,. 2004). Bacterial and fungal contamination are able to provoke deformation in mushrooms (Bessete et al,. 1985, Terashima et al,. 2002) and fungal contamination may reduce mushroom yield (López-Averalo et al,. 1996); (ii) fly eggs survive the alkaline treatment and seems to be a frequently problem (Léon-Monzón et al,. 2004), (iii) studies carried out so far used substrates not available through the year in temperate climate countries and performed evaluations only with P. ostreatus and P. pulmonarius (Léon-Monzón et al,. 2004, Contreras et al,. 2004, Avendaño-Hernandez and Sánchez

2013, Bernabé-González and Cayetano-Catarino 2009), restricting the application of this method.

Moreover, different soaking time and lime concentration have been used to prepare the substrate (Avendaño-Hernandez and Sánchez 2013, Contreras et al,. 2004, Léon-Monzón et al,. 2004), without any information related to the efficiency of the concentration used and also time spent, which is not desirable to standardize the method and, consequently, to solve the problems currently observed.

Finally, given the importance role that minerals play in human metabolism, is important in deprived regions, the consumption of food that can provide meaningful amounts of daily requirements of most important minerals. Several studies have analyzed mineral contents in different species, showing mushrooms contain high levels of some important minerals (Kalač 2009) and can be a good source of minerals. However, there is still not enough information about mineral composition of edible mushrooms and the interdependences between elements accumulated in fruit bodies and substrate composition. Mineral composition in mushrooms is mostly characteristic for the species (Falandysz and Borovička 2013), however, has been shown that some species have predilection to accumulate toxic element and several mushrooms are specifically abundant in certain elements, showing species-specific accumulation (Brzostowski A et al,. 2011, Borovička et al,. 2010, Drewnowska M et al,. 2012). Among the factor that can influence minerals sequestration in mushrooms are: (1) differences in substrate composition (Aloupi et al,. 2012, Borovička et al,. 2010), (2) time of interaction between fungi and substrate/soil as well as competition between substrate/soil and mycelia for minerals binding sites in fungal fruit bodies (Chudzyńskia et al,. 2013, Zhang et al,. 2010), and (3) the acidic and organic matter content in substrate/soil (Gast et al,. 1988).

Therefore, in this study we tested the effect of different lime concentration and immersion time on pH and microbiota in the substrate based on sawdust and rice bran, objecting to standardize this alkaline method. Sawdust/rice bran was chosen because it is a common substrate used in mushroom cultivation and can be easily found through the year in many countries. Moreover, relationship between pH of lime solution and substrate after alkaline treatment were evaluated to determine if the pH of the solution could be a useful parameter to monitor the mushroom cultivation process. Finally, we evaluated the technical viability of substrate preparation using alkaline method for mushroom production of Pleurotus species and the effect of these methods on mineral sequestration in mushrooms. The emergence of antagonistic microorganism during the mycelial run was also evaluated.

2. Material and Methods

2.1. pH of the solution

To evaluate the effect of lime concentration on pH of the solution, lime in different concentration (0.01%, 0.03%, 0.06%, 0.12%, 0.25%, 0.50%, 1.0% and 2.0%) were added to ultrapure water (18.2 MΩ·cm at 25 ± 2°C). Next, pH was measured (pH meter, Model B—212, Horiba, Japan) at intervals of 0, 1, 2, 3 and 4 h. Before each measurement, solution was homogenized. Two replicates were analyzed.

2.2. Substrate and pH

The substrate was based on oak sawdust and rice bran (2.62:1; w/w). Sixty grams of substrate was soaked in 600 mL of lime solution prepared as describe above. At intervals of 0, 1, 2, 3 and 4 h, 20 g of samples were added to 100 mL of ultrapure water (18.2 MΩ·cm at 25 ± 2°C) and shaken for 1 h. After settlement of the particles, pH of the substrate solution was recorded. Three replicates were analyzed.

It was observed that pH of the substrate was more homogeneous after soaking during 4 h in lime solution (Figure 1). Considering this, another important parameter was tested: the relation of substrate/water. The amount of water is very important, because many rural areas where the method could be used have restrict water availability and also, after use, the lime-water solution is dumped, so less water use is desirable. We used 4 h in lime solution to evaluate the effect of substrate mass on pH. Whereas only concentrations higher than 1.0% of lime efficiently alkalized the substrate, so it was used 1.0, 1.5 and 2.0% of lime solution in this experiment. Different amount of substrate were soaked in 5 L of lime solution for 4 h: 20, 40, 80, 160, 320 or 500 g. When it was used lime-solution ratio higher than 500:5 (w/v) we observed that some parts of substrate were not completed cover up by the solution, so we did not tested ratio higher than this. The soaked substrates were centrifuged at 1800 rpm to remove the solution before samples collection. Then, 20 g of samples were placed in 100 mL of ultrapure water and shaken for 1 h. After settlement of the particles, pH of the substrate solution was recorded. Four replicates were analyzed.

2.3. Microbial estimation

Sixty grams of substrate was soaked in 600 mL of lime solution with different concentrations (0.0, 0.5, 1.0 or 2.0%) for 4 h. Then, 20 g of substrate were placed in 100 mL of sterile NaCl-peptone water solution (0.85% NaCl, 0.1% Peptone, w/v) for 10 min without shaking to avoid microbial growth. Before suspensions collection the solution was homogenized. These suspensions were then used for microbial counts by cell enumeration using the determination of the total number of unit forming colonies (UFC). Serial decimal dilutions of each suspension (10^{-1} to 10^{-5}) were plated using spread plate technique on different agar media: Plate Count Agar (PCA, Eiken Chemical, Tochigi, Japan) containing 2.5 μg mL^{-1} nystatin (MP Biomedicals) for the

total aerobic bacteria, incubated at 25 °C for 2 d; Rose Bengal Agar Medium (10.0 g L^{-1} dextrose, 5.0 g L^{-1} peptone, 1.0 g L^{-1} KH_2PO_4, 0.5 g L^{-1} $MgSO_4.7H_20$, 10 mL L^{-1} 0.033% Rose Bengal, 15.0 g L^{-1} agar) containing 1 μg mL^{-1} streptomycin (Wako Pure Chemical Industries) for total fungi, incubated at 25 °C for 4 d; Actinomycetes Isolation Agar (10.0 g L^{-1} glycerol, 15.0 g L^{-1} agar, 1.0 g L^{-1} KH_2PO_4, 1.0 mL L^{-1} nutrient solution I [1.38 g L^{-1} $MnCl_2.4H_20$, 1.17 g L^{-1} H_3BO_3, 0.10 g L^{-1} $(NH_4)_6Mo_7O_{24}.4H_2O$, 0.57 g L^{-1} $ZnSO_4.7H_20$, 0.01 g L^{-1} $CuSO_4.5H_2O$], 0.5 mL L^{-1} nutrient solution II [13.36 g L^{-1} $FeCl_3$, 30.71 g L^{-1} EDTA]) containing 1 μg mL^{-1} streptomycin and 2.5 μg mL^{-1} nystatin for total actinobacteria, incubated at 25 °C for 6 d. The pH of each culture medium was adjusted to three values: 7.0, 8.0 and 10.0. This procedure was done to mimic pH values found in the substrate immersed in the lime solution, which can give an indication if pH exerts a selective pressure in microbial community. Ten replicates were evaluated. Microbial counts were expressed as units forming colony (UFC) per gram of dried substrate.

2.4. Mushroom production

The mushrooms forming fungi tested were P. ostreatus isolate PLO 06, P. ostreatus isolate PO-04, P. djamor, P. sajor-caju, P. pulmonarius, P. cornucopiae and P. eryngii. Those fungi belong to the mycological collection of the Forest Resource Science (Faculty of Agriculture, Hokkaido University, Japan), expect, fungi PLO 06 that belong to mycological collection of the Laboratório de Associações Micorrízicas / Departamento de Microbiologia / BIOAGRO / UFV. Oak sawdust/rice bran at a 2.62:1 (w/w) ratio was used as substrate for mushroom cultivation. This substrate composition was choosing because it is commonly used for mushroom cultivation of many fungi species.

Three types of substrate treatment were tested.: (1) boiling it in water containing 0.5% $Ca(OH)_2$ for 30 min; (2) or submerging it in cold tap water containing 2% $Ca(OH)_2$ for 4 h or (3) sterilization.

Five hundred grams of the substrate were added to 5 L of lime solution. In our laboratory, previous studies showed that boiling substrate in water containing 0.5% $Ca(OH)_2$ for 30 min can be used to produce mushroom (unpublished data). This method was chosen because using lime and temperature to treat the substrate, allow preparing substrate in a short time. This procedure may give an indication if combination of the two methods (lime and temperature) that can be an interesting approach to decrease the time required to prepare substrate. After treatment, the substrates were centrifuged to remove excess of water. For sterilization treatment, the substrates were moistened with water to 55% of the retention capacity, and 260 g of each substrate was placed in a plastic bottle. The bottles were then autoclaved at 121 °C for 1 h. Next, 260 g of each sample was packaged in a plastic bottle and inoculated with spawn of different isolates of fungi. The bottles were closed and incubated at 25 °C for 10 to 35 d. After incubation period, the bottles were transferred to a fruiting room with controlled temperature and humidity of 15 °C and 90%, respectively. There were six bottles for each fungus and treatment method.

Two harvests of mushrooms were performed. The fresh weight of the mushrooms was recorded to determine the biological efficiency (BE), calculated as BE = (fresh weight of mushrooms/dry weight of substrate) x 100.

After mushroom harvesting, the diameter and length of stipe and diameter of cap were measured. The cap diameter was determined by the medium of two directions measures that were perpendicular to each other.

2.5. Mineral content

For digestion, ETHOs One microwave close system (maximum pressure 1450 psi, maximum temperature 300 °C) was used. Samples (500 mg) of dry mushrooms were subjected to microwave digestion with a mixture of nitric acid and hydrogen peroxide (7:1, v:v) at 200 °C (1000 W) for 15 min, as recommended by the manufacturer, and diluted to 50 ml with ultra-pure water (Milli-Q system, Millipore, Japan). Five blank digestion was carried out in the same way.

The concentration of Cu, P, Ca, Fe, Mn, Mo, K, Mg, Na, Zn and Pb were measured by inductively coupled plasma (ICP-OES). Metal standard solutions were prepared by diluting 100 or 1000 mg/L stock multi-element standard solution (High-purity standards, Charleston) immediately before use. All samples and standards were dilute acid solutions of 0.1- 1M. Limit of detection was defined as the concentration corresponding to three times the standard deviation of 5 blanks. Detection limit values of elements (mg l^{-1}) in ICP-OES were found to be 0.01 for Ca, 0.02 for Cu, 0.01 for Fe, 0.05 for Mg, 0.002 for Mn. 0.002 for Mo, 0.09 for Na, 0.2 for P, 0.003 for Pb and 0.004 for Zn.

2.6. Ranking mineral content data

We ranked the mineral content data as describes above (Table 1).

Table 1: Description of how the mineral composition data of mushroom were ranked.

Description	Action	Example	Rank
When there is no statistical differences between data groups	All groups are ranked as 0	12702.26 ± 403.65 13125.45 ± 964.65 12353.94 ± 476.36	0 0 0
When all data groups are statistically different	Groups are ranked as -1;0;1	14866.61 ± 309.72 a 11584.44 ± 786.88 c 13834.57 ± 362.25 b	1 -1 0
When one data group is	Groups are ranked as -	11.84 ± 1.28 a	1

statistically equal to the others groups	1;0;1	9.28 ± 2.34 ab 8.64 ± 0.74 b	0 -1
When two data groups are statistical equal and one group has lower measurements	Groups are ranked as -1; 0;	14181.77 ± 1454.23 a 14126.12 ± 1330.10 a 11628.56 ± 283.57 b	0 0 -1
When two data groups are statistical equal and one group has higher measurements	Groups are ranked as 0;1	15281.86 ± 1309.14 a 11329.90 ± 787.40 b 11613.70 ± 341.42 b	1 0 0

2.5. Statistical analysis

This experiment was conducted using a completely randomized design. Non-linear regression analyses were used to predict the relationship among variables and lime concentration and the Pearson's correlation coefficients were used to perform correlation analyses at the significance level of 5%. The results of mushroom production and mineral content were expressed as the mean values with standard deviation. This data were subjected to analysis of variance (ANOVA), and the averages were compared using Tukey's test ($P < 0.05$). This analysis was choosing because comparisons were done within the fungi strains.

3. Results

3.1. pH of the solution

No effect of immersion time on pH was observed in 0.06% of lime solution (Figure 1). Moreover, modified hyperbola III regression ($R^2 = 0.965$, $F(4.18) = 130.97$, $p < 0.001$) clearly shows pH solution is hyperbolic positive related to lime concentration, and pH did not increase in 0.5% of lime solution (Figure 1).

3.2. Substrate and pH

Then, we analyzed the effect of lime proportion on pH of the substrate to verify if pH of the substrate and solution presented a similar pattern. Likewise, immersion time showed no effect on pH of the substrate, but it was observed higher pH homogeneity in substrate when it was soaked in 2.0% of lime solution for 4 h (Figure 1). Sigmoidal

regression (R^2 = 0.980, F (4.24) = 405.57, $p < 0.001$) shows pH of the substrate quickly increases from 0.5% to 1% of lime, but has a slight increases from 1% of lime. Moreover, we found a low correlation between pH of the solution and substrate (r = 0.550, p = 0.018), showing that pH of the solution is not a good predictor of substrate pH.

Furthermore, it should be considered that the mass of substrate immersed in lime solution might influence the pH of the substrate. Thus, based on earlier results, it was evaluated the effect of substrate mass on pH of the substrate immersed in lime solutions from 1% to 2%. The increasing in the mass have a small effect on pH of the substrate (Figure 2). Thus, even when the highest amount of substrate was used the lime solution was able to infiltrate in the substrate and efficiently increase the pH.

3.3. Microbial estimation

Then, an important question must be answered: the lime addition has antimicrobial activity? Additionally, pH increases influence the antimicrobial activity? In order to answer those questions, plate count method was performed to estimate the cultivable cells numbers presented in the substrate after immersion in the lime solution. Level of cells decreased drastically due to lime addition (Figure 3), clearly showing that lime solution has antimicrobial activity. However, the pH seems to have a small influence in the cells growth (Figure 3). Only fungi presented higher CFU count in pH 10 than pH 7, indicating that immersion in lime solution for 4 h offered a strong selective pressure to alkaliphiles fungi.

3.4. Mushroom production

The contamination evaluation clearly shows that boilling is the treatment with highest contamination rate (Figure 4). Also, the growth of the antagonist fungi was

faster than other treatments. The variation of contamination rate among the species showed a diverse competitive ability of fungi studied. Indeed, P. eryngii was not able to be cultivated using the alkaline treatments (Figure 4)

Alkaline treatments influenced all technical parameters of mushrooms production evaluated ($P < 0.05$). The mycelium run and days to reach the harvest day revealed a slight increase in the time required to achieve the first flush when lime treatment is used ($P < 0.05$; Table 1). In general, the number of mushrooms, in the first flush, was highest in the sterilized substrate (Table 1). Moreover, it was observed a decrease of mushroom yield in the first flush, but with compensation in the second flush, resulting in a similar total mushroom yield for most fungi species studied. The exception was P. cornucopia, which was not able to flush in boiled substrate and only 66% of the bottles flush when lime treatment was used, being excluded from the others evaluations.

The mushroom morphology data revealed a different trend among the fungi (Figure 5). In general, stipe length and stipe and pileus diameter seems to increase when alkaline techniques were used. However, wide variations were observed showing a specie-specific response to the environment conditions.

3.5. Mineral content

The mineral contents of the samples are given in the Table 3 and 4. All the metal concentrations were determined on a dry weight basis. The contents of trace metals in the samples ranged from 0.87–2.80, 00.08–0.9, 4.18–15.05, 0.07–0.3 and 11.05–15.28 mg/g dw for Mg, Ca, K, Na and P, and 48.26–124.39, 39.18–85.07, 5.49–28.23, 7.01–24.69 and 0.04–0.88 mg/kg dw for Zn, Fe, Cu, Mn and Mo, respectively. The most abundant element was found to be phosphorus followed by potassium and magnesium,

while the most variable mineral was calcium. Mushrooms contained a wide range of minerals, particularly phosphorus and potassium, which is in agreement with the literature (Sarikurkcu et al,. 2011). The ranked data (Table 5) showed it seems to be a tendency of alkaline methods to remove some minerals, mainly, P, Mn, Cu and Zn. On the other hand, we can observe an increase in level of Ca and Na. Thus, it seems that alkaline methods lixiviate some minerals from the substrate, decreasing the level found of these minerals in the mushrooms, but simultaneously, induces bioaccumulation of Ca and Na.

4. Discussion

The use of immersion of substrate in lime solution as treatment for Pleurotus cultivation increased in the last decade because it is a cheap and simply method. However, problems with contamination (Léon-Monzón et al,. 2004, Contreras et al,. 2004) and lack of standardization raise the questions about how long the substrate needs to be soaked, which concentration of lime should be used and how to monitor the cultivation process. Furthermore, which parameters of mushroom production are affected by immersion of substrate in lime solution?

First, it was observed that pH of the lime solution is not a useful parameter to monitor the cultivation process on sawdust based substrate. The buffering capacity of sawdust (Raviv et al,. 2002) influences the loss of H^+ to the alkaline solution, which likely explain the small correlation between pH of the substrate and solution. However, it needs to be considered that buffer capacity will vary depending on physic-chemical composition of each substrate, thus justifying investigations with others substrates. Development of control practices and procedures is an interesting approach to improve alkaline immersion methods.

Many rural areas have problems with water availability, so the volume of lime-water solution should be reduced as much as possible, in other words, it should be used the volume required only to cover up the substrate. Furthermore, better water utilization is important nowadays. Here, the maximum proportion of substrate and solution tested was 500 g to 5 L. Use of higher proportions could lead to a poor infiltration of the alkaline solution, which could adversely affect the pH homogeneity and microbiota reduction. However, water absorption will vary depending on substrate type and amount of water present in substrate. So, when another substrate is used the maximum ratio of substrate/lime-water solution needs to be determined to avoid water waste. Another way to save water can be the reuse of lime-water solution; however, further studies should evaluate if this reused solution will be efficiently as well as the originally solution.

In this study, it was showed that soaking in 2.0% of lime solution for 4 h is a good method to prepare sawdust/rice bran for Pleurotus spp. cultivation. Besides the substrate pH increasing in a homogeneous way (Figure 1), which is important to avoid growth of antagonistic microorganisms, this method also decreases up to 99% of resident microbiota (Figure 3) and showed mycelium run, days to reach the harvest time and mushroom yields comparable to sterilized substrate (Table 1). Therefore, it is possible to reach to two conclusions. First, solutions of lime lower than 2% should not be used because this decreases the disinfestation efficiency of the substrate (Figure 3). Second, even considering that physic-chemical composition of others substrates are different and that this might affect the parameters evaluated in this study, it is reasonable to conclude that long soaking times (e.g. 12 – 48 h), as described in others studies (Bernabé-González and Cayetano-Catarino 2009, Contreras et al,. 2004, Léon-Monzón et al,. 2004), are not required.

We observed growth of contaminants when alkaline methods were used, mainly with boilling method (Figure 4), showing that this method seems to be not suitable for mushroom production. The contaminants were mainly Trichoderma sp., Penicillium sp. and Aspergillus sp., all of which are common contaminants in Pleurotus spp. mushroom cultivation (Avendaño-Hernandez and Sánchez 2013, López-Averalo et al,. 1996). Similar contamination results were reported by Léon-Monzón et al,. (2004) when alkaline methods were used. Growth of antagonistic microorganism should be avoided because some common contaminants, in mushroom production, produce harmful substances for human health, as mycotoxins (Bennett and Klich 2003, Waśkiewicz 2014). Due to the capacity to accumulate substances found in the substrate in the fruiting bodies, it is possible that, in the presence of contaminants occur accumulation of harmful substance in mushrooms, which is not desirable and need to be avoided.

The contamination growth observed here inhibited P. eryngii mycelium run and flush of P. cornucopiae (Figure 4; Table 1), showing that some Pleurotus species are sensitive to the alkaline conditions and/or growth of antagonistic microorganism. Confirming that influence of antagonistic microorganism on mushroom production seems to vary depending on mushroom species (Hernández et al,. 2003, Jaramillo Mejía and Albertó 2013, Léon-Monzón et al,. 2004, Fletcher and Gaze 2007). On the other hand, others studies did not observed fungal contamination when alkaline methods were used (Contreras et al,. 2004, Hernández et al,. 2003), at least macroscopically. Besides using the Pleurotus ostreatus and similar alkaline techniques, those studies used different substrate for mushroom cultivation, which will reflect in a distinct diversity and initial number of microorganisms present in the substrate, influencing the risk of contamination. Therefore, despite the good potential of immersion in lime-water solution for being used in mushroom cultivation, the selection of appropriate substrates

and fungus species well adapt to alkaline conditions and with fast mycelium growth seems to be essential to use this method.

The mushrooms morphology measurements showed a change in mushrooms shape when alkaline methods were used (Figure 5). This may have implications in market value of the mushrooms because customers used to buy mushrooms tend to base the judgment about the quality of mushrooms on shape and color. Changes in shape can be associated with bad quality of mushrooms and lead to rejection of the product. On the other hand, this problem will not occur in new markets, but the challenge will be to introduce the new product to the customer. Thus, mushrooms growers need to possess the knowledge and information in terms of customers' needs and preferences, in each region, to define the best approach to commercialize the mushrooms produced by alkaline methods.

The elements concentration in the fruiting bodies revealed influence of substrate treatments and different sensitivity to uptake metals ions among the fungi (Table 3 and 4). We also observed Mo levels lower than 1 mg kg^{-1}. The differences were significant for all metal analyzed ($P < 0.05$) and wide variations were observed, showing a species-specific sensitivity to uptake metals ions. Also, table 5 showed it seems to be common to occur leaching or unavailability to mineral sequestration of P, Cu, Zn and Mn from the substrate, decreasing the levels found in the mushrooms together with bioaccumulation of Ca and Na. Indeed, previous studies showed that organic acid can increase the availability of some minerals (Mendes et al,. 2013, Mendes et al,. 2012, Gast et al,. 1988); therefore, it is reasonable to conclude that alkalization of substrate decreases availability of some minerals. Thus, although the specie-specific sensitivity to uptake metals ions, the mineral values found are comparable with others studies in the literature (Çağlarırmak 2007, Reis et al,. 2012, Nnorom et al,. 2013), showing that

mushrooms produced by alkaline methods during two flushes can provide a significant proportion of dietary requirement for macronutrients and micronutrients.

It is expected that due to high concentration of Ca in lime solution a Ca-enrichment in the substrate occurs. Indeed, bioaccumulation of Ca in mushrooms was observed for most fungi tested (Table 3 and 4) and also in the table 5, which is in agreement with previous study (Chapter 1 and 2). Thus, these mushrooms can be an alternative source of calcium in deprived regions and development countries where milk or milk derivatives, the primary source of calcium (Poliquin et al,. 2009, Singh et al,. 2007, Guéguen and Pointillart 2000), are unavailable or an expensive food. Also, studies already showed that calcium from others foods than green vegetables have higher possibility to be more available for humans (Heaney et al,. 1988). However, should be pointed out that only Ca-enriched mushrooms consumption will be not enough to reach the calcium recommended daily intake and complementation with others food is necessary (Singh et al,. 2007, Ross et al,. 2011).

We can conclude that immersion in 2% lime solution for 4 h is an effective method to cultivate mushrooms of different Pleurotus species. This is important in deprived regions and increases the perspective to disseminate these methods, creating a cheap and fast way to produce high quality food. However, an appropriate selection of fungi species and substrate is required.

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Tables and Figures

Table 2: Mycelium run, days to reach harvest, number of mushrooms and mushroom yield of 6 fungi strains using 3 methods to prepare the substrate (mean ± SD).

Species	Treatment	Days of mycelium run	Days to reach harvest	Number of mushrooms		Mushroom yield (g)		
				1º Flush	2º Flush	1º Flush	2º Flush	Total
P. sajor—caju	Autoclave	14.00 ± 1.00	10.60 ± 0.55 ^{c}	7.40 ± 1.34 ^{a}	3.00 ± 1.58	45.46 ± 2.66 ^{a}	19.54 ± 5.26 ^{ab}	65.00 ± 6.09 ^{a}
	Boil	15.17 ± 2.56	14.83 ± 1.32 ^{a}	3.16 ± 1.32 ^{b}	2.50 ± 1.04	32.93 ± 5.88 ^{b}	17.63 ± 4.47 ^{b}	50.57 ± 7.55 ^{b}
	Lime	14.33 ± 0.81	12.66 ± 0.82 ^{b}	4.33 ± 1.86 ^{b}	3.33 ± 1.03	36.00 ± 7.09 ^{b}	25.67 ± 5.51 ^{a}	61.67 ± 7.05 ^{a}
Pleurotus ostreatus (PLO 06)	Autoclave	13.60 ± 1.14 ^{a}	11.00 ± 0.00 ^{b}	6.60 ± 0.89	5.60 ± 1.51 ^{b}	24.72 ± 2.45 ^{b}	17.98 ± 1.36	42.70 ± 3.36 ^{b}
	Boil	11.00 ± 0.89 ^{b}	12.00 ± 0.63 ^{b}	6.33 ± 1.36	11.00 ± 4.80 ^{ab}	34.68 ± 9.66 ^{a}	27.62 ± 7.83	64.40 ± 2.83 ^{a}
	Lime	15.16 ± 1.47 ^{a}	17.33 ± 1.21 ^{a}	7.50 ± 1.51	11.67 ± 3.88 ^{a}	29.38 ± 3.37 ^{ab}	28.20 ± 10.61	57.58 ± 10.15 ^{a}
P. djamor	Autoclave	14.80 ± 1.48 ^{a}	9.80 ± 0.44	16.40 ± 4.22 ^{a}	3.60 ± 1.14	35.00 ± 3.61 ^{a}	17.00 ± 4.83 ^{b}	52.00 ± 6.75 ^{b}
	Boil	12.00 ± 0.89 ^{b}	12.16 ± 1.47	5.66 ± 1.96 ^{b}	6.40 ± 3.21	24.28 ± 4.36 ^{b}	29.90 ± 6.80 ^{a}	53.17 ± 3.29 ^{b}
	Lime	16.16 ± 1.60 ^{a}	11.83 ± 2.64	9.00 ± 2.00 ^{b}	5.66 ± 1.75	33.18 ± 5.03 ^{a}	33.20 ± 8.68 ^{a}	66.38 ± 10.79 ^{a}
Pleurotus ostreatus (PO -04)	Autoclave	14.40 ± 1.51 ^{b}	13.00 ± 0.00 ^{b}	14.00 ± 3.00 ^{a}	3.60 ± 1.51	43.38 ± 4.68 ^{a}	15.00 ± 2.71 ^{b}	58.38 ± 6.42
	Boil	16.85 ± 1.46 ^{ab}	17.85 ± 1.34 ^{a}	8.00 ± 3.42 ^{b}	5.14 ± 1.57	28.81 ± 7.22 ^{b}	23.14 ± 5.05 ^{ab}	51.96 ± 8.34
	Lime	18.00 ± 2.90 ^{a}	18.50 ± 1.22 ^{a}	8.83 ± 2.56 ^{b}	5.33 ± 1.63	31.05 ± 4.26 ^{b}	25.23 ± 7.71 ^{a}	56.28 ± 7.61
P. pulmonarius	Autoclave	14.00 ± 0.70 ^{b}	10.40 ± 1.94 ^{b}	9.20 ± 2.28 ^{a}	2.40 ± 1.14	44.24 ± 5.54	24.20 ± 7.86	68.44 ± 7.23
	Boil	13.00 ± 1.41 ^{b}	10.50 ± 1.51 ^{b}	6.50 ± 2.51 ^{ab}	1.80 ± 0.44	42.73 ± 5.57	27.28 ± 8.58	68.36 ± 5.92
	Lime	18.67 ± 3.08 ^{a}	14.67 ± 2.73 ^{a}	5.00 ± 2.36 ^{b}	2.25 ± 1.25	43.97 ± 7.22	28.90 ± 9.06	76.15 ± 3.36
P. cornucopiae *	Autoclave	14.40 ± 0.54 ^{b}	9.60 ± 0.54 ^{b}	9.60 ± 2.07 ^{b}	nd	29.08 ± 6.40	nd	29.08 ± 6.40
	Boil **	19.50 ± 0.83 ^{ab}	nd	nd	nd	nd	nd	nd
	Lime ***	24.17 ± 6.40 ^{a}	19.75 ± 6.18 ^{a}	14.00 ± 2.94 ^{a}	nd	29.77 ± 3.70	nd	29.77 ± 3.70

* This strain flushes just once; ** After mycelium run, no flush was observed; *** Only 66% of the bottles produced mushrooms.

Means with different letters within a column, for each Pleurotus specie, are significantly different ($P < 0.05$).

nd = not determined

Table 3: Mineral content in mushrooms of the first flush using different substrate treatments (mean ± SD; mg Kg^{-1} of dry mass).

Fungi specie	Disinfestation methods	P	Ca	Fe	Mn	Mo
Pleurotus sajor-caju	Autoclaved	12702.26 ± 403.65	102.10 ± 48.07 b	60.22 ± 5.53 b	09.11 ± 0.75 b	0.24 ± 0.08 a
	Boil	13125.45 ± 964.65	373.27 ± 111.76 a	64.67 ± 11.39 a	14.43 ± 2.38 a	0.04 ± 0.04 b
	Lime	12353.94 ± 476.36	281.04 ± 81.38 a	66.19 ± 2.82 a	13.02 ± 1.19 a	0.21 ± 0.11 a
Pleurotus ostreatus (PLO 06)	Autoclaved	14866.61 ± 309.72 a	381.95 ± 132.42 a	57.45 ± 4.59 b	20.90 ± 1.31 a	0.11 ± 0.03 b
	Boil	11584.44 ± 786.88 c	200.27 ± 82.08 b	42.46 ± 6.12 c	10.28 ± 1.64 c	0.20 ± 0.06 a
	Lime	13834.57 ± 362.25 b	285.64 ± 76.60 ab	73.65 ± 2.40 a	15.10 ± 1.64 b	0.15 ± 0.06 ab
Pleurotus djamor	Autoclaved	12335.55 ± 450.22	344.55 ± 23.11 b	76.72 ± 5.39 a	23.77 ± 3.19 a	0.66 ± 0.99 a
	Boil	12166.03 ± 226.76	997.32 ± 159.61 a	58.01 ± 4.84 b	16.95 ± 1.10 b	0.15 ± 0.05 b
	Lime	12386.19 ± 1689.72	943.38 ± 82.55 a	39.18 ± 1.98 c	15.52 ± 4.51 b	0.88 ± 0.33 a
Pleurotus ostreatus (PO-04)	Autoclaved	14181.77 ± 1454.23 a	80.68 ± 12.19 b	54.57 ± 6.09	13.05 ± 0.64	0.12 ± 0.09 b
	Boil	14126.12 ± 1330.10 a	290.84 ± 124.66 a	51.89 ± 6.63	12.51 ± 2.56	0.33 ± 0.17 a
	Lime	11628.56 ± 283.57 b	190.47 ± 75.22 a	47.83 ± 10.16	11.34 ± 1.60	0.24 ± 0.11 ab
Pleurotus pulmonarius	Autoclaved	15281.86 ± 1309.14 a	106.37 ± 18.13 b	65.61 ± 4.48 a	11.84 ± 1.28 a	0.18 ± 0.14 b
	Boil	11329.90 ± 787.40 b	237.92 ± 107.76 a	45.02 ± 5.76 b	9.28 ± 2.34 ab	0.14 ± 0.11 b
	Lime	11613.70 ± 341.42 b	146.38 ± 40.11 b	65.93 ± 7.25 a	8.64 ± 0.74 b	0.44 ± 0.17 a
Fungi specie	Disinfestation methods	K	Mg	Cu	Na	Zn
Pleurotus sajor-caju	Autoclaved	10197.83 ± 299.48 b	1599.44 ± 27.12 c	10.74 ± 1.41 a	109.59 ± 28.21 b	55.50 ± 5.75 b
	Boil	10857.57 ± 495.89 a	2028.06 ± 136.72 a	9.65 ± 3.36 b	183.38 ± 44.48 a	64.78 ± 4.77 a
	Lime	10066.53 ± 652.14 b	1768.72 ± 73.78 b	7.05 ± 1.51 b	168.19 ± 11.93 a	61.52 ± 10.20 b
Pleurotus ostreatus (PLO 06)	Autoclaved	10005.19 ± 202.46 a	1780.62 ± 50.12	28.23 ± 1.20 a	140.08 ± 8.75 b	82.08 ± 5.15 a
	Boil	9006.18 ± 377.57 b	1676.65 ± 165.25	13.24 ± 4.04 b	165.03 ± 66.43 b	48.26 ± 2.29 c
	Lime	10094.94 ± 524.22 a	1781.71 ± 84.70	15.75 ± 2.29 b	288.05 ± 8.75 a	66.75 ± 4.37 b
Pleurotus	Autoclaved	9891.77 ± 278.50 c	1955.01 ± 80.35 b	9.90 ± 1.87	79.03 ± 8.80 b	124.39 ± 9.90 a

djamor	Boil	11356.94 ± 417.20 a	2594.58 ± 117.78 a	10.23 ± 0.44	186.03 ± 26.41 a	113.38 ± 4.40 a
	Lime	10427.87 ± 658.27 b	2535.13 ± 576.81 a	8.58 ± 1.43	217.95 ± 60.54 a	90.92 ± 12.43 b
Pleurotus	Autoclaved	11599.66 ± 1112.88 a	1619.03 ± 158.37 b	17.76 ± 1.07	196.25 ± 37.23 b	86.78 ± 2.14 a
ostreatus	Boil	4188.29 ± 1480.99 c	2472.95 ± 350.98 a	16.69 ± 1.71	233.59 ± 50.93 a	59.60 ± 6.84 b
(PO-04)	Lime	6979.06 ± 337.07 b	872.11 ± 19.26 c	15.30 ± 3.21	230.92 ± 33.27 a	51.79 ± 4.92 c
Pleurotus	Autoclaved	15050.33 ± 1695.37 a	1915.16 ± 171.77 a	10.88 ± 1.28 a	155.77 ± 28.80	82.47 ± 1.38 a
pulmonarius	Boil	10146.65 ± 857.82 b	1683.64 ± 89.62 b	6.61 ± 1.81 b	174.97 ± 24.53	49.93 ± 1.92 c
	Lime	10790.02 ± 293.41 b	1642.02 ± 90.69 b	6.72 ± 0.85 b	160.04 ± 25.60	59.00 ± 0.74 b

Table 4: Mineral content in mushrooms of the second flush using different substrate treatments (mean ± SD; mg Kg^{-1} of dry mass).

Fungi specie	Disinfestation methods	P	Ca	Fe	Mn	Mo
Pleurotus sajor-caju	Autoclaved	13007.70 ± 558.52	181.14 ± 108.90 c	78.81 ± 12.29 a	14.23 ± 4.95	0.26 ± 0.12
	Boil	12657.27 ± 1117.04	465.79 ± 149.87 b	69.97 ± 4.09 a	15.74 ± 4.95	0.12 ± 0.08
	Lime	12801.75 ± 1110.57	617.82 ± 58.22 a	58.22 ± 11.75 b	15.41 ± 3.34	0.32 ± 0.53
Pleurotus ostreatus (PLO 06)	Autoclaved	14308.78 ± 608.66 a	164.67 ± 113.28	85.07 ± 14.61	14.50 ± 3.08 a	0.18 ± 0.05 ab
	Boil	12399.42 ± 1089.43 b	228.50 ± 95.35	70.57 ± 21.12	10.73 ± 2.96 b	0.27 ± 0.11 a
	Lime	12961.26 ± 1148.81 b	159.64 ± 107.91	75.59 ± 8.10	10.62 ± 2.28 b	0.12 ± 0.09 b
Pleurotus djamor	Autoclaved	12895.22 ± 518.00	399.19 ± 112.52 b	48.48 ± 4.04 c	24.69 ± 3.65 a	0.37 ± 0.14
	Boil	11935.83 ± 1845.60	289.30 ± 48.02 b	62.77 ± 6.26 a	13.95 ± 7.20 b	0.38 ± 0.30
	Lime	12369.22 ± 1851.32	869.05 ± 485.98 a	55.45 ± 6.26 b	14.40 ± 4.11 b	0.32 ± 0.22
Pleurotus ostreatus (PO-04)	Autoclaved	13479.35 ± 864.25 a	98.81 ± 16.89 b	53.08 ± 6.69 ab	8.99 ± 0.54	0.17 ± 0.13
	Boil	11059.87 ± 490.25 b	105.50 ± 32.90 b	44.09 ± 10.41 b	7.01 ± 1.75	0.23 ± 0.13
	Lime	12344.19 ± 1517.93 ab	301.72 ± 119.00 a	56.26 ± 10.09 a	9.98 ± 2.30	0.31 ± 0.20
Pleurotus pulmonarius	Autoclaved	14047.29 ± 530.58 b	194.84 ± 82.55 b	56.47 ± 0.88 ab	12.32 ± 0.77	0.87 ± 0.54 a
	Boil	12849.62 ± 704.51 c	245.47 ± 11.00 ab	60.43 ± 9.24 a	9.24 ± 1.54	0.33 ± 0.11 b
	Lime	15148.09 ± 602.12 a	343.33 ± 146.51 a	47.99 ± 10.45 b	10.56 ± 4.18	0.22 ± 0.09 b
Fungi specie	Disinfestation methods	K	Mg	Cu	Na	Zn
Pleurotus sajor-caju	Autoclaved	9045.22 ± 312.68 b	1773.67 ± 126.15 b	11.10 ± 3.55 a	121.83 ± 59.30 b	80.11 ± 10.13 a
	Boil	9812.91 ± 1126.74 ab	1982.85 ± 245.83 a	7.00 ± 1.72 ab	146.63 ± 58.22 b	56.06 ± 5.49 b
	Lime	10525.62 ± 1373.65 a	2116.55 ± 61.45 a	5.49 ± 1.61 b	242.60 ± 71.16 a	50.02 ± 4.63 b
Pleurotus ostreatus (PLO 06)	Autoclaved	9651.85 ± 387.12	1701.52 ± 96.26	18.15 ± 3.08 a	145.02 ± 38.82 b	86.10 ± 2.16 a
	Boil	8540.73 ± 886.16	1711.80 ± 119.90	10.18 ± 4.31 b	288.91 ± 88.50 a	53.55 ± 13.81 b
	Lime	9327.54 ± 983.22	1709.51 ± 134.75	6.62 ± 2.39 c	163.30 ± 27.40 b	54.01 ± 5.88 b
Pleurotus	Autoclaved	8748.90 ± 373.92 b	2389.91 ± 203.54	8.22 ± 0.50	75.24 ± 10.29	106.23 ± 6.63 b

djamor	Boil	9853.52 ± 503.13 a	2803.85 ± 904.50	11.21 ± 6.38	112.52 ± 63.80	121.21 ± 13.15 a
	Lime	9961.00 ± 396.79 a	2495.11 ± 723.83	6.84 ± 1.65	118.12 ± 65.86	102.45 ± 5.26 b
Pleurotus	Autoclaved	6098.06 ± 4036.12 a	1896.32 ± 116.25 b	13.16 ± 2.74 ab	114.61 ± 5.81 b	86.78 ± 2.14 a
ostreatus	Boil	1866.71 ± 539.61 b	1830.51 ± 129.41 b	8.77 ± 1.97 b	130.51 ± 14.25 b	59.60 ± 6.84 b
(PO-04)	Lime	6173.74 ± 1545.35 a	2369.03 ± 342.19 a	14.58 ± 6.36 a	301.72 ± 94.54 a	51.79 ± 4.92 c
Pleurotus	Autoclaved	10034.88 ± 342.34 b	1623.67 ± 74.85 b	15.41 ± 0.55 a	257.58 ± 139.80	64.39 ± 8.91
pulmonarius	Boil	10410.25 ± 868.53 b	1827.32 ± 24.65 b	8.80 ± 0.99 b	317.03 ± 100.17	60.76 ± 3.85
	Lime	13034.56 ± 684.69 a	2634.21 ± 590.02 a	7.70 ± 0.99 b	233.36 ± 37.42	54.26 ± 6.71

Table 5: Mineral content of mushrooms data from first and second flush using different substrate treatments ranked according to the approach describe in table 1 of chapter 3.

	P	Ca	Fe	Mn	Mo	K	Mg	Cu	Na	Zn
						1º Flush				
Autoclaved	2	-2	0	2	-2	1	-1	3	-3	3
Boil	-1	0	-2	-1	0	0	2	0	0	-1
Lime	-1	0	0	-1	1	0	-1	0	1	-2
						2º Flush				
Autoclaved	2	-2	-1	2	1	-2	-1	3	0	3
Boil	-2	0	1	0	1	-1	0	0	1	1
Lime	1	4	0	0	-1	2	2	-1	2	-1
						Total				
Autoclaved	4	-4	-1	4	-1	-1	-2	6	-3	6
Boil	-3	0	-1	-1	1	-1	2	0	1	0
Lime	0	4	0	-1	0	2	1	-1	3	-3

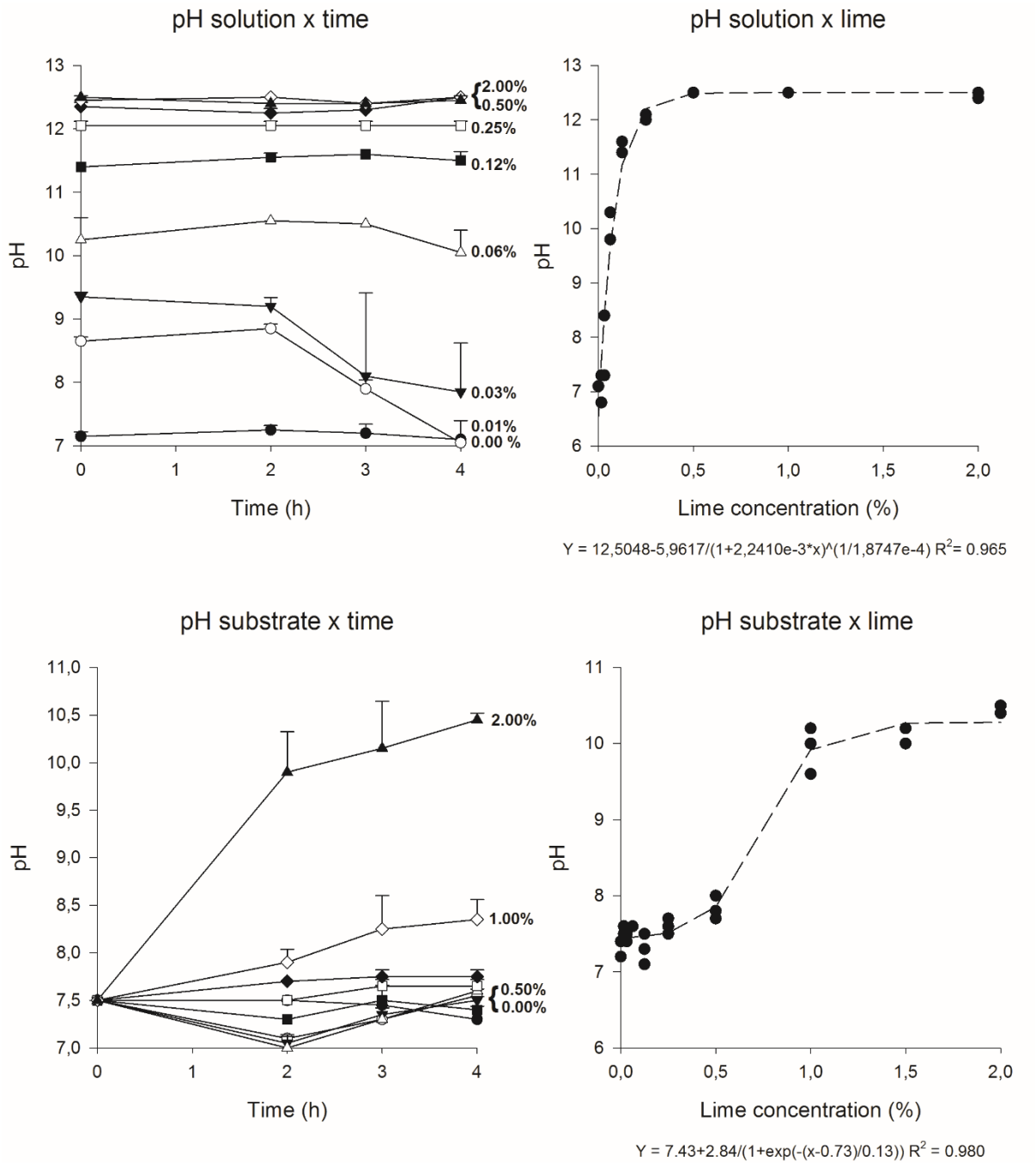

Figure 1: Effect of immersion time and lime concentration on pH of solution and substrate pH. The percentages in the graphs (pH solution x time; pH substrate x time) are lime concentration in each lime solution evaluated. pH measures in graphs (pH solution x lime; pH substrate x lime) were done after 4 h of immersion.

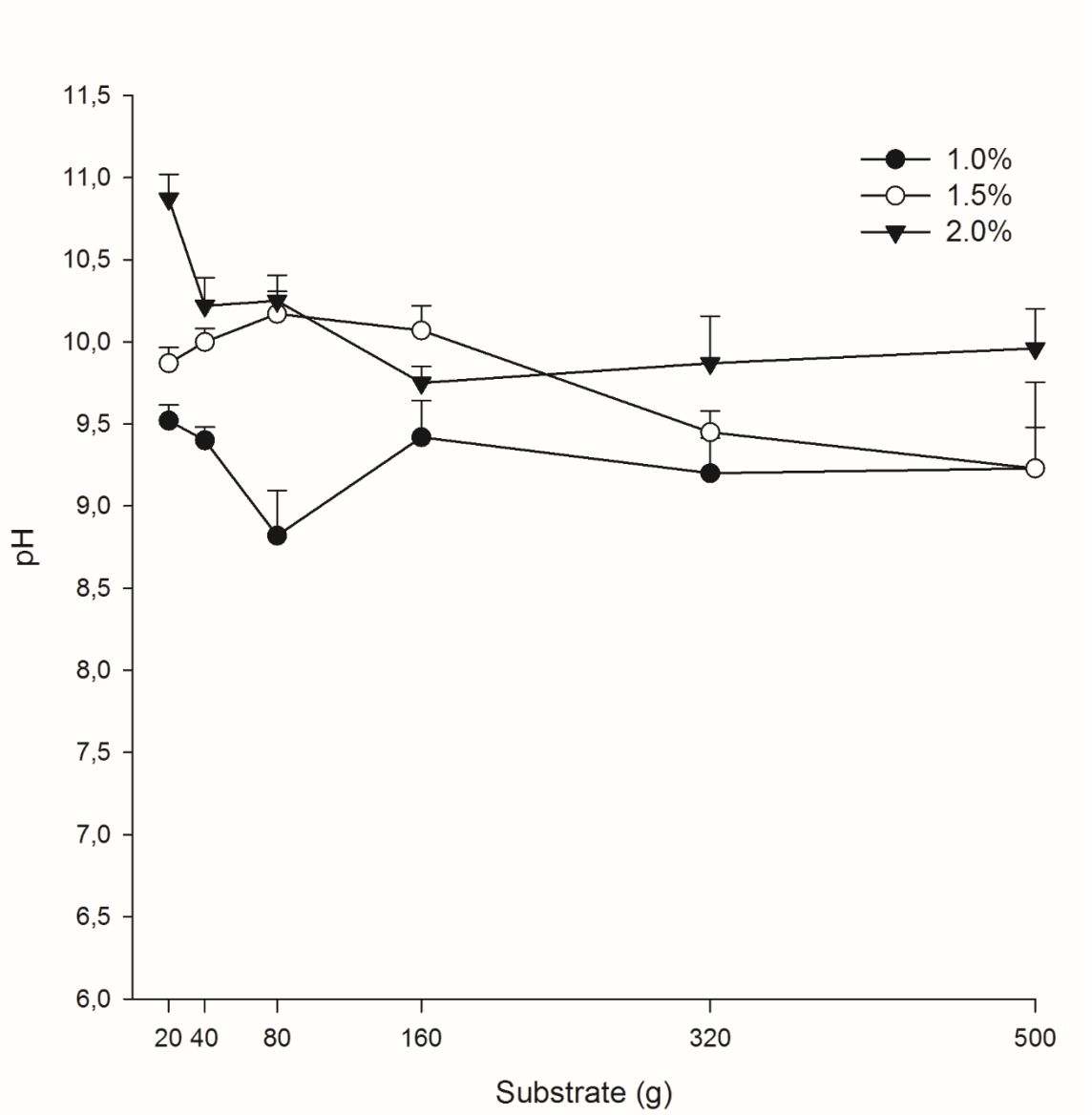

Figure 2: Effect of mass substrate soaked for 4 h in 5 L of 1.0% (dark circle), 1.5% (white circle) and 2% (dark triangle) lime-water solution on pH of substrate.

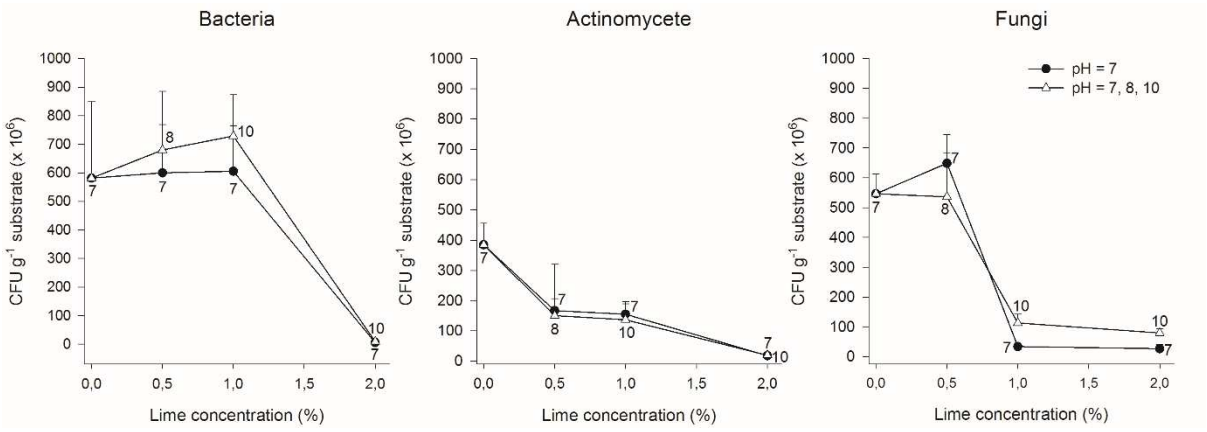

Figure 3: Effect of lime immersion and pH on microbial population in substrate. CFU is colony-forming units. pH of the agar culture media for counting is similar to pH found in the substrate after soaking in lime solution for 4 h. pH 7 (dark circles) are the controls and the others pH (white triangle) were selected to mimic the pH measured at each lime concentration in the substrate after soaking for 4 h.

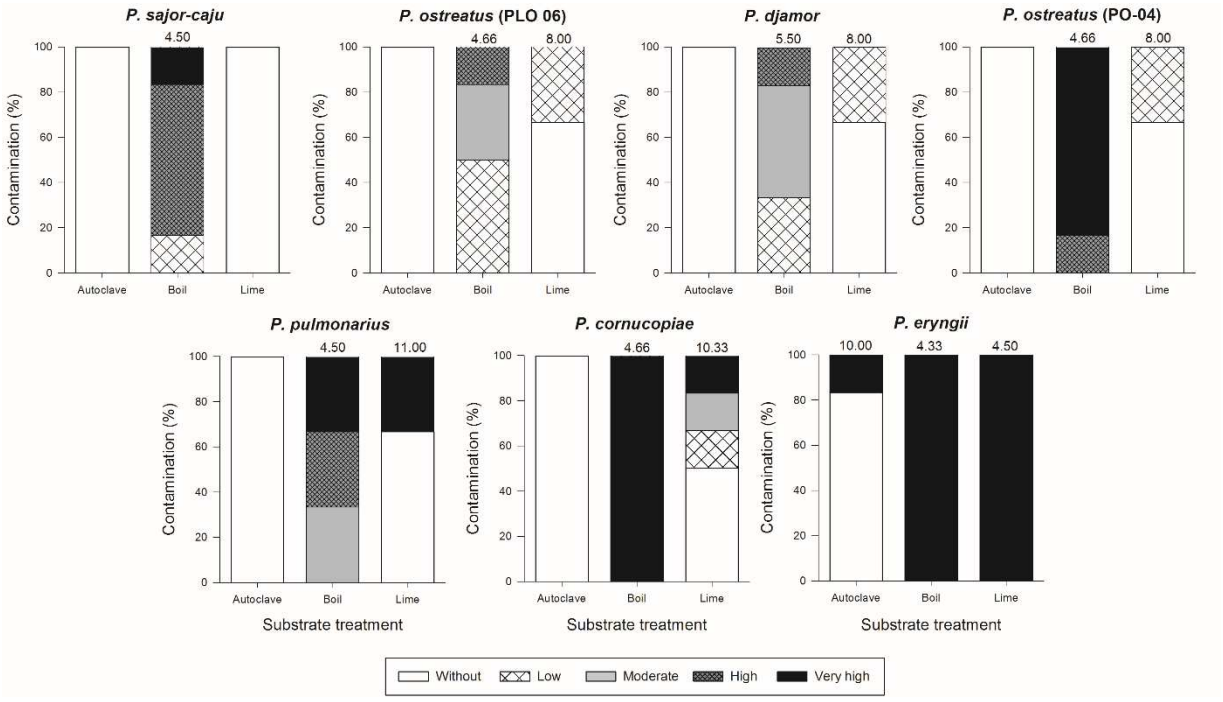

Figure 4: Growth of antagonistic fungi in substrate prepared by following methods: boil (boiled for 30 min in 5 L of a 2% lime-water solution), lime (soaked for 4 h in 5 L of a 2% lime-water solution) and autoclave (sterilized for 60 min at 121 ºC) The numbers in the top of the bars indicate the average days necessary to observe contamination growth. .

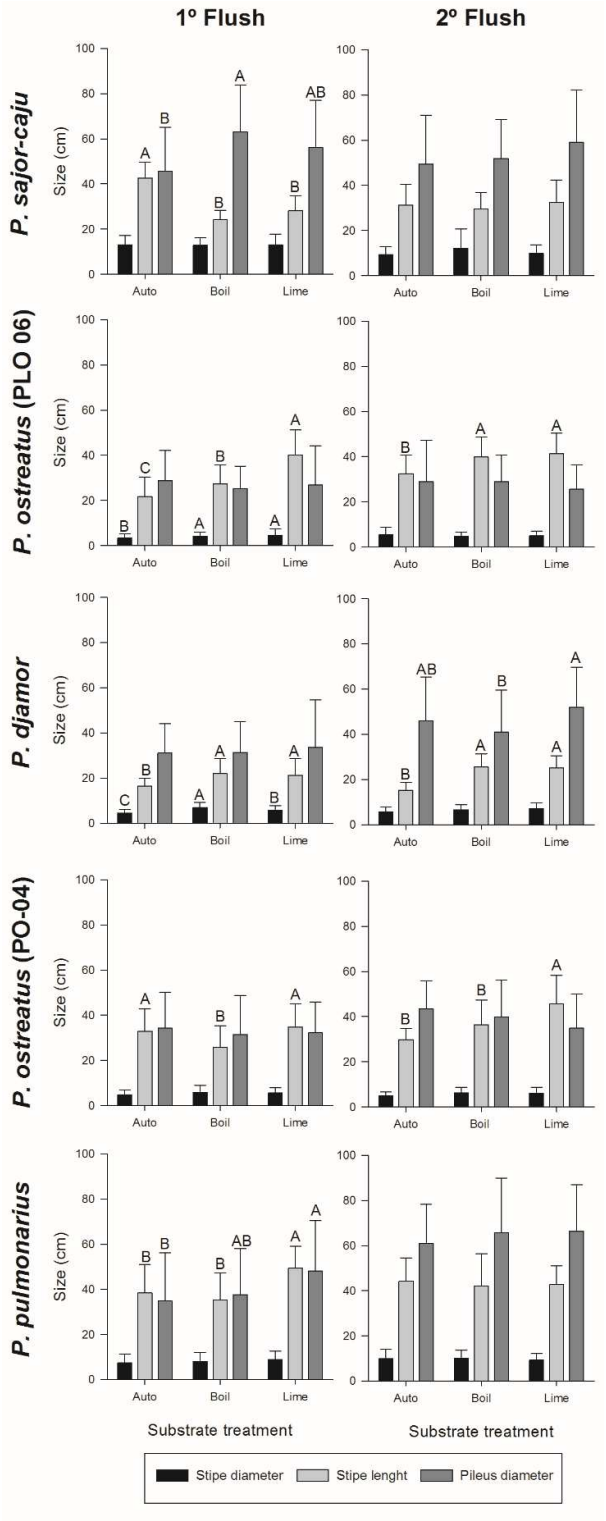

Figure 5: Diameter and length of stipe and diameter of cap of mushrooms cultivated in substrates prepared by following methods: boil (boiled for 30 min in 5 L of a 2% lime-water solution), lime (soaked for 4 h in 5 L of a 2% lime-water solution) and autoclave (sterilized for 60 min at 121 ºC). The data are means (± SD) and were compared by repeated measures one-way ANOVA followed by Tukey's test ($P < 0.05$), where bars not sharing a same letter are significantly different.

CONSIDERAÇÕES FINAIS

Neste estudo foi possível demonstrar que os métodos alcalinos para tratamento de substratos são uma alternativa viável e financeiramente mais acessível para produção de cogumelos de diferentes espécies de cogumelos do gênero Pleurotus. Dessa forma, foi possível concluir que:

1- Os métodos alcalinos são capazes de produzir quantidades de cogumelos tão bem quanto o método tradicional de esterilização do substrato, utilizando cerca de um terço dos recursos financeiros utilizados pelo método convencional. Além disso, foi observado que os ciclos de produção foram similares ao cultivados utilizando a esterilização.
2- Diferentes substratos podem ser utilizados para o cultivo de cogumelos utilizando os métodos alcalinos. Entretanto, a composição do substrato demonstrou ser mais relevante para a produtividade e para o aparecimento de contaminantes que o método de desinfestação, principalmente, quando se utiliza substratos mais difíceis de cultivar cogumelos, como, por exemplo, casca de café.
3- Apenas 4 h de imersão do substrato em uma solução de 2% de cal hidratada são suficientes para a alcalinização do substrato e redução de 99% da microbiota residente, com ressalva de se utilizar uma proporção peso/volume de substrato e solução de cal de 10%. Concentrações menores que 2% de cal hidratada apresentaram menor eficiência para redução da microbiota residente, portanto, concentrações menores que 2% não são aconselháveis.
4- Diferentes espécies de Pleurotus podem ser cultivadas utilizando os métodos alcalinos. Entretanto, observamos que duas espécies não foram possíveis cultivar utilizando esses métodos, demonstrando que esses métodos apresentam restrições. Portanto, a seleção de substratos e isolados de fungos adaptadas às condições alcalinas é necessária para utilização dos métodos alcalinos para produção dos cogumelos.
5- Apesar de os métodos alcalinos terem afetado a composição química dos cogumelos, o perfil da composição demonstrou que os mesmos se encontram na dentro da faixa de variação observada em outros estudos, ou seja, estão com potencial nutricional mantido. Observamos que as variações são espécies-especificas.

Portanto, métodos alcalinos, principalmente imersão em solução de cal 2% durante 4 h, apresentam enorme potencial para serem utilizadas para cultivo de cogumelos, inclusive em regiões carentes. Entretanto, é importante ressaltar a necessidade de se selecionar substratos e estirpes de fungos adaptadas a condições alcalinas.