UNIVERSIDADE FEDERAL DO PARANÁ

GABRIEL LUCAS DE JESUS

PRODUÇÃO DE *Pleurotus spp.* EM SUBSTRATO PÓS-CULTIVO DE COGUMELOS

CURITIBA

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Tese apresentada ao Programa de Pós-Graduação em Agronomia – Produção Vegetal, Setor de Ciências Agrárias, Universidade Federal do Paraná, como requisito parcial à obtenção do título de Doutor em Ciências.

Orientadora: Profª. Drª. Francine Lorena Cuquel

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Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação AGRONOMIA (PRODUÇÃO VEGETAL) da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **GABRIEL LUCAS DE JESUS** intitulada: **PRODUÇÃO DE *Pleurotus* spp. EM SUBSTRATO PÓS-CULTIVO DE COGUMELOS** , sob orientação da Profa. Dra. FRANCINE LORENA CUQUEL, que após terem inquirido o aluno e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de doutor está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

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RESUMO

A produção mundial dos cogumelos *Pleurotus* spp. (shimeji ou cogumelo ostra) e *Lentinula edodes* (shiitake) está crescendo, devido as suas características gastronômicas, nutricionais e medicinais. A mesma tendência se observa no Brasil, entretanto, a produtividade e a eficiência biológica do cultivo nacional são inferiores aos maiores produtores mundiais. O manejo dos substratos, usualmente selecionados de acordo com as matérias primas de menor custo disponíveis na região de cultivo, é uma das justificativas das diferenças encontradas na produtividade média. Estima-se que, por ano, 42 milhões de toneladas de substrato exaurido da produção de *Lentinula edodes* (SMS) são geradas no mundo. Esse resíduo orgânico possui características que possibilitam seu retorno ao sistema produtivo de forma ecológica, sobre a qual se sabe muito pouco. Este trabalho visa fornecer subsídios para aumentar a produtividade e eficiência biológica de *Pleurotus* spp produzidos com SMS de *Lentinula edodes*, através da compreensão da dinâmica nutricional, bioquímica e enzimática envolvida no processo produtivo. Desta maneira, quatro capítulos foram desenvolvidos onde: i) O efeito da utilização de SMS de *Lentinula edodes* na formulação de substrato com uma relação C/N de 45/1 na produção de *Pleurotus ostreatus var florida* 'MB, *Pleurotus ostreatus* 'SB', *Pleurotus sajor-caju* e *Pleurotus djamor,* proporcionou uma produtividade média de 20,5% e eficiência biológica média de 84%. ii) O efeito da aplicação de doses de Mn exógeno, na forma de sulfato de Mn ($MnSO_4$) na produção de *Pleurotus ostreatus* 'MB', aumentou a atividade da enzima manganês peroxidase e diminuiu a produtividade e a eficiência biológica. iii) A suplementação do cultivo com *P. ostreatus* 'MB' com 15% de SMS proporcionou produtividade e eficiência biológica superiores (25% e 107%, respectivamente) em comparação com a produtividade e eficiência biológica sem suplementação (15,6% e 66,5%). iv) Ao comparar diferentes matérias-primas, a formulação do substrato com palha de trigo foi superior em termos de produção de *Pleurotus ostreatus* 'MB' em relação à serragem de eucalipto. Em relação a suplementação destas matérias primas, o SMS apresentou resultados superiores em termos produtivos na palha de trigo, enquanto o farelo de arroz foi superior para a matéria-prima serragem. Portanto, a utilização de SMS de *Lentinula edodes* para produzir *Pleurotus* ostreatus é viável, pode substituir a suplementação normalmente utilizada aumentando a produtividade e eficiência biológica média, além de significar a reciclagem de um material que é considerado resíduo para produtores de cogumelos shiitake.

Palavras-chaves: shiitake; shimeji; cogumelo ostra; substrato exaurido de cogumelos; produção ecológica; resíduos lignocelulósicos, matéria-prima, macronutrientes, micronutrientes, manganês peroxidase.

ABSTRACT

The world production of *Pleurotus* spp. (shimeji or oyster mushroom) and *Lentinula edodes* (shiitake) is growing due to gastronomic, nutritional and medicinal characteristics. The same trend is observed in Brazil, however, the productivity and biological efficiency are lower than the largest world producers. Substrate management, usually selected according to the lowest cost raw materials available in the growing region, is one of the justifications for the productive differences found. It is estimated that, per year, 42 million tons of spent mushroom substrate from *Lentinula edodes* production (SMS) are generated in the world. This organic product is one of the possible primaries to return in an ecological way to the production system, about which very little is known. This work provides subsidies to increase the productivity and biological efficiency of *Pleurotus* spp produced with SMS from *Lentinula edodes*, with the better understanding of the nutritional, biochemical and enzymatic dynamics involved in production process. In this way, four chapters were developed where: i) The effect of using *Lentinula edodes* SMS in the substrate formulation with a 45/1 C/N ratio on the production of *Pleurotus ostreatus* var florida 'MB', *Pleurotus ostreatus* 'SB'*, Pleurotus sajor-caju* and *Pleurotus djamor* provided an average productivity of 20.5% and average biological efficiency of 84%. ii) On the other hand, when testing doses of exogenous Mn, in the form of Mn sulfate ($MnSO_4$) for *Pleurotus ostreatus* 'MB', increased the activity of the manganese peroxidase enzyme and decreased productivity and biological efficiency. iii) Supplementation of P. ostreatus 'MB' with 15% of SMS provided superior productivity and biological efficiency (25% and 107%, respectively) compared to productivity and biological efficiency without supplementation (15.6% and 66.5%, respectively). iv) When comparing different raw materials, the substrate formulation with wheat straw is superior in terms of production of *Pleurotus ostreatus* 'MB' compared to sawdust. Regarding the supplementation of these raw materials, SMS presents high results in terms of production in wheat straw, while rice bran is superior for the sawdust raw material. Therefore, the use of SMS from *Lentinula edodes* to produce *Pleurotus ostreatus* is viable, it can replace the supplementation normally used without impacting productivity and biological efficiency, in addition to meaning the recycling of a material that is considered waste for producers of shiitake mushrooms.

Index terms: shiitake; shimeji; Oyster mushroom; spent mushroom substrate; ecological production; lignocellulosic residues, raw material, macronutrients, micronutrients, manganese peroxidase.

CONTENTS

1. GENERAL INTRODUCTION

Pleurotus ostreatus (Jacq. ex Fr. P.Kumm), popular known as shimeji or oyster mushroom, and *Lentinula edodes* (Berk.), popular known as shiitake, worldwide production have been expanding due to their gastronomic, nutritional and medicinal properties. The same trend in the increase in production is observed in Brazil, however, the productivity and biological efficiency of national cultivation are lower than those of the largest world producers. They respectively production correspond to 19% and 22% of the worldly mushroom production (Royse et al., 2017). Both are widely produced in Brazil (9,647 tons), representing 61% of the volume of all edible mushroom species produced in the country (Sanchez at al., 2018).

Edible mushrooms are saprophytic fungi that have a huge environmental importance. They have the ability of degrade lignocellulosic residues (cellulose, hemicellulose and lignin) from the substrate, extracting from it essential nutrients for their growth, such as carbon (C), nitrogen (N), phosphorus (P), calcium (Ca), magnesium (Mg), potassium (K), iron (Fe), copper (Cu), zinc (Zn), manganese (Mn) and molybdenum (Mo), which are crucial for several metabolic functions (Miles and Chang, 2004). The ability of mushrooms makes it possible to convert residues from agronomic and forestry activities, which can be potential environmental pollutants, into nutritious foods with high added value. Several lignocellulosic residues can be used to compose the substrate to growth *Pleurotus* spp., such as, straw from annual and perennial crops (rice straw, beans, soybeans, corn, wheat); sawdust, from different types of wood; banana leaves; grasses; coconut husks; corn cob and sugarcane bagasse (Sturion & Oetterer, 1995; Royse, 1996; Dias et al., 2003; Ragunathan and Swaminathan, 2003; Urben, 2004; Webster and Weber, 2007; Donini et al. 2009; Philippoussis et al., 2009; Sánchez, 2010; Vieira and De Andrade, 2016; Urben, 2017).

The composition of substrates affects the physicochemical characteristics of mushrooms and are determinant in their productivity and biological efficiency (Ragunathan and Swaminathan, 2003; Chang and Miles, 2004; Pedra and Marino, 2006; Oyetayo and Ariyo, 2013; Urben, 2017; Bellettini et al., 2019). This is mainly because the raw materials used in the substrate formulation are the main source of nutritional variation. Wheat straw and sawdust have been the major lignocellulosic

residues of *Pleurotus* substrate's formulations for a long time in the world (Philippoussis et al., 2009). Other cereal straws (rice, oat, barley) are used as raw material in other countries. In Brazil, sugarcane bagasse and grasses (Brachiaria straw and others) are used, mainly due to the local availability of these agro-waste. The choice of substrate according to the availability of raw material makes it difficult to compare production systems around the world. Since little information is published and according to reports from local producers, this variability generates oscillations in *Pleurotus* productivity and biological efficiency, where the average local biological efficiency is below that recorded worldwide.

An appropriated C/N ratio on the substrate is essential to allows obtaining high mushrooms productivity and biological efficiency. In such way substrate must be supplemented, besides the lignocellulosic residues, with cereal bran (wheat, soybean, corn, rice, etc.), which increase N in the substrate (Samuel and Eugene, 2012; Cogorni et al., 2014; Bellettini et al., 2019). Lignocellulosic degradation and bioabsorption by edible mushrooms is possible because they have a complex enzymatic extracellular apparatus, involving oxidative enzymes (lignin peroxidase (LiP), laccase (Lac), manganese peroxidase (MnP)) and hydrolytic enzymes (cellulase (Cel), xylanase (Xyl)). Mushrooms enzymatic activity depends on fungus species and the substrate composition (Stajić et al., 2006; Elisashvili et al., 2008; Elisashvili and Kachlishvili, 2009). Very little is known about the mainly enzymatic co-factor of the lignocellulosic enzymes. To study this will provide subsidies to improve the *Pleurotus* spp productivity and biological efficiency. Manganese is one of those, which affect the *Pleurotus* spp. mycelial growth and production as a co-factor of MnPs (Niess and Grabbe, 1990; Lelley and Janssen, 1993; Curvetto et al., 2002; Cohen et al., 2001; Rodriguez-Estrada and Royse, 2007).

At the end of the mushrooms production a residue, which contains organic matter (C and N), macro and micronutrients, named spent mushroom substrate (SMS) is generated (Mohd Hanafi et al., 2018). For each mushroom kilogram produced, about five kilograms of SMS is generated (Lau et al., 2003; Ma et al., 2014; Gao et al., 2021). Taking into account an annual *Lentinula edodes* production of 7 million tons (Royse et al., 2017), it is estimated that 42 million tons of SMS are generated annually. SMS is as agricultural waste normally incorrectly disposed on opened land. Recycling SMS inside the mushrooms productive system might be an effective method in mitigating

this solid waste Rinker (2017), which may represent an economy in the production of the substrate since such waste is readily available and without costs. However, few studies reported how to growth *Pleurotus* spp. by using SMS as a raw matter without compromising the productivity and biological efficiency (Royse, 1992; Siddhant, 2009; Pardo-Giménez et al., 2012; Ashrafi et al., 2014; Wang et al., 2015; Picornell-Buendia et al., 2016; Economou et al., 2017).

Therefore, this work aims to provide subsidies to increase the productivity and biological efficiency of *Pleurotus* spp produced with SMS from *Lentinula edodes*, with the better understanding of the nutritional, biochemical and enzymatic dynamics involved in the *Pleurotus* spp. production process.

2. *Pleurotus spp.* PRODUCTION BY USING SPENT *Lentinula edodes* SUBSTRATE

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Summary

The worldwide mushrooms industry is growing fast with emphasis on the shimeji (*Pleurotus* spp.) and shiitake (*Lentinula edodes*). The most *Pleurotus* genera worldly commercialized species are *Pleurotus ostreatus, Pleurotus sajor-caju*, and *Pleurotus djamor*, which are available in the market in different strains to allows growers to choose the best one adjusted to his growth conditions to reach high productivity and biological efficiency. Edible mushrooms after harvesting generates a bulky underutilized residue, known as spent mushroom substrate (SMS). This residue has been discarded in dumps or even underused as a layer of organic soil, where it could be destinated to produce a new cycle of mushrooms. Very little is known if the best C/N ratio depends on the *Pleurotus* specie and strain, and if the SMS from *Lentinula edodes* might be used as substrate' raw material for *Pleurotus* growth. Thus, the objective of this work was contribute to the production system of *Pleurotus* spp in substrates containing SMS with information that allows achieve high productivity and biological efficiency. In such way *Pleurotus ostreatus* (strains MB and SB), *Pleurotus sajor-caju* and *Pleurotus djamor* cultivated in substrates containing SMS with 30/1, 35/1, 40/1, 45/1 C/N ratios were compared with a substrate without SMS with 50/1 C/N ratio (control). It was concluded that the highest *P. ostreatus* 'MB', *P. ostreatus* 'SB', *Pleurotus sajor-caju*, and *Pleurotus djamor* productivities and biological efficiencies were obtained with 45/1 C/N ratio, and the SMS is a substrate' raw material that can be used to product these *Pleurotus* species and strains.

Index words: *Pleurotus spp.; Lentinula edodes*; Oyster mushroom; C/N ratio; spent mushroom substrate.

2.1 INTRODUCTION

Edible mushrooms production has been expanding worldwide due to their gastronomic, nutritional and medicinal features. *Pleurotus ostreatus,* known as shimeji or oyster and *Lentinula edodes*, known as shiitake, are the mainly mushrooms grown, with respectively 19% and 22% of the worldly market (Royse et al., 2017). Within the same species of *Pleurotus*, there are different genetic materials, named as strains, known to respond differently to environmental factors, substrates, supplementation and mineral composition (Visscher, 1989; Uhart et al. 2008; Uddin et al. 2010; Bellettini et al. 2019; Mleczek et al. 2021).

After mushrooms harvest, a substrate residue is generated, which usually is known as spent mushroom substrate (SMS). Each kilogram of mushroom produced results in an average of 5 to 6 kg of SMS (Gao et al., 2021). In the recent decade, the rapid development of global edible mushroom industry has resulted in annual production of more than 60 million tons SMS (Atallah et al., 2021). This amount of material has been discarded in dumps or even underused as a layer of organic soil because if the producer accumulates SMS, it can be a source of contamination. Researchers have put in effort to device strategies for efficient valorization of SMS, including as alternative for fertilizers and soil amendments, food for animals and in vermicomposting (Perez-Chavez et al., 2019). Since SMS contains organic matter (C and N), macro and micronutrients, it might be an option to produce a new cycle of *Pleurotus* spp. mushrooms. Several authors report the feasibility of using SMS from different mushrooms to produce *Pleurotus* (Royse, 1992; Siddhant, 2009; Pardo-Giménez et al., 2012; Ashrafi et al., 2014; Wang et al., 2015; Picornell-Buendia et al., 2016; Economou et al., 2017). However, the main challenges of this reuse approach arise from maintaining the quantity and quality of the mushrooms produced.

Since substrates' C/N ratio are among the main conditions that influence the *Pleurotus* spp productivity and biological efficiency and little is known if the SMS from *Lentinula edodes* might be used as substrate' raw material for *Pleurotus* growth, this work aimed to evaluate *Pleurotus ostreatus* (strains MB and SB), *Pleurotus sajor-caju* and *Pleurotus djamor* productivity and biological efficiency cultivated in substrates containing *Lentinula edodes*' SMS under several C/N ratios.

2.2 MATERIAL AND METHODS

SMS was supplied from a local *Lentinula edodes* (Berk.) (shiitake) grower. Substrates were prepared with the raw material described on the Table 1, as recommended by Urben, (2017). Three samples for each substrate were collected analyzed following the methodology adapted (Malavolta et al., 1989). The analysis of total carbon and nitrogen of the substrates was performed using the Walkey-Black method to organic carbon and Semi-micro-Kjeldahl method to nitrogen. For the K, Ca, Mg, P and Fe the contents were obtained through nitric-perchloric digestion and analyzed in atomic absorption spectrophotometry. Data were expressed in g of mineral kg^{-1} of substrate for macronutrients evaluated and in mg of mineral kg^{-1} of substrate for micronutrients. Neutral detergent fiber (NDF), acid detergent fiber (FDA) and lignin (LIG) were used to determine the hemicellulose and cellulose content of the substrates (Van Soest et al., 1991). Hemicellulose was calculated using FDN and FDA and cellulose was determined using FDA and LIG (hemicellulose = FDN-FDA; cellulose = FDA-LIG) (Zadrazil and Brunnert, 1981).

Table 1. Characterization of the spent shiitake substrate (SMS) from *Lentinula edodes* and the other raw materials used to prepare substrates for *Pleurotus ostreatus var florida* 'MB', *Pleurotus ostreatus* 'SB', *Pleurotus sajor-caju* and *Pleurotus djamor* grown.

Raw material	C	N	Lig	Cel	Hem	C/N	Lig/N
	% (dry weight)						
Spent shiitake substrate (SMS)	43.59	2.03	8.09	19.98	15.87	21.47	3.98
Cynodon dactylon (L.) Pers	42.91	0.68	4.66	32.14	38.88	63.10	6.85
Rice Bran	49.54	2.01	4.55	9.17	21.50	24.64	2.26

Carbon (C), Nitrogen (N), Lignin (Lig), Cellulose (Cel), Hemicellulose (Hem), Spent Substrate shiitake (SMS).

Substrates composition is on the Table 2. The control treatment, just with *Cynodon dactylon* and rice bran (without SMS), with 50/1 C/N ratio, attended the Urben, (2017) recommendation. One hundred sixty (160) polypropylene bags (2000g)

were equally stuffed and separated in 32 bags for each one of the five substrates (Table 2). Bags were inoculated with four mushrooms materials: *Pleurotus ostreatus var. florida* “MB”, *Pleurotus ostreatus* “SB”, *Pleurotus sajor-caju* and *Pleurotus djamor*, provided by the mycelium company Funghi & Flora®, formed the first factor to be evaluated in the present study. The grass was cut to a size of 2cm ±1, mixed together with rice bran, $CaCO_3$, SMS and hydrated to the recommended humidity of 60%. As substrate treatment, severe pasteurization of 95ºC for 14h was used. After cooling to room temperature, inoculation was carried out in an aseptic environment, with "spawns" (mycelium inoculated in wheat grains), corresponding to 5% of the fresh weight of the substrate. The culture bags containing each species were incubated at 25ºC, in a controlled environment, in the darkness. After the colonization period, 2 cm holes, equidistant, were made on all sides of each bag and taken for fruiting induction, in a controlled environment, at 22ºC ± 1 for *P. ostreatus var florida* ‘MB’ and *P. ostreatus* ‘SB’ and at 24ºC ± 1 for *P. sajor-caju* and *P. djamor*, with relative humidity of 85% ± 2, lighting of 700 lux (12 h / day-1, fluorescent lamps), keeping the control of the CO_2 level low (<1200 ppm).

For the evaluation of mycelial coverage (%) and average mycelial growth (cm^2/day), external bags’ pictures were taken within seven-day intervals (7, 14, 21 and 28 days) inside a paper frame (10 cm x 10 cm) demarcated on the four faces of the cultivation bag. These images mycelial coverage areas were calculated with help of the ImageJ software (Abramoff et al., 2004). In addition, the colonization period (days) was counted from the inoculation (Day 0) until complete colonization of the culture bag by the mycelium. After fructification induction, productivity (weight of fresh mushrooms x 100. $weight^{-1}$ of fresh substrate) and biological efficiency (weight of fresh mushrooms x 100 $weight^{-1}$of dry substrate) were calculated (Royse et al., 2004).

A completely randomized design, with four mushrooms materials (*Pleurotus ostreatus var. florida* “MB”, *Pleurotus ostreatus* “SB”, *Pleurotus sajor-caju* and *Pleurotus djamor*), five substrates’ ratios (50/1, 45/1, 40/1, 35/1, 30/1) and eight replicates, totaling 160 bags. Data were analyzed concerning with homogeneity, ANOVA, regression analysis for N and Tukey (HSD) with the Software for Data Variance Analysis - Sisvar (Ferreira, 2011).

Table 2. Raw material proportions of substrates' C/N ratios used to growth *Pleurotus ostreatus var florida* 'MB'*, Pleurotus ostreatus* 'SB'*, Pleurotus sajor-caju* and *Pleurotus djamor* before their grown.

Substrates' C/N Ratios	*C. dactylon*	Rice bran	SMS	$CaCO_3$	pH* (w)	N	Lig	Cel	C	Hem	Lig/ N Ratio	K	Mg	Ca	P	Fe
	% (dry weight)					% (dry weight)						g.kg^{-1}				mg.kg^{-1}
50/1 (Control)	82.00	15	0	3	6.47	0.86	4.50	27.73	42.62	35.11	5.23	11.98	2.08	27.76	3.42	0.54
45/1	77.00	0	20	3	6.19	0.94	5.25	26.90	42.19	31.88	5.58	10.96	2.69	27.05	3.18	1.80
40/1	68.00	9.00	20	3	6.30	1.07	5.34	27.64	43.65	32.71	4.99	10.81	2.90	25.69	3.95	1.96
35/1	54.00	23.00	20	3	6.27	1.26	5.32	24.42	44.58	25.34	4.22	10.18	3.16	22.59	5.07	2.19
30/1	37.00	40.00	20	3	6.24	1.48	5.30	20.52	45.70	18.73	3.58	9.41	3.47	18.82	6.44	2.47

* Carbon (C), Nitrogen (N), Lignin (Lig), Cellulose (Cel), Hemicellulose (Hem), Spent Substrate shiitake (SMS), pH (w) in water, potassium (K), Magnesium (Mg), Calcium (Ca), Phosphorus (P), Iron (Fe).

2.3 RESULTS AND DISCUSSION

P. ostreatus var florida 'MB'*, P. ostreatus* 'SB'*, P. sajor-caju* and *P. djamor* mycelium coverage (%) was influenced by the substrates' C/N ratios (Table 3). This can be explained because mycelium growth is related to the fungus' ability to secrete oxidative (lignin peroxidase, laccase, manganese peroxidase) and hydrolytic (cellulase, xylanase) enzymes that will provide energy for the mycelium development, which are mainly mediated by the mushrooms' species, and substrates' composition (Stajić et al., 2006; Elisashvili et al., 2008; Singh et al., 2008; Elisashvili and Kachlishvili, 2009; Knop et al. 2015). *P. ostreatus var florida* 'SB', *P. ostreatus* 'MB' and *P. djamor* mycelium coverage was higher in substrates containing C/N ratio was higher than 40/1, and *P. sajor-caju*, it was higher when C/N ratio than higher than 35/1 (Table 3). Such results show the differences of each species and strains for the concentration of N level on the substrates (Donini et al., 2009).

Despite the important role played by N, in the highest doses (Table 2), due to the addition of SMS and rice bran, both sources of organic nitrogen (lower C/N ratios), delayed the mycelial coverage for all species and strains (Table 3). Due to this increase in N, the average mycelium growth of the species of *Pleurotus* was stimulated up to 1.26% of N (substrates' C/N ratio of 35/1), with emphasis on strain 'SB' being higher than the other species (Table 3). Concentrations equal to or higher than 1.5% of N can inhibit mycelium growth, affecting the degradation of lignin or causing plasmolysis of hyphae due to high concentrations of amino acids (Tshinyangu and Hennebert, 1996; Silva et al., 2007; Urben, 2017; Bellettini et al., 2019). In the present study, the negative effect of high concentrations of N (above 1.26% - substrates' C/N ratio 35/1) on the average mycelium growth of *Pleurotus* spp is observed, corroborating with D'Agostini et al., 2011 in which demonstrated that substrates' C/N ratios below 30/1 reduced the mycelium growth of *Pleurotus ostreatus*. According to Economou et al., 2017, the mycelium growth in a petri dish of P. *ostreatus* and P. *pulmonarius* was higher in the culture medium containing SMS and substrates' C/N ratio of 30/1, compared to the culture medium containing substrates' C/N ratio of 20/1 and 10/1.

Table 3. Average mycelium growth (cm²/day), colonization period (days), productivity and biological efficiency of *Pleurotus ostreatus var florida* 'MB', *Pleurotus ostreatus* 'SB', *Pleurotus sajor-caju* and *Pleurotus djamor* produced on five substrates with different substrates' C/N ratios containing spent mushroom substrate (SMS).

Characteristics	Substrates' C/N ratio	*P. ostreatus var florida* "MB"	*P. ostreatus* "SB"	*Pleurotus sajor-caju*	*Pleurotus djamor*
Average mycelium growth (cm²/day)	50/1	4.67 a A	5.08 a AB	4.41 a B	4.72 a AB
	45/1	4.35 b A	5.28 a AB	4.23 b B	4.81 ab AB
	40/1	4.98 bc A	5.88 a A	4.51 c B	5.51 ab A
	35/1	4.17 b AB	4.59 ab BC	5.45 a A	4.47 b B
	30/1	3.36 b B	4.13 ab C	4.41 a B	3.91 ab B
Colonization period (days)	50/1	21.50 a A	20.12 a AB	23.11 a AB	21.50 a A
	45/1	23.25 b A	19.25 a AB	23.63 b B	21.25 ab A
	40/1	20.25 ab A	17.25 a A	23.00 b AB	18.87 a A
	35/1	24.12 b A	21.87 ab BC	19.26 a A	22.75 ab AB
	30/1	29.89 b B	24.5 a C	23.11 a AB	25.75 a B
Productivity (%)	50/1	20.23 a AB	19.62 ab B	16.56 b AB	18.00 ab A
	45/1	22.11 ab A	23.65 a A	18.11 c AB	19.68 bc A
	40/1	17.08 b B	22.49 a AB	15.95 b B	17.01 b A
	35/1	19.49 ab AB	21.67 a AB	19.51 ab A	17.35 b A
	30/1	8.88 c C	14.78 a C	10.49 bc C	13.53 ab B
Biological efficiency (%)	50/1	86.41 a AB	81.96 a A	77.77 a A	77.78 a A
	45/1	89.49 a A	90.14 a A	80.56 a A	80.54 a A
	40/1	73.91 b BC	86.43 a A	75.65 ab A	74.24 b A
	35/1	66.02 b C	68.19 b B	86.59 a A	59.42 b B
	30/1	27.51 b D	40.65 a C	24.76 b B	31.49 ab C

Means followed by same lowercase in the same row and the same uppercase letter in the same column do not differ statistically by the Tukey test at 5% probability.

Nitrogen (N), phosphorus (P), potassium (K), magnesium (Mg), iron (Fe), zinc (Zn), manganese (Mn), selenium (Se), molybdenum (Mo) and copper (Cu) are important nutrients to grows mushrooms, possibly playing several roles in growth and development (Chang and Miles, 2004). P, Mg and Fe in the substrate increased with the addition of SMS (Table 2), comparing with substrate without SMS, indicating a possible influence of these elements for the growth of *Pleurotus* spp. According to Oyetayo and Ariyo, 2013, who measured proximal and mineral analyzes of *P.*

ostreatus grown in different agro-industrial residues, they found that phosphorus had the highest value among all analyzed minerals.

Among the main nutrients already mentioned, the balance between C and N in the substrate is important, since cellulose, hemicellulose and lignin are the main carbonaceous components used by *Pleurotus* spp. as energy sources for growth and development (Bonatti et al., 2004). In the vegetative period of growth, hemicellulose and lignin are degraded in greater intensity (Li et al., 2001). In this period, the average mycelium growth of the *Pleurotus* was higher in the substrate containing the C/N ratio of 40/1, consequently, requiring a shorter period of colonization. In this case, the initial concentration of hemicellulose decreased by 6.83% and, on the other hand, the initial concentration of lignin increased by 15.70% due to the application of SMS. Although lignin is a more complex polymer to be metabolized by mycelia, Platt et al., 1984 observed that the mycelium *P. ostreatus* had more capacity to degrade lignin and its degradation played an important role in its development. Possibly the results of the present work corroborate with such authors, where the addition of SMS provided a higher concentration of lignin in the substrate, comparing with substrate without SMS (Table 2).

Productivity and biological efficiency of the species of *Pleurotus* were affected by the different substrates' C/N ratios (Table 3). According Donini et al., 2009 that reported a productivity of 18.69% and biological efficiency of 74.79% for one of the three strains of *P. ostreatus* grown in elephant grass (*Pennisetum purpureum* Schum.) + 20% rice bran (substrates' C/N ratio of 47/1), similar results are found in the present work with substrates' C/N ratio of 45/1 (whose substrate contained SMS, without addition of rice bran). These authors showed the differences in productivity between strains in relation to the different substrates used. On the other hand, Siqueira et al., 2012 achieved productivity of 19.1% and lower biological efficiency (54.5%) of *P. pulmonarius* grown in *Cynodon dactylon* (L.) Pers + soy straw and corn straw. Possibly, this difference is related to differences between species and in the concentrations of N (0.71%) used by such authors. Likewise, Mohamed et al. (2012) reported low biological efficiency for *P. ostreatus* (15.8%) and P. *ostreatus var. columbinus* (13.7%) produced in *Cynodon dactylon* (L.) Pers, without the addition of any organic nitrogen source.

Nitrogen doses affected productivity and biological efficiency of all species and strains evaluated (Figure 1). In the same way as mycelial growth, the productivity and biological efficiency of all species were also negatively affected above the 1.2% of N. Similarly, Kurt and Buyukalaca (2010) showed that *P. ostreatus* productivity and N content of substrates were positively correlated, while they observed higher laccase and carboxymethylcellulase activities in media supplemented with wheat bran. In addition, they suggested that high N values (over a specific threshold, in their case: N > 1.4%) could be associated with a decrease in productivity. Our results corroborate the aforementioned authors, demonstrating that above 1.2% N, productivity and biological efficiency were compromised.

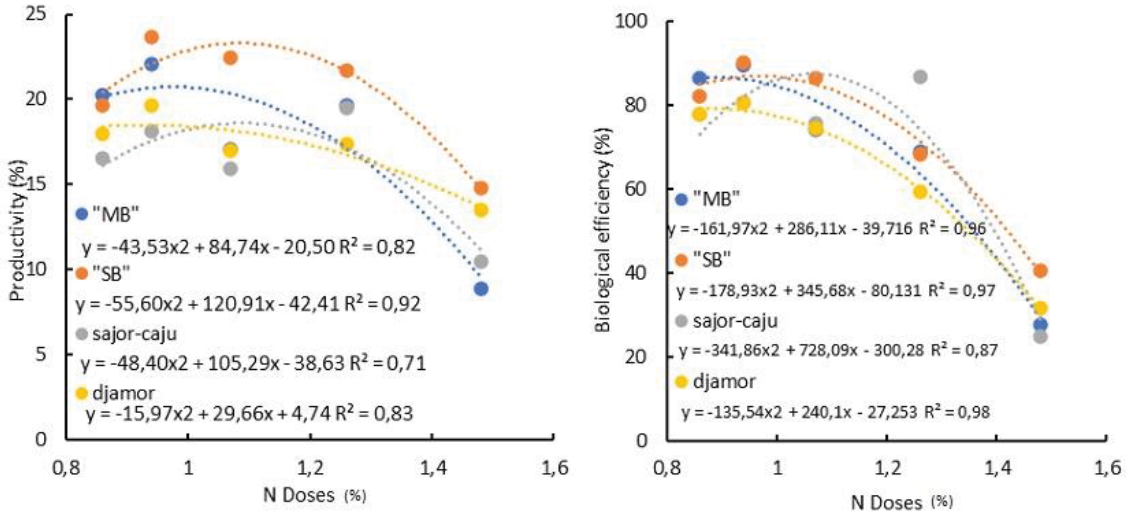

Figure 1. Productivity (%) and biological efficiency (%) of *Pleurotus* species on substrates supplemented with spent mushroom substrate of *Lentinula edodes* in different N concentrations.

Since a good substrate must provide rapid colonization by *Pleurotus*, it will serve as a nutritional base for the reproductive or fruiting period. In fruiting period, the nutritional demand occurs in greater intensity for cellulose and lignin (Li et al., 2001). Substrate containing the 45/1 substrates' C/N ratio showed a 1.0% reduction in cellulose concentration, while the lignin concentration increased by 14.28%, when comparing with the control substrate (without SMS) (Table 2). Some authors positively correlate the increase in productivity of *Pleurotus* spp and the rates of lignin degradation (Obodai et al., 2003; Zhai and Han, 2018). First, the promotion of productivity and biological efficiency can be attributed to the fact that hemicellulose

and lignin in SMS are partially degraded, facilitating the use of these components by the mycelium. Second, SMS contains organic nitrogen, which is easily absorbed by mycelia, reflecting a high growth rate.

2.4 CONCLUSIONS

It is possible produce *Pleurotus ostreatus var florida* ‘MB’, *Pleurotus ostreatus* ‘SB’, *Pleurotus sajor-caju* and *Pleurotus djamor* with SMS from *Lentinula edodes* and the best substrates’ C/N ratio is 45/1.

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3. MANGANESE APPLICATION IN OYSTER MUSHROOM PRODUCTION

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Abstract

*Pleurotus ostreatu*s is one of the main edible mushrooms produced worldwide. Substrate composition (lignocellulosic materials, macro and micronutrients) plays an important role in productivity and biological efficiency, mediated through hydrolytic and oxidative enzymes, including manganese peroxidase (MnP). The aim of this study was to improve *Pleurotus ostreatus* 'SB' productivity and biological efficiency by apply manganese, a co-factor of MnP enzymatic activity, in the substrate. Manganese sulfate was added to the substrate generated 0, 2, 4, 20 and 80 mg of manganese. Kg^{-1}. MnP enzymatic activity was carried out in 0, 5, 10, 15, 20 and 30 days after inoculation. Results obtained showed that manganese application linearly increased the MnP enzymatic activity, but compromised productivity and biological efficiency. This demonstrates the importance of performing substrate's chemical analysis before recommending any application of exogenous Mn to produce *Pleurotus ostreatus.*

Index terms: Manganese peroxidase (MnP); oxidative enzymes; *Pleurotus ostreatus*; enzymatic activity; Shimeji.

3.1 INTRODUCTION

Worldwide edible mushrooms production and consumption has been expanding over the years, due to their nutritional, gastronomic and medicinal potential. The main mushrooms species are *Lentinula edodes* (Berk.) (shiitake) (22%) and *Pleurotus* spp. (Shimeji) (19%) (Royse et al., 2017).

Mushrooms are grown on substrates mainly composed by lignocellulosic residues, their energetic source (Sánchez, 2009). Substrates composition is determinant to reach high mushrooms productivity and biological efficiency (Ragunathan and Swaminathan, 2003; Chang and Miles, 2004; Pedra and Marino, 2006; Urben, 2017; Bellettini et al., 2019). This is mainly because the raw materials used in the substrate formulation are the main source of nutritional variation. Wheat straw and sawdust have been the major lignocellulosic residues of *Pleurotus* substrate's formulations for a long time in the world (Philippoussis et al., 2009). Other cereal straws (rice, oat, barley) are used as raw material in other countries. In Brazil, sugarcane bagasse and grasses (Brachiaria straw and others) are used, mainly due to the local availability of these agro-waste. A potential and very little explored raw material to produce *Pleurotus* spp. substrate is the spent mushroom substrate (SMS). It is a residue generated at the end of the mushrooms production, which contains organic matter (C and N), macro and micronutrients. SMS is normally incorrectly disposed on open land. Recycling SMS inside the mushrooms productive system might be an effective method in mitigating this solid waste (Rinker, 2017).

Pleurotus spp. mushrooms are able to degrade the lignocellulosic compounds with their oxidative (lignin peroxidase (LiP), laccase (Lac), manganese peroxidase (MnP)), and hydrolytic (cellulase (Cel), xylanase (Xyl)) enzymes. These enzymes activity depend on substrate composition, which supply enzymatic cofactors (Stajić et al., 2006; Elisashvili et al., 2008;Elisashvili and Kachlishvili, 2009). One of these cofactors is manganese (Mn) for MnP. Results still are conflicting, and not clear, if manganese level on the substrate might increase *Pleurotus* spp. productivity and biological efficiency (Niess and Grabbe, 1990; Lelley and Janssen, 1993; Curvetto et al., 2002; Cohen et al., 2001; Rodriguez-Estrada and Royse, 2007). The aim of this study was to evaluate the enzymatic activity of MnP by *Pleurotus ostreatus* 'SB' and improve the productivity and biological efficiency through the exogenous application of manganese in the substrate.

3.2 MATERIAL AND METHODS

Substrate was prepared as recommended by Urben, 2017, contained 770g of hay (*Cynodon dactylon* (L.) Pers), 200g of SMS from *Lentinula edodes*, and 30g of gypsum ($CaCO_3$). Manganese sulfate was added to the substrates in 1000 mL of hydration water, up to the recommended humidity of 65%, in the doses: 0; 6,15; 12,31; 61,57; 246,30 mg. These concentrations generated 0, 2, 4, 20 and 80 mg of manganese per kilogram of substrate. Control treatment, instead receiving manganese, was moisture only with water at the same volume the other treatments.

Bags containing the substrate were pasteurized for 14 hours at approximately 95ºC. After cooling the bags at room temperature, *Pleurotus ostreatu*s 'SB' (Funghi and Flora®) spawn was inoculated, in an aseptic environment, at the proportion of 5% of the fresh substrate The culture bags were then incubated at 25ºC in a controlled environment, in the dark, for approximately 30 days. After the colonization period, eight equidistant 3 cm holes were pierced in each bag, and the bags were taken to a controlled environment at 20ºC±1 with a relative humidity of 85% ± 2 for fruiting induction.

All raw materials were evaluated for Neutral detergent fiber (NDF), acid detergent fiber (FDA) and lignin (LIG) to determine the hemicellulose and cellulose content of the substrates (Van Soest et al., 1991). Hemicellulose was calculated using FDN and FDA and cellulose was determined using FDA and LIG (hemicellulose = FDN-FDA; cellulose = FDA-LIG) (Zadrazil and Brunnert, 1981). Three samples for each substrate were collected analyzed following the methodology adapted (Malavolta et al., 1989). The analysis of total carbon and nitrogen of the substrates was performed using the Walkey-Black method to organic carbon and Semi-micro-Kjeldahl method to nitrogen. For the K, Ca, Mg, Zn, Mn, Cu and Fe the contents were obtained through nitric-perchloric digestion and analyzed in atomic absorption spectrophotometry. Data were expressed in g of mineral kg^{-1} of substrate for macronutrients evaluated and in mg of mineral kg^{-1} of substrate for micronutrients.

Table 1. Macronutrient, micronutrient and chemical analysis of raw materials and complete substrate (mixture) used in the cultivation of *Pleurotus ostreatus* 'SB'.

Raw material	Ca	P	K	Mg	Mn	Zn	Cu	Fe	pH	N	Lig	Cel	C	Hem
	g.Kg^{-1}				mg.Kg^{-1}					% (dry mass)				
Formulated substrate	32.9	7.5	13.6	4.3	158.2	27.9	8.0	699.5	6.19	0.94	5.25	26.90	42.19	31.88
Spent substrate of Shiitake (SMS)	10.1	9.7	6.4	4.2	153.8	21.8	5.2	948.4	6.30	2.03	8.09	19.98	43.59	15.87
C. dactylon	7.5	2.7	20.5	2.7	123.6	44.7	9.7	682.4	6.24	0.68	4.66	32.14	42.91	38.88

* Carbon (C), nitrogen (N), lignin (Lig), cellulose (Cel), hemicellulose (Hem), spent substrate from the production of shiitake (*Lentinula edodes*) (SMS), pH (w) in water, potassium (K), magnesium (Mg), calcium (Ca), phosphorus (P), iron (Fe).

External mycelial coverage (%) and mycelial average growth (cm²/day) were measured through bags' pictures taken each five-day intervals (5, 10, 15, 20 and 25 days) with a paper frame (10 cm x 10 cm) demarcated on the four faces of the cultivation bag. These images mycelial coverage areas were calculated with the help of the ImageJ software (Abràmoff et al., 2004). In addition, the *Pleurotus ostreatu*s 'SB' production period, number of clusters, number of pileus, length and width (cm) of pileus were evaluated. Productivity (weight of fresh mushrooms x 100/ weight of fresh substrate) and the biological efficiency (mass of fresh mushrooms x 100/ dry substrate mass) were calculated according to Royse et al, 2004.

The MnP enzyme activity analysis was carried out according to methodology adapted from Kuwahara et al., (1984). Samples were collected at 0, 5, 10, 15, 20 and 30 days after spawn inoculation. For each date, three homogeneous samples were collected from a culture bag of each substrate. Crude protein extract (CPE) was performed with 50 mL of phosphate buffer (0.2M pH 7.5) and 25 g of substrate/mycelium. This mixture was homogenized in a porcelain mortar for 5 min and centrifuged at 250 rpm for 60 min. The supernatant was filtered through filter paper (Whatman nº 1). The CPE was used for the analysis of the enzymatic activity of manganese peroxidase (MnP). Manganese peroxidase (MnP) activity was determined using 500 µL of CPE, 100 µL of phenol red (1 g L^{-1}, ε = 3162 M^{-1} cm^{-1}), 100 µL of sodium lactate (250 mmol L^{-1}, pH 4.5), 200 µL bovine albumin (1% w/v), 50 µL hydrogen peroxide and 50 µL manganese sulfate. This reaction was incubated in a water bath at 37 °C for 10 min. Then, 40 µL of sodium hydroxide (2 mmol L^{-1}) were added to stop the reaction and absorbance was determined at 610 nm.

Experiment was conducted in completely randomized design, with five treatments and eight replications. The homogeneity of variances was tested (Bartlett's test), and posteriorly the analysis of variance and regression analysis were performed using the statistical software 'Data Variance Analysis – Sisvar' (Ferreira, 2008).

3.3 RESULTS AND DISCUSSION

Lignin is one of the lignocellulosic constituents of the raw material used to prepare the *Pleurotus ostreatu*s substrate (Table 1). The degradation of lignocellulosic compounds (lignin, hemicellulose and cellulose) occurs through the enzymatic complex produced by fungi, named white rot fungi, including *Pleurotus* (Sanchez, 2009). Micronutrients, such as manganese (Mn), plays a role in the activation of many enzymes, including those of the TCA (tricarboxylic acid) cycle and nucleic acid synthesis (Walker and White 2005). However, one of the main activities of Mn is the role of co-factor of manganese peroxidase (MnP) (Gold and Alic 1993; Menezes and Barreto, 2015). MnP activity is essential and responsible for breaking the lignin molecule, fractionating and making the C chains available into smaller molecules so that the mycelium can absorb and complete its mycelial development and growth (Menezes et al., 2015). Manganese application provided higher enzymatic activity (Figure 1), in agreement with some previous studies (Racz and Tasnadi, 1998; Cohen et al., 2001; Alemawor et al., 2009). However, its application, compared to the substrate that was not supplemented with manganese, did not favor the mycelial coverage, nor the average mycelial growth, and neither reduced the colonization period ($P<0.05$).

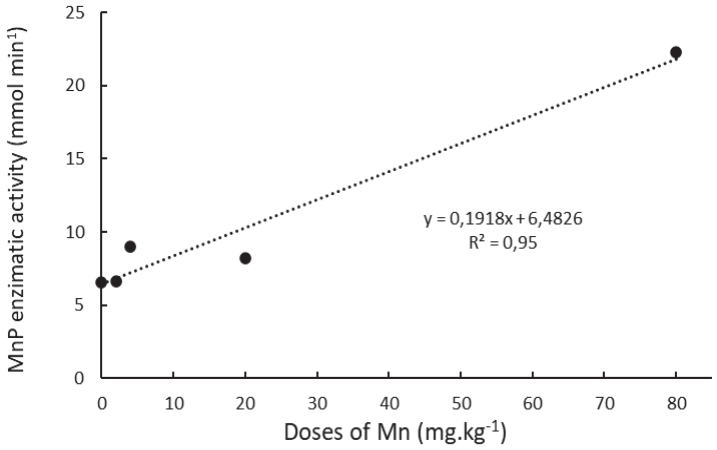

Figure 1. Mean enzymatic activity of manganese peroxidase (MnP) during the first 30 days of cultivation of *Pleurotus ostreatus* 'SB' on substrates containing SMS supplemented with different doses of manganese (Mn).

It was expected that the application of Mn would increase the degradation of lignin and consequently, the availability of short chain polysaccharides, influencing the mycelial growth. However, it is believed that the short period of mycelium growth was not enough for these differences to be observable since the nature of the substrate, the concentration and bio-availability of Mn and fermentation period are potential factors that affect the lignin degradation by *Pleurotus* (Alemawor, 2009).

Manganese supplementation did not affect the number of pileus (13.1±0.58), the number of clusters (5.2±0.34), neither the average length (3.2±0.12) and width (3.1±0.11) of *Pleurotus ostreatus* 'SB' pileus ($P<0.05$). In this way, the application of Mn does not directly affect the morphology of the fruiting bodies, in agreement with previous studies that, in addition to the genetics of the species, the composition of substrates affects the physicochemical characteristics of mushrooms (Ragunathan and Swaminathan, 203; Chang and Miles, 2004; Oyetayo and Ariyo, 2013).

The manganese supplementation caused a negative effect over the productivity and biological efficiency (Figure 2). Possibly the high doses of Mn interfered in the degradation of lignin, an important source of carbon for the development of basidiocarps, agreeing with Bermek and Eriksson, 2009 cultivating *Phlebia radiata*, another white rot fungus, showed that high concentrations of Mn provided less efficient degradation of synthetic lignin. Since lignin is degraded in vegetative and reproductive phases, the efficiency of *Pleurotus* spp. in degrading lignocellulose depends in MnP acting on the cell wall of the raw materials used in the growth substrate (Li et al., 2001; Rytioja et al., 2014).

In our work, manganese level in the substrate, previously to the exogenous manganese application, was 158.2 $mg.Kg^{-1}$ (Table 1). That is, above the minimum level of manganese in the substrates (150 to 620 $mg.Kg^{-1}$) for *Pleurotus* spp. growth reported by other authors (Chiu et al., 1998; Lelley and Janssen, 1993; Curvetto et al., 2002; Baldrian et al., 2005; Rodriguez-Estrada and Royse, 2007; Elhami et al., 2008). Such big manganese interval, among those authors, certainly is due to the distinct raw materials composition used to prepare the

substrates (cotton residue, sugarcane bagasse, sawdust, wheat straw and sunflower seed husk etc.). This range of Mn found in different raw materials is probably the reason for the conflicting results. This is because few authors consider the initial Mn concentration of the substrate before evaluating the application of doses. For instance, regarding studies that applied exogenous doses of manganese are between 50 and 400 $mg.kg^{-1}$ and concentrations above 200 $mg.kg^{-1}$ inhibit the growth and production of *P. ostreatus* (Lelley and Janssen, 1993; Curvetto et al., 2002; Rodriguez-Estrada and Royse, 2007).

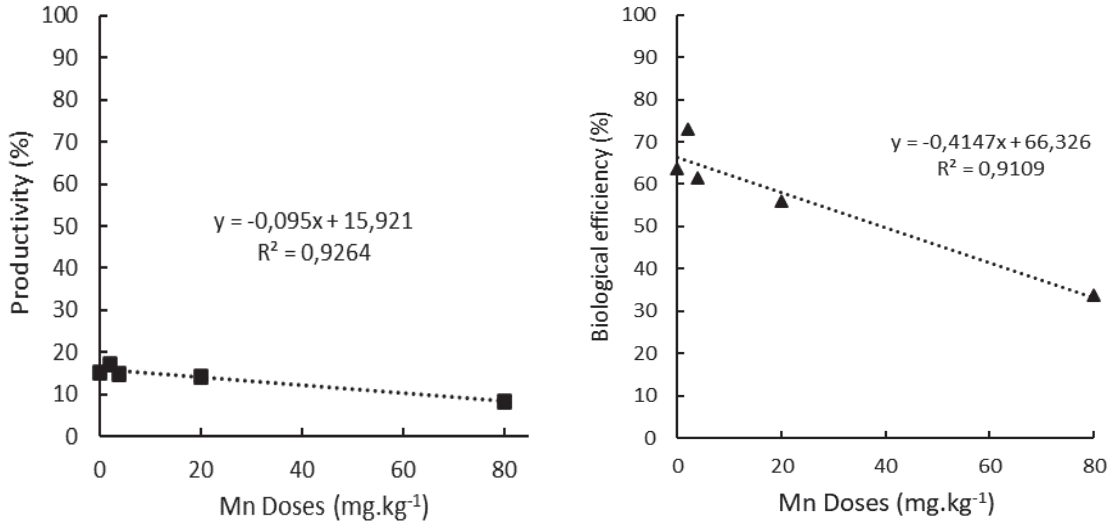

Figure 2. Productivity (%) and biological efficiency (%) of the cultivation of *Pleurotus ostreatus* 'SB' on substrates containing SMS supplemented with different doses of manganese (Mn).

Nitrogen affects *Pleurotus* spp. enzymatic activity, with low levels being able to stimulate the production of ligninolytic enzymes, while a high content may have an inhibitory effect on the enzymatic activity (Singh et al., 2008; Bellettini et al., 2019). There are species that adapt to substrates with nitrogen content in the range of 0.03% to 1.5% (Machado et al., 2016). In the substrate used in this research, the amount of nitrogen was 0.94% (Table 1). Therefore, the high concentration of N in the substrate was probably not a limiting factor for productivity and biological efficiency. However, it may have favored the continuity of mycelial growth. During the growth of *Pleurotus* spp. mycelium, in addition to the consumption of carbon content, there is an increase in the concentration of nitrogen in substrate, through accumulation of proteins and enzymes produced

by the fungus (Alananbeh et al., 2014). In the present study, the evaluation of MnP was carried out only during the vegetative period, that is, during mycelial growth, where the highest production of this enzyme normally occurs and decreases during the formation of primordia and fruiting bodies, corroborated with Savoie et al., 2007. Since MnP activity was not evaluated during the production period, it is believed that higher Mn doses induced the continuity of mycelial growth to the detriment of the production of fruiting bodies.

The Mn concentration used in our substrates was among the range reported in the literature and below the 200 $mg.kg^{-1}$ content considered a limit. Such deleterious effect, caused by Mn excess, can be explained by the enzymatic imbalance. According to Velazquez-Cedeno et al. (2002), high endoglucanase activity was important in the fruiting phase of *Pleurotus* spp., implying a mobilization of nutrients to produce new elements necessary for the formation of fruiting bodies, with preferential degradation of certain polysaccharides with accumulation of proteins in the substrate. Possibly, the stimulus to produce large amounts of MnP continued during the reproductive phase, prejudice the activity of other enzymes essential to fruiting (cellulase, endoglucanase and xylanase).

Results showed that even if manganese did not compromise micellization, high manganese doses can seriously compromise *Pleurotus* spp. fruiting bodies production depending on the previous manganese level on the substrate. In such way, before to recommend to apply manganese, it is necessary to know how much manganese there is in the substrate. Taking into account the substrates' heterogeneity used for *Pleurotus* spp. growers and that few of them carry out substrate's chemical analysis before to cultivate the mushrooms it is a high risk to recommend to apply manganese.

3.4 CONCLUSIONS

The manganese application linearly increased the MnP enzymatic activity and did not increase the productivity and biological efficiency of *Pleurotus ostreatus*.

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4. NUTRIENT UPTAKE IN SUPPLEMENTED SUBSTRATE BY OYSTER MUSHROOM

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ABSTRACT

Spent mushroom substrate (SMS) can be a good alternative for the supplementation of the Oyster mushroom substrate, replacing the cereal bran normally used. Therefore, the objective was to evaluate the production of *Pleurotus ostreatus* supplemented with *Lentinula edodes'* SMS, through the nutritional analysis of the substrate. Wheat straw was used as substrate and supplemented with rice bran (RB) or SMS in 0%, 7%,15% and 30%. Ca, K, Mg, Mn, Zn, Cu and Fe contents of the cultivation substrates (before and after harvest) were determined. Mycelial growth (cm²/day), mycelial time colonization (days), number of clusters, number of pileus, average clusters weight (g), pileus lenght (cm) and width (cm), productivity (1st, 2nd and 3rd flush) (%), biological efficiency (%) of mushrooms were evaluated. Results indicated mycelial growth was higher when the substrate was supplemented regardless of the source. The same tendency followed for second flush (%), third flush (%), average clusters weight (g), productivity (%) and biological efficiency (%). The proportions of 15% of SMS achieved the highest biological efficiency. All the nutrients analyzed had a decrease in their concentrations. The only nutrients that showed differences in absorption according to each substrate used were Ca, K and Mn. This difference was also noticed according to the type of supplement, in which substrates supplemented with SMS had greater absorption of Ca, while substrates supplemented with RB absorbed more K. It is noteworthy that the mineral contents in substrates directly affect the production of *P. ostreatu*s, which implies that the SMS are a nutritional source in terms of mineral contents and thus can be recycled and used to replace rice bran and produce Oyster mushroom.

Index terms: Shimeji mushroom; macronutrients; micronutrients; spent mushroom substrate; nutrient absorption.

4.1. INTRODUCTION

Edible mushrooms are saprophytic fungi that have great culinary and medicinal importance, in addition to being considered a nutritional food. At a global scale, consumption of mushrooms has increased from 1 to 4.7 kg of cultivated edible mushrooms per capita in the period 1997 to 2013 (Royse et al., 2017). The genus *Pleurotus*, popularly known as Oyster or Shimeji mushroom, are the second most produced in the world constitutes about 19% of the world's output (Royse et al., 2017). Wheat straw and sawdust have been the major lignocellulosic residues of *Pleurotus* substrate's formulations for a long time in the world (Philippoussis et al., 2009). Other cereal straws (rice, oat, barley), sugarcane bagasse and grasses are used as raw material in other countries, when these agro-wastes are locally available.

The ability to degrade lignocellulosic materials, through its enzymatic apparatus, allows the extraction of nutrients from the medium to obtain essential elements for its growth, such as carbon (C), nitrogen (N), minerals and vitamins. Phosphorus (P), calcium (Ca), magnesium (Mg) and potassium (K) are also taken into consideration as mushroom macronutrients, and trace elements such as iron (Fe), copper (Cu), zinc (Zn), manganese (Mn) and molybdenum (Mo) appear to be crucial for various metabolic functions (Chan and Miles, 2004).

The composition of substrates affects the physicochemical characteristics of mushrooms and are determinant in their productivity and biological efficiency (Ragunathan and Swaminathan, 203; Chang and Miles, 2004; Pedra and Marino, 2006; Oyetayo and Ariyo, 2013; Urben, 2017; Bellettini et al. 2019). To obtain good yields of *Pleurotus* spp., nutritional adjustment and availability must be considered generally supplementing the substrate with cereal bran (wheat, soybean, corn, rice, etc.). A possible alternative to use as a supplement is by applying spent mushroom substrate (SMS), which can be defined as the biomass produced by mushroom production that remains after harvesting a mushroom crop (Mohd Hanafi et al., 2018).

For one kilogram of mushroom produced, about five kilograms of SMS is generated (Lau et al., 2003; Ma et al., 2014). With an annual production of 7 million tons of shiitake, it is estimated that 35 to 42 million tons of SMS are generated annually from this mushroom (Royse et al., 2017). SMS are considered as agricultural waste and are normally disposed on open land, burned or disposed incorrectly. Reusing or recycling could serve as an effective method in mitigating this solid waste, providing new economic opportunities and positive environmental consequences. Since SMS is an available material, containing organic matter (C and N), macro and micronutrients, a viable option for destination is the addition to the substrate to produce a new cycle of mushrooms.

Few reports in the literature inform the chemical composition of SMS and which mushroom species it was produced (Rinker, 2017). Some works that produced different *Pleurotus* strains, different raw materials and concentrations of SMS in the substrate reported productivities and biological efficiency equal to or higher than the substrate without the addition of SMS (Royse, 1992; Siddhant, 2009; Pardo-Giménez et al., 2012; Ashrafi et al., 2014; Wang et al., 2015; Picornell-Buendia et al., 2016; Economou et al., 2017). Unpublished data from previous works showed that it was possible to use SMS of *Lentinula edodes* in the substrate to produce *P. ostreatus*. In view of the above, the present study sought to increase the productivity and biological efficiency of *Pleurotus ostreatus* supplemented with *Lentinula edodes'* SMS, in addition to understanding the nutritional dynamics of the main nutrients consumed.

4.2 MATERIAL AND METHODS

4.2.1 Substrate preparation and experimental conditions

Substrate's preparation was made according Urben (2017) with modifications. *Pleurotus ostreatus* "MB" was provided by the mycelium by Funghi and Flora®. Seventy (70) polypropylene bags (2000g) were equally stuffed with 10 bags of each seven substrate's formulations (Table 1). Wheat straw was cut to a size of 3±2cm, mixed with $CaCO_3$, supplemented with rice bran or SMS and hydrated to the recommended humidity of 60%. SMS originated from a commercial production of the *Lentinula edodes* mushroom. After filling the cultivation bags, severe pasteurization was carried out at 95ºC for 14 hours. After cooling the culture bags to room temperature, inoculation was carried out in an aseptic environment, with "spawns" (mycelium inoculated in sorghum grains), corresponding to 5% of the fresh weight of the substrate. The culture bags were incubated at 25ºC, in a controlled environment, in the darkness. After the colonization period, six holes with 3 cm, equidistant, were made in each bag and taken to fructification induction, in a controlled environment, at 22ºC ± 1, with relative humidity of 85% ± 2, lighting of 700 lux (12 h / day^{-1}, fluorescent lamps), keeping the control of the CO_2 level low (<1200 ppm).

Table 1. Proportion and chemical composition of raw materials used to grow *P. ostreatus* mushroom.

Substrates	Wheat straw	Rice Bran	Spent Mushroom Substrate	$CaCO_2$	Nitrogen	Carbon	C/N Ratio
			%				
Control	97	0	0	3	0.51	40.87	79/1
7% RB	90	7	0	3	0.58	41.28	72/1
7% SMS	90	0	7	3	0.59	41.14	70/1
15% RB	82	15	0	3	0.64	41.75	65/1
15% SMS	82	0	15	3	0.73	45.66	63/1
30% RB	67	30	0	3	0.78	42.63	55/1
30% SMS	67	0	30	3	0.84	42.03	50/1

RB: Rice bran; SMS: spent mushroom substrate from *Lentinula edodes*.

4.2.2 Elements analysis

Calcium (Ca), potassium (K), magnesium (Mg) manganese (Mn), zinc (Zn), copper (Cu) and iron (Fe) contents were measured in the substrates, before and after the mushroom crop cycle. Three samples for each substrate were collected analyzed following the methodology adapted (Malavolta et al., 1989). Samples were collected before inoculation of the *P. ostreatus* mycelium and after the 70-day culture cycle. For the K, Ca, Mg, Zn, Mn, Cu and Fe the contents were obtained through nitric-perchloric digestion and analyzed in atomic absorption spectrophotometry. Data were expressed in g of mineral kg^{-1} of substrate for macronutrients evaluated and in mg of mineral kg^{-1} of substrate for micronutrients. Mineral consumption by *P. ostreatus* was calculated by subtracting the initial concentration in the substrate from the final concentration in the substrate.

Table 2. Macro and micronutrients analysis in the substrates before *P. ostreatus* mushroom growth.

Substrates	Ca	K	Mg	Mn	Zn	Cu	Fe
		$g.Kg^{-1}$			$mg.Kg^{-1}$		
Control	5.82	10.47	4.12	48.21	24.07	4.59	459.01
7% RB	5.78	10.43	4.28	59.97	28.01	4.73	451.54
7% SMS	6.80	10.34	4.23	57.09	25.23	4.70	473.70
15% RB	5.62	10.28	4.89	107.01	43.76	5.24	421.65
15% SMS	10.39	9.88	4.61	89.65	29.44	5.06	527.54
30% RB	5.61	10.45	6.02	183.41	69.65	6.24	392.67
30% SMS	15.39	8.78	5.01	135.07	34.61	5.41	587.49

RB: Rice bran; SMS: spent mushroom substrate from *Lentinula edodes*.

4.2.3 Vegetative features

For the analysis of the micelial growth rate (cm^2/day), pictures of the culture bags were taken at seven-day intervals (5, 10, 15 and 20 days). Pictures were taken in a paper square (10 cm x 10 cm) demarcated on the four faces of

the cultivation bag, the average of the four faces was calculated for each evaluation period and interpreted using the ImageJ software (Abràmoff et al., 2004). In addition, mycelial colonization time (days) was counted from the inoculation (Day 0) until complete colonization of the culture bag.

4.2.4 Quality e productive parameters

During the production, number of clusters, number of pileus, average clusters weight (g), average pileus length (cm) and width (cm) was recorded. Productivity (%) as well as its distribution in flush (1st, 2nd and 3rd flush (%)) was calculated from the weight of fresh mushrooms x 100. weight^{-1} of fresh substrate. Biological efficiency (%) was calculated by weight of fresh mushrooms x 100 weight^{-1} of dry substrate (Royse et al, 2004).

4.2.5 Statistical analysis

Experiment was conducted in completely randomized blocks design, with seven treatments and ten replications. The homogeneity of variances was tested (Bartlett's test), and posteriorly, the analysis of variance and orthogonal contrasts for production and nutrients analysis were performed using the statistical software 'Data Variance Analysis – Sisvar' (Ferreira, 2011). The orthogonal contrasts performed were:

a) Control X (7% RB + 7% SMS + 15% RB + 15% SMS + 30% RB + 30% SMS);

b) (7% RB +15% RB + 30% RB) X (7% SMS + 15%SMS + 30% SMS);

c) 15% SMS X (7% SMS + 30%SMS);

d) 7% SMS X 30% SMS;

e) 15%RB X (7% RB + 30% RB);

f) 7% RB x 30% RB.

4.3 RESULTS AND DISCUSSION

4.3.1 *Productivity*

Mycelial growth features (vegetative phase), such as mycelial growth rate (cm^2/day) and mycelial colonization time (days), were influenced by the supplements ($p<0.05$). Production features such as number of clusters, number of pileus, first flush (%), average pileus length and width (cm) were not affected by the supplements ($p<0.05$). On the other hand, second flush (%), third flush (%), average clusters weight (g), productivity (%) and biological efficiency (%) were influenced by the supplementation ($p<0.05$). Orthogonal contrasts showed that mycelial growth was higher when the substrate was supplemented regardless of the source (Table 3). The same tendency followed for second flush (%), third flush (%), average clusters weight (g), productivity (%) and biological efficiency (%) (Table 3). Comparing the sources of supplements used (RB or SMS), productivity and biological efficiency was higher when 15% SMS is applied (C/N Ratio – 63/1).

Mushrooms need a well-balanced nutritional complex within the substrate, such as a C/N ratio and nutrients. C/N ratio is important because C source acts like a key component of storage and structural compounds in the cell and it comes in many forms: monosaccharides, disaccharides, and polysaccharides. The mycelium also has the ability to utilize C through larger polymers such as lignin, hemicellulose and cellulose. In this way, the substrates were carefully chosen to create a range of C/N ratios (Table 1) (Chang and Milles, 2004). Unpublished data from previous works showed that it was possible to use SMS of *Lentinula edodes* in the substrate to produce *P. ostreatus*. Although a wide range C/N ratio is possible to produce Oyster mushroom, in our studies N contents higher than 1.2% did not show satisfactory productivity results. Thus, it was decided to limit the C/N range between 50/1 to 80/1 (Table 1).

The nitrogen seems to play an important role in mushrooms growth. Nitrogen is a crucial element needed by all fungi to synthesize compounds containing nitrogen, such as pyrimidines, purines, protein and for chitin, which is the cell wall component composed of b (1-4)-linked unit of N- acetylglucosamin (Hoa et al, 2015). Curvetto et al. (2002) observed that supplementation of

sunflower seed husk with ammonium (NH_4^+) to produce *P. ostreatus* increased the productivity of this species in up to 50%, as it promotes mycelium development. In the same way, the supplementation of this work promoted greater mycelial growth (Table 4). This increase in mycelial growth through supplementation corroborate with Wang et al., 2015, in which the mycelial growth rate was progressively increased and the mycelial colonization time was shortened with SMS supplementation. In fact, the addition of supplements (RB and SMS) promoted mycelial growth, consequently decreasing mycelial time colonization (Table 4).

In addition to the nitrogen, C/N ratio is another important factor in determining mushrooms productivity (Ritota and Manzi, 2019). Since C and N contents directly impact mycelial development and fruiting body formation, the supplementation chosen altered this C/N ratio. In the orthogonal contrasts performed, supplementation with SMS reached the highest productivity and biological efficiency, compared to substrate without supplementation, as well as showed higher second and third flush and average clusters weight (g) (Table 3). Nitrogen plays an important role in the synthesis of lignocellulolytic enzymes, which will act on cellulose, hemicellulose and lignin polymers. Decreasing the C/N ratio, through organic nitrogen compounds, probably allowed the mycelium to consume a greater amount of short-chain sugars, enhancing its growth and later greater production of fruiting bodies. Similarly, Kurt and Buyukalaca (2010) showed that *P. ostreatus* productivity and nitrogen content of substrates were positively correlated, while they observed higher laccase and carboxymethylcellulase activities in media supplemented with larger amount of wheat bran.

The formulation employed for the design of the substrate deeply influences the yield and quality of the mushrooms harvested, in addition to supplementation has also been reported as a way to improve the biological efficiency (Moonmoon et al., 2010; Sánchez, 2010; Gaitán-hernández et al., 2014; Pardo- Giménez et al. 2016; Xie et al., 2017; He et al., 2018; Liang et al., 2019). In this case, the quality of *P. ostreatus* was different comparing wheat straw with 0% supplement versus addition of RB or SMS (Table 3). With RB or SMS in the substrate, *P. ostreatus* was able to increase the average clusters weight (g) (Table 4), in which

heavier clusters corroborate the increase in productivity and biological efficiency. In addition, production according to flushes was also influenced. The application of SMS increased the amount (%) of mushrooms produced in the second flush, while in the third flush the addition of RB also increased this percentage (Table 4). Such results corroborate with Donini et al. (2009), where the amount and the kind of bran may vary depending on the mushroom species as well as the mushroom growth stage.

Different supplements were used to enhance oyster mushroom production, for example, wheat bran (Yildiz et al., 2002; Al-Momany and Ananbeh, 2011), soya bean (Upadhyay et al., 2002), cottonseed hull (Fanadzo et al., 2010), olive mill waste (Ruiz-rodriguez et al., 2010), rice bran and maize powder (Alam et al., 2010). Corroborating with the presented results (Table 4), *P. ostreatus* has an average biological efficiency of 64% when produced only with wheat straw (Chitamba et al., 2012; Koutrotsios et al., 2014; Hossain, 2018). Supplementing the substrate with the sources mentioned above, the biological efficiency achieved can reach 114%, similar to the results obtained with 15% SMS (BE 107%) (Table 4).

Our results emphasize the importance of the substrate supplementation to increase *P. ostreatus* productivity and biological efficiency. The addition of nitrogen sources, influencing the C/N ratio, is a crucial factor for a good mycelial development and consequently a good production. Probably, the nutritional complex present (cellulose, hemicellulose, lignin, nitrogen, minerals) was favorable with the supplementation with 15% of SMS (C/N Ratio – 63/1). This amount of added supplement provided the best gains in the vegetative and reproductive parts, allowing absorption and interaction with the other nutrients.

Table 3. Value of *Pr>F* from orthogonal contrasts of growth rate (cm²/day), mycelial colonization time (days), second flush (%), third flush (%), weight (g), productivity (%) and biological efficiency (%) of *P. ostreatus* cultivated on wheat straw with different supplementation. RB: Rice bran; SMS: spent mushroom substrate from *Lentinula edodes.*

Variable	Ortogonal contrasts					
	a	*b*	*c*	*d*	*e*	*f*
	---------- *Pr>F* ----------					
Mycelial growth rate (cm²/day)	0.0001	*ns*	*ns*	*ns*	*ns*	0.0195
Mycelial time colonization (days)	0.0001	*ns*	*ns*	*ns*	*ns*	0.0139
First flush (%)	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
Second flush (%)	*ns*	*ns*	*0.0001*	*0.0065*	*0.0042*	*ns*
Third flush (%)	0.0448	*ns*	*0.0008*	*ns*	*ns*	*ns*
Number of pileus	ns	*ns*	*ns*	*ns*	*ns*	*ns*
Number of clusters	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
Average leght cap (cm)	ns	*ns*	*ns*	*ns*	*ns*	*ns*
Average widht cap (cm)	ns	*ns*	*ns*	*ns*	*ns*	*ns*
Average clusters weight (g)	0.0197	*ns*	0.0001	*ns*	0.0009	*ns*
Productivity (%)	0.0025	*ns*	0.0001	*0.0014*	*0.0001*	*ns*
Biological efficiency (%)	0.0149	*ns*	*0.0001*	0.0005	*0.0001*	*ns*

Ortogonal contrast: a) Control X (7% RB + 7% SMS + 15% RB + 15% SMS + 30% RB + 30% SMS); b) (7% RB +15% RB + 30% RB) X (7% SMS + 15%SMS + 30% SMS); c) 15% SMS X (7% SMS + 30%SMS); d) 7% SMS X 30% SMS; e) 15%RB X (7% RB + 30% RB); f) 7% RB x 30% RB.

Table 4. Mycelial Growth rate (cm²/day), mycelial colonization time (days), second flush (%), third Flush (%), weight (g), productivity (%) and biological efficiency (%) means of *P. ostreatus* cultivated on wheat straw with different supplementation.

Substrates' supplementation	Mycelial rowth rate (cm²/day)	Mycelial colonization time (days)	Second flush (%)	Third flush (%)	Average clusters weight (g)	Productivity (%)	Biological efficiency (%)
Control	4.64± 0.17	21.84± 0.84	3.86± 0.32	2.34± 0.37	30.86± 3.16	15.68 ± 0.81	66.50 ± 4.26
7% RB	5.55± 0.36	18.92± 1.55	3.90± 0.34	3.14± 0.50	31.39± 3.17	17.84 ± 0.47	68.26 ± 2.68
7% SMS	5.51± 0.22	18.41± 0.75	5.28± 0.33	3.00± 0.50	31.25± 2.86	18.42 ± 0.81	73.69 ± 3.26
15% RB	5.75± 0.13	17.49± 0.44	6.15± 0.33	3.99± 0.45	48.10± 1.83	22.25 ± 1.46	97.21 ± 3.49
15% SMS	5.86± 0.14	17.18± 0.43	7.58± 0.41	4.88± 0.45	54.40± 2.01	25.03 ± 1.21	107.02 ± 5.09
30% RB	6.24± 0.13	16.11± 0.38	4.18± 0.42	2.80± 0.43	38.10± 1.98	15.52 ± 0.52	67.49 ± 4.40
30% SMS	5.94± 0.11	16.88± 0.33	2.98± 0.41	2.60± 0.44	30.59± 1.84	14.02 ± 9.83	47.64 ± 3.10

RB: Rice bran; SMS: spent mushroom substrate from *Lentinula edodes*. Mean:n=10, ±standard error.

4.3.2 *Nutrient dynamics*

Mushrooms are a good source of minerals, once they are taken up by the growing mycelium and translocated to the fruiting body. While the importance of C and N is limiting, no less important are other elements, which may be needed at lower concentrations. SMS or cereal bran such as the rice bran used in this work, are sources of NH_4^+, NO_3^-, P, K, Ca, Mg, Na, Fe, Cu, Mn, and Zn (Fujihara et al. 1995; Cheung, 1997; Medina et al., 2012; Zhu et al., 2013).

According orthogonal contrasts, the only nutrients that showed differences in absorption according to each substrate used were K, Ca and Mn (Table 5 and Table 6). This difference in absorption was also noticed according to the type of supplement. Substrates supplemented with SMS had greater absorption of Ca, while substrates supplemented with RB absorbed more K. These results reinforce the importance of supplementation to produce *P. ostreatus*. Both types of supplements were essential to achieve the greatest biological efficiencies. Furthermore, while the 15% RB or SMS supplementation provided the necessary conditions in terms of C and N, this scenario was probably also favorable for a preference of *P. ostreatus* for K, Ca and Mn absorption occurs.

Table 5. Ca, K and Mn consumption means by *P. ostreatus* cultivated on wheat straw with different supplementation.

Substrates' supplementation	K ($g.kg^{-1}$)	Ca ($g.kg^{-1}$)	Mn ($mg.kg^{-1}$)
Control	3.74± 0.09	1.94± 0.05	9.06± 0.55
7% RB	4.52± 0.03	1.91± 0.03	10.75± 0.37
7% SMS	4.87± 0.11	2.27± 0.04	9.22± 1.10
15% RB	6.56± 0.20	3.75± 0.44	19.78± 2.40
15% SMS	5.76± 0.21	5.37± 0.34	18.02± 0.85
30% RB	4.86± 0.16	1.81± 0.29	29.59± 1.83
30% SMS	3.78± 0.33	2.10± 0.37	28.60± 2.79

RB: Rice bran; SMS: spent mushroom substrate from *Lentinula edodes*. Mean:n=3, ±standard error.

Table 6. Value of *Pr>F* from orthogonal contrasts of K, Ca, Mn, Cu, Fe, Mg and Zn consumption by *P. ostreatus* cultivated on wheat straw with different supplementation. RB: Rice bran; SMS: spent mushroom substrate from *Lentinula edodes.*

Variable	Orthogonal contrasts					
	a	*b*	*c*	*d*	*e*	*f*
	---------- *Pr>F* ----------					
K	0.0001	0.0052	0.0001	0.0011	0.0001	*ns*
Ca	0.0084	0.0052	0.0001	*ns*	0.0001	*ns*
Mn	0.0022	*ns*	*ns*	0.0001	ns	0.0001
Cu	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
Fe	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
Mg	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
Zn	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*

Orthogonal contrast: a) Control X (7% RB + 7% SMS + 15% RB + 15% SMS + 30% RB + 30% SMS); b) (7% RB +15% RB + 30% RB) X (7% SMS + 15%SMS + 30% SMS); c) 15% SMS X (7% SMS + 30%SMS); d) 7% SMS X 30% SMS; e) 15%RB X (7% RB + 30% RB); f) 7% RB x 30% RB.

Several studies claim that K is the element found in the highest concentration in the fruiting body (Manzi et el., 1999; Mattila et al., 2001; La Guardia et al., 2005; Oyetayo, 2005; Lee et al, 2009; Oyetayo and Ariyo, 2013). Potassium has a role as a cofactor in some enzyme systems, is involved in carbohydrate metabolism and is important in the maintenance of ionic balance. Loss of potassium from cells can be detrimental to living organisms, including the fungi (Chang and Milles, 2004). Due to this importance in metabolism, probably to support a greater production of fruiting bodies, *P. ostreatus* chose to absorb larger amounts of this nutrient in substrates supplemented, mainly with RB (Table

5), probably due to the availability of this element in these substrates. On the other hand, the substrates that were supplemented with SMS had lower initial concentrations of K than the others, probably because they had already gone through a cycle of cultivation of the shiitakes' mushroom, indicating a probable preference for absorbing higher levels of K when supplemented with RB.

Regarding Ca, the amount absorbed was higher when the substrate was supplemented with 15% SMS (Table 6). Despite the initial Ca content increasing as more SMS is applied, the amount absorbed by *P. ostreatus* did not follow the same trend (Table 5). According Thongsook and Kongbangkerd (2011), *P. ostreatus* did not appear to accumulate more calcium when its substrate was supplemented with high amounts of Ca. Cardoso et al. (2020) studied the influence of calcium silicate on *P. ostreatus* and found no relationship with Ca and productive parameters. They argue that it seems the sawdust-based cultivation substrate itself was able to provide the necessary minerals and there was probably a saturation level in relation to the calcium levels in the substrate. For Koutrotsios et al. (2020) no correlation was detected in Ca concentration values was measured in mushrooms versus concentrations in the cultivation substrates. It is noted that these authors correlated the amounts of Ca applied with the levels in the fruiting bodies. For example, when Ca is applied with the aim of conserving the fruiting bodies, they influenced the physiological state of the tissue, indicating a positive effect of hyphae compaction, basic structural components of mushroom tissues, in relation to the control (Thongsook and Kongbangkerd, 2011).

Our results demonstrate that the increase in Ca concentrations in the substrate is not proportional to the increase in the amount absorbed by the fungus. However, our results possibly indicate a relationship between the amount of absorbed Ca x productivity of the fruiting bodies (figure 1). Pearson's correlation demonstrates the influence of Ca, presenting an r value of 0.64 for productivity and 0.60 for biological efficiency. Since no studies were found in the literature that evaluated the Ca concentration after the *Pleurotus* cycle, and the consulted literature evaluated the Ca concentration in the fruiting body, the association becomes difficult. Supplementation with 15% of SMS provided the indispensable conditions in terms of C and N, this scenario was probably also

favorable for the occurrence of a preference of *P. ostreatus* for the absorption of Ca. The physiological and metabolic role of Ca in mushrooms is not fully understood. Therefore, more experimental evidence is needed to clarify the mechanism of Ca absorption, as well as possible interactions with other elements (such as ionic charge density, ion electrical charge/ion size) (Koutrotsios et al., 2020). Under conditions of higher productivity and biological efficiency (15% SMS or RB) it was where there was the highest consumption of Ca and K (contrasts c and e, table 6). This demonstrates that they are important nutrients for mushroom cultivation, in agreement with Chang and Milles, 2004, suggesting that future studies with these nutrients may favor productive traits. However, under our knowledge, its application in the cultivation of *Pleurotus* has not yet been investigated.

The nutrient Mn also had different absorption rates according to each substrate. Unlike Ca and K, as the amount of supplement increased, absorption mean of Mn increased (Table 5). Although there was a higher absorption rate, according to a greater availability of Mn, it did not reflect in an increase in productivity and biological efficiency. Micronutrients, such as manganese plays a role in the activation of many enzymes, including those of the TCA (tricarboxylic acid) cycle and is involved in nucleic acid synthesis (Walker and White, 2011). Mn acts as a co-factor of MnP, may favor mycelial growth and production of *Pleurotus* spp., through stimulation and enzymatic activation of MnPs. These extracellular oxidative enzymes can degrade lignocellulosic compounds (Niess and Grabbe, 1990; Lelley and Janssen, 1993; Curvetto et al., 2002; Cohen et al., 2001; Rodriguez-Estrada and Royse, 2007).

The results with Mn application in the *Pleurotus* substrate are conflicting, probably due to the initial concentration of Mn in the substrate, which in most works with this nutrient is not measured. According to Lelley and Janssen, 1993, *P. ostreatus* grown in a substrate supplemented with manganese at 100 $mg.Kg^{-1}$ was more productive than at 400 $mg.kg^{-1}$. In another study, Rodriguez-Estrada and Royse, 2007 obtained higher *P. eryngii* production and biological efficiency at the lowest supplemented dose of Mn (50 $mg.Kg^{-1}$), compared to the highest applied dose (250 $mg.Kg^{-1}$). Moreover, Curvetto et al., 2002 demonstrated that high doses of Mn (above 200 $mg.kg^{-1}$) inhibit the growth and production of *P.*

ostreatus in a substrate with sunflower seed husk. According to the results of the present work, supplementation with 15% RB and 15% SMS added 58.8 and 41.44 $mg.g^{-1}$ of Mn, respectively, compared to the substrate without supplement. In this example, this increase in Mn provided an increase in BE of 46.1% and 60.9%, respectively. Further increasing the concentrations of supplement and, consequently Mn, did not have the same effect, on the contrary, decreasing BE to the same levels a substrate without supplement. Probably this concentration of Mn is enough for the fungus perform his vital functions and enzymatic activations, while concentrations above these probably provided a favorable environment for it to continue in the vegetative stage, not producing large amounts of fruiting bodies.

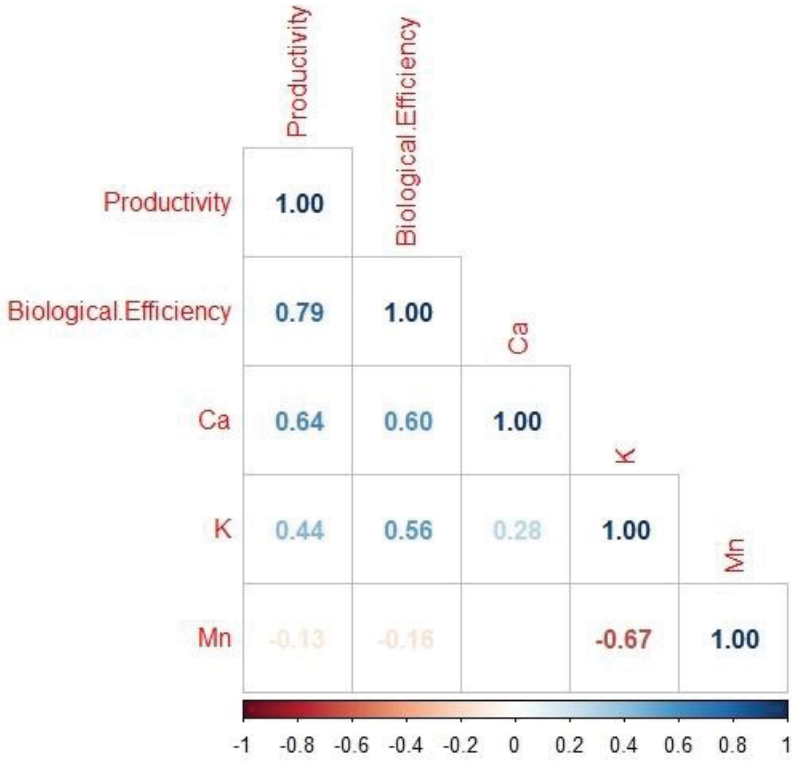

Figure. 1 Pearsons' correlation between nutrients absorbed by *Pleurotus ostreatus* versus Productivity and Biological efficiency. The data presented correspond to the value of r (correlation). *Data had a p value (probability) less than 0.05. Values between 0.6 and 0.8 have high correlation and values between 0.8 and 1.0 have extremely high correlation.

Despite the clear importance of the nutrients mentioned above, the other elements that did not show differences in the contents absorbed by *P. ostreatus* play important roles. This is the case of the nutrients (Mg, Cu, Zn and Fe), which also are important elements for enzymatic cofactors. Equally important, although they are required only in trace amounts, are called minor elements. For example, many enzymes are activated by magnesium, and it is important in ATP metabolism. Iron is a constituent of catalase and of the cytochromes. Zinc is a constituent or activator of many enzymes including alcohol dehydrogenase (Chang and Milles, 2004). Copper as a micronutrient has a key role as a metal activator. It induces laccase transcription, and also plays an important role in laccase production (Palmieri et al., 2000; Ikehata et al., 2004). Therefore, there is still uncertainty about the minimum requirements of these and other elements, as determining the minimum concentrations for absorption physiology in small amounts for fungal growth is a challenge.

Supplementation of the growing substrate for *P. ostreatus* is essential to increase productivity and biological efficiency. The use of SMS would reduce costs for the producer, replacing the supplementation normally used, in addition to meaning a recycling of a material that is considered waste for producers of shiitake mushrooms. Once the mycelium colonizes the substrate in search of these nutrients for development, the presence and availability of all these elements in this complex matrix becomes important. Therefore, the presence and combination of all these nutrients is the main factor for good productions. The nutrition physiology of edible mushrooms is advancing in the literature, and future studies that encompass the enzyme complex during the entire cultivation phase are the key to a better understanding of this topic.

4.4 CONCLUSION

Supplementation with 15% SMS provided productivity and biological efficiency of 25% and 107% in *P. ostreatus*, respectively, where under these conditions the most consumed nutrients were Ca and K.

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5. ATIVIDADE ENZIMÁTICA DO COGUMELO OSTRA CULTIVADO EM DIFERENTES MATÉRIAS-PRIMAS SUPLEMENTADAS

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Resumo

A produção e consumo de cogumelos comestíveis vem se expandindo ao longo dos anos, devido ao valor nutricional, gastronômico e medicinal. A composição química do substrato é um dos fatores decisivos na produção de cogumelos. Palha de trigo (WS) e serragem (SD) de diversas espécies têm sido as principais matérias-primas empregadas na produção do cogumelo Ostra, entretanto quando utilizadas sozinhas, resultam em baixa produtividade e eficiência biológica. A suplementação do substrato potencializa a produção de cogumelos alterando alguns fatores, dentre eles a dinâmica enzimática e produção de compostos bioquímicos imprescindíveis para o metabolismo de *Pleurotus*. Entretanto, não existe um claro entendimento de como a suplementação influencia esta síntese enzimática e bioquímica. Portanto o presente estudo buscou aumentar a produtividade e eficiência biológica de *Pleurotus ostreatus* através da compreensão da dinâmica bioquímica envolvida no processo produtivo. Palha de trigo (*Triticum aestivum*) (WS) e serragem de eucalipto (*Eucalyptus urograndis*) (SD) foram suplementadas com 15% de SMS de shiitake, 15% de farelo de arroz (RB) e 15% SMS+RB. As proteínas totais (PT), os açúcares totais (AT) e a atividade enzimática da peroxidase de manganês (MnP) foram avaliados durante o ciclo de cultivo de *P. ostreatus*. Neste trabalho são apresentados os efeitos da adição de nitrogênio no substrato, através dos suplementos, promovendo maior atividade das enzimas lignocelulósicas, ocorrendo a bioconversão de celulose, hemicelulose e lignina em subprodutos responsáveis pelos incrementos na PR, EB, número de cachos e peso médio de cachos. Em particular, a formulação do substrato com a matéria-prima WS é superior em termos produtivos do que SD para *P. ostreatus*. Em relação ao suplemento, a combinação de RB + SD demonstrou ser mais produtivo, enquanto SMS + WS é recomendado para alta eficiência biológica de *P. ostreatus*.

Termos indexadores: Shimeji, manganês peroxidase, atividade enzimática, enzimas oxidativas, substrato exaurido de cogumelo.

5.1 INTRODUÇÃO

A produção e consumo de cogumelos comestíveis vem se expandindo ao longo dos anos, devido ao valor nutricional, gastronômico e medicinal, sendo o gênero *Pleurotus* (nome popular de Cogumelo Ostra ou Shimeji) o segundo gênero mais produzido no mundo (Royse et al., 2017). Palha de trigo (WS) e serragem (SD) de diversas espécies têm sido as principais matérias-primas empregadas na produção do cogumelo Ostra, entretanto quando utilizadas sozinhas, resultam em baixa produtividade e eficiência biológica (Philippoussis, 2009). Buscando alcançar maiores produtividades e eficiência biológica, normalmente utilizam-se outras matérias-primas chamadas de suplementos sendo os farelos de cereais (trigo, arroz, soja, etc.) os mais utilizados.

Uma possível alternativa como suplementação é o substrato exaurido de cogumelo (SMS). O SMS é constituído de substrato e micélio após o ciclo de produção de outros cogumelos (Mohd Hanafi et al., 2018). Apesar do SMS ser fonte de macro e micronutrientes, normalmente é subutilizado ou descartado de forma incorreta, podendo se tornar um problema para o produtor (Medina et al., 2012; Rinker, 2017). Diversos autores relatam a viabilidade da utilização de SMS para a produção de *Pleurotus* (Royse, 1992; Siddhant, 2009; Pardo-Giménez et al., 2012; Ashrafi et al., 2014; Wang et al., 2015; Economou et al., 2017). No entanto, poucos estudos informam a composição química do substrato e qual espécie de cogumelo o SMS foi derivado (Rinker, 2017).

A composição química do substrato é um dos fatores decisivos na produção de cogumelos, afetando diretamente a produtividade, eficiência biológica e a qualidade (Ragunathan e Swaminathan, 2003; Chang e Miles, 2004; Pedra e Marino, 2006; Urben, 2017;Bellettini et al., 2019). As matérias-primas que compõem o substrato são a base nutricional para o fungo, fornecendo carbono (C), nitrogênio (N) e minerais. Os suplementos, por serem fontes de nitrogênio (N), alteram a relação C/N do substrato e são responsáveis por estimular a síntese de proteínas e enzimas. Os cogumelos produzem uma bateria de enzimas extracelulares (oxidativas: por exemplo a manganês peroxidase (MnP) e hidrolíticas: como a celulase) que degradam as matérias-

primas lignocelulósicas (lignina, hemicelulose e celulose), formando subprodutos facilmente assimiláveis, como açúcares de cadeia curta (glicose, manose, trealose etc.), para a nutrição fúngica (Stajić et al., 2006; Elisashvili et al., 2008; Singh et al., 2008; Elisashvili e Kachlishvili, 2009).

Diversos autores estabeleceram correlações entre a degradação de compostos lignocelulósicos e a síntese de enzimas lignocelulolíticas (Cho et al., 2002; Elisashvili et al., 2003; Alemawor et al., 2009; Isikhuemhen e Mikiashvilli, 2009; Da Luz et al., 2012). No entanto, não existe uma clara compreensão de que maneira a suplementação altera a síntese destas enzimas e compostos bioquímicos, além de como estas enzimas afetam diretamente a produtividade e eficiência biológica de *P. ostreatus*. Deste modo, o entendimento destes fatores forneceria suporte para a reutilização do SMS para produzir cogumelos Ostra, trazendo novas oportunidades ao produtor através da ampla disponibilidade deste resíduo e da redução do tradicional aporte de farelo de cereais. Portanto, o presente estudo buscou aumentar a produtividade e eficiência biológica de *Pleurotus ostreatus* cultivado em palha de trigo e serragem de eucalipto, suplementados com farelo de arroz e SMS de *Lentinula edodes*, através da compreensão da dinâmica bioquímica envolvida no processo produtivo.

5.2 MATERIAL E MÉTODOS

5.2.1 Preparo do substrato

Pleurotus ostreatus “MB”, na forma de “spawns” (micélio inoculado em grãos de sorgo), foi fornecido pela empresa “Funghi e Flora”. Cento e vinte e seis (126) sacos de polipropileno foram igualmente preenchidos com um volume médio de 2.0 kg de substrato em seis (6) diferentes formulações (Tabela 1), confeccionados conforme Urben, 2017 com modificações. Palha de trigo (*Triticum aestivum*) (WS) e serragem de eucalipto (*Eucalyptus urograndis*) (SD) foram utilizadas como matérias-primas básicas para a formulação dos substratos (Tabela 2). O SMS foi adquirido de uma produção comercial de *Lentinula edodes*, após o ciclo de cultivo do cogumelo shiitake. A granulometria da SD e WS utilizada foi de 3cm ±2, misturada com 3% $CaCO_3$, 15% farelo de arroz (RB), 15% SMS ou 15% de RB+SMS (MIX) e hidratada até a umidade recomendada de 60% (Table 1).

Tabela 1. Proporção e composição de cada substrato utilizado para o cultivo de *Pleurotus ostreatus*.

Substratos	SD	WS	RB	SMS	$CaCO_3$	N	C	Relação C/N
	(%)							
SD + RB	82	0	15	0	3	0,50	37,34	75
SD + SMS	82	0	0	15	3	0,43	37,04	86
SD + MIX	82	0	7,5	7,5	3	0,47	37,19	80
WS + RB	0	82	15	0	3	0,80	42,02	52
WS + SMS	0	82	0	15	3	0,74	41,72	57
WS + MIX	0	82	7,5	7,5	3	0,77	41,87	54

*SD: Serragem; WS: palha de trigo; RB: Farelo de arroz; SMS: substrato exaurido de cogumelo.

Fibra detergente neutro (FDN), fibra detergente ácido (FDA) e lignina (LIG) foram usados para determinar o teor de hemicelulose e celulose dos substratos (Van Soest et al., 1991). A hemicelulose foi calculada usando FDN e FDA e a celulose foi determinada usando FDA e LIG (hemicelulose = FDN-FDA; celulose = FDA-LIG) (Zadrazil e Brunnert, 1981).

Tabela 2. Composição bromatológica de cada matéria-prima utilizada para o cultivo de *Pleurotus ostreatus.*

Matérias primas	Celulose	Hemicelulose	Lignina	N	C	C/N
	%					
SD	49,02	14,11	18,23	0,25	37,20	149
WS	33,74	37,28	4,36	0,62	42,91	69
RB	11,41	20,45	4,55	1,95	45,54	23
SMS	18,78	15,87	7,29	1,52	43,59	29

*SD: Serragem; WS: palha de trigo; RB: Farelo de arroz; SMS: substrato exaurido de cogumelo

5.2.2 Condições experimentais

Após a montagem dos sacos de cultivo, realizou-se a pasteurização severa à 95ºC por 14h. Após o resfriamento dos sacos de cultivo à temperatura ambiente, a inoculação foi realizada em ambiente asséptico, com spawns, correspondendo a 5% da massa fresca do substrato. Os sacos de cultivo foram incubados a 25ºC±1, em ambiente controlado, na ausência de luz. Após o período de colonização, seis furos de 3 cm, equidistantes, foram feitos em cada saco e levados para indução de frutificação, em ambiente controlado, a 22ºC±1, com umidade relativa de 85% ± 2, iluminação de 700 lux (12 h/ dia-1, lâmpadas fluorescentes), mantendo o controle do nível de CO_2 baixo (<1200 ppm).

5.2.3 Análises bioquímicas e enzimáticas

Seis datas de coleta foram realizadas: 0; 7; 14; 40; 60 e 80 dias após a inoculação. Para cada data, três amostras homogêneas foram coletadas, a partir de um saco de cultivo de cada substrato. O extrato de proteína bruta (CPE) foi realizado com 50 mL de tampão fosfato (0,2M pH 7,5) e 25 g de substrato/micélio. Essa mistura foi homogeneizada em almofariz de porcelana por 5 min e centrifugada a 250 rpm por 60 min. O sobrenadante foi filtrado em papel filtro (Whatman nº 1). O CPE foi utilizado para a análise da atividade enzimática de manganês peroxidase (MnP), determinação de proteínas totais (PT) e açúcares totais (AT).

5.2.4 *Manganês peroxidase (MnP)*

A atividade de manganês peroxidase (MnP) foi determinada usando 500 µL de CPE, 100 µL de vermelho de fenol (1 g L^{-1}, ε = 3162 M^{-1} cm^{-1}), 100 µL de lactato de sódio (250 mmol L^{-1}, pH 4,5), 200 µL albumina bovina (1% p/v), 50 µL de peróxido de hidrogênio e 50 µL de sulfato de manganês. Esta reação foi incubada em banho-maria a 37 ºC por 10 min. Em seguida, 40 µL de hidróxido de sódio (2 mmol L-1) foram adicionados para interromper a reação. A absorbância foi determinada a 610 nm (Kuwahara et al., 1984).

5.2.5 *Proteínas totais (PT)*

O teor de proteína total (PT) em CPE foi realizado pela metodologia descrita por Bradford em 595 nm (Bradford, 1976). A curva padrão foi feita com albumina bovina (1% p/v) com concentração variando de 0 a 1100 µg mL^{-1}. Para cada reação, 2,5 mL de reagente de Bradford, 1,5 mL de CPE. Esta reação foi incubada por 10 min a 25 ºC e realizada a leitura.

5.2.6 *Açúcares totais (AT)*

A quantificação dos açúcares totais (AT) foi realizada pelo método fenol-sulfúrico (Dubois et al.,1956). Para isso, primeiramente, 0,1 mL de CPE foi diluído com 0,5 mL de solução de fenol (5%). Posteriormente, 2,5 mL de H_2SO_4 (95,5%) foram adicionados às amostras. Os tubos de ensaio foram mantidos à temperatura ambiente por 20 minutos para o desenvolvimento da cor. Finalmente, a absorbância foi registrada usando espectrofotômetro em um comprimento de onda de 490 nm. A curva padrão para a determinação dos açúcares solúveis totais foi construída utilizando soluções de glicose, cujas concentrações variaram entre 0 a 1,0 mg/mL.

5.2.7 Período de colonização do P. ostreatus

Após a inoculação do micélio de *P. ostreatus*, o crescimento micelial médio (cm²/dia) foi avaliado através de fotografias tiradas em intervalos de cinco dias (0, 5, 10, 15 e 20 dias). As fotografias foram tiradas com auxílio de um molde (10 cm x 10 cm), demarcado nas quatro faces do saco de cultivo, processadas e interpretadas pelo software ImageJ (Abràmoff et al. 2004). Além disso, o período de colonização (dias) foi contado desde a inoculação (Dia 0) até a colonização completa do saco (100%) pelo micélio.

5.2.8 Período de produção do P. ostreatus - qualidade

As seguintes variáveis de qualidade foram avaliadas: número médio de cachos, número médio de píleos, peso médio de cacho (g), comprimento (cm) e largura (cm) média do píleo.

5.2.9 Período de produção do P. ostreatus - produtividade

A produtividade total (PR) (%) (massa de cogumelos frescos x 100. massa de substrato fresco^{-1}), bem como a produtividade em fluxos (1º, 2º e 3º fluxo (%)) e eficiência biológica (EB) (%) (massa de cogumelos frescos x 100 massa de substrato seco^{-1}) foram avaliados segundo Royse et al. (2004).

5.2.10 Análise estatística

O experimento consistiu em seis tratamentos com 15 repetições cada, distribuídos em delineamento inteiramente casualizado (DIC). Testou-se a homogeneidade de variâncias (teste de Bartlett), e posteriormente realizou-se a análise de variância e polinômios ortogonais para as variáveis de produção e o modelo de regressão para as variáveis bioquímicas utilizando o software *Statistical Analysis System* (SAS®). Os seguintes contrastes ortogonais foram analisados:

a) SD (RB + SMS + MIX) versus WS (RB + SMS + MIX);
b) SD (RB + SMS) versus SD + MIX;
c) SD + RB versus SD + SMS;
d) WS (RB+ SMS) versus WS + MIX;
e) WS + RB versus WS + SMS.

5.3 RESULTADOS E DISCUSSÃO

As concentrações da enzima manganês peroxidase (MnP), proteínas totais (PT) e açúcares totais (AT) entre os substratos foram diferentes ($p<0.05$) (Tabela 3). Diversos estudos indicam que a matéria-prima utilizada nos substratos, além da espécie e linhagem do fungo, induzem de forma diferente a produção das enzimas hidrolíticas e oxidativas (Elisashvili et al. 2008; Han et al. 2017; Leite et al. 2019). Por exemplo, de acordo com Elisashvili et al., 2006, a presença de substrato lignocelulósico é obrigatória para a produção de MnP por *P. dryinus*, pois quando cultivado em meio sintético com diferentes fontes de carbono (C), não houve a produção da enzima. Desta maneira, ambos os substratos utilizados (SD e WS) são matérias-primas lignocelulósicas, produzindo concentrações de MnP diferentes.

A atividade da MnP nos substratos compostos de serragem (SD) foi superior no início do cultivo (7 dias) (Tabela 3, contraste *a*, Figura 1). Possivelmente a maior concentração de MnP em SD indiretamente deve-se ao conteúdo elevado de lignina, em comparação com os substratos de WS (Tabela 2). Isto porque os fungos do gênero *Pleurotus* degradam matérias-primas ricas em celulose, hemicelulose e lignina com objetivo de utilizar compostos de C como fonte de energia para seu metabolismo. O micélio, com objetivo de acessar a celulose e hemicelulose, degrada a lignina (polímero de cadeia aromática que recobre a celulose e hemicelulose) através de diferentes enzimas oxidativas como: lacase (Lac), manganês peroxidase (MnP) e peroxidase versátil (VP) (Ruhl er al., 2008). Comparativamente aos nossos resultados, Elisashvili et al., 2008 descobriram que a produção enzimática oxidativa (Laccase e MnP) em substratos lignocelulósicos foi maior do que em substratos com resíduos de frutas. Segundo os autores, a composição química dos resíduos de frutas (alta concentração de açúcares e polissacarídeos) induziu a produção de enzimas hidrolíticas (celulase e xilanase), enquanto os substratos lignocelulósicos (palha de trigo e folhas de árvores) induziram a produção de enzimas oxidativas (Laccase e MnP). Em nosso caso, provavelmente a SD por possuir uma composição química com maior teor de lignina (Tabela 2) e uma baixa concentração de AT (Tabela 3, contraste *a*, Figura 3) em comparação com a WS, induziu uma maior atividade de enzimas oxidativas (MnP), corroborando com o estudo citado. Desta maneira, a produção de MnP nesta etapa, preferencialmente em substratos com SD, é indicada para que o fungo consiga degradar uma maior quantidade de lignina em busca de compostos de C mais simples.

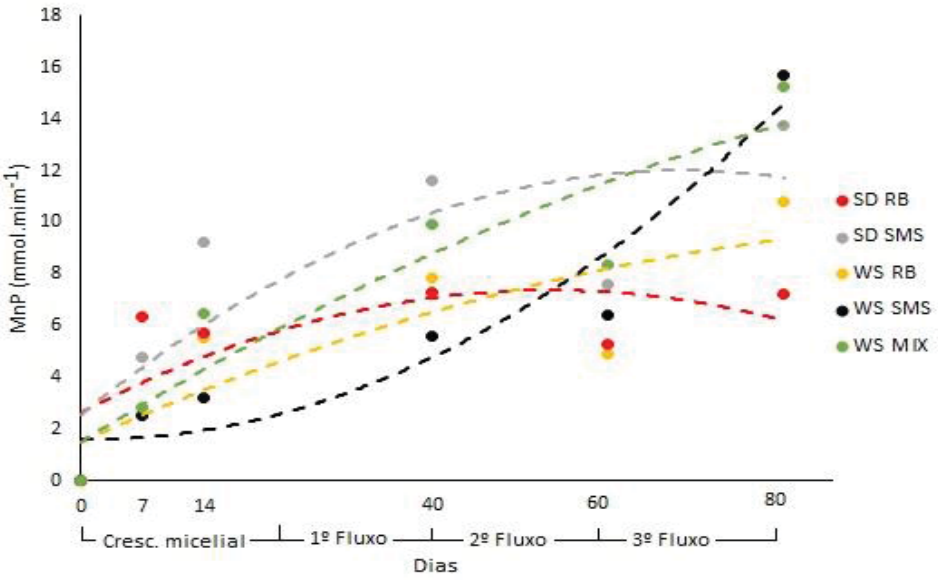

Figura 1. Atividade da MnP produzida por *Pleurotus ostreatus* cultivado em SD e WS suplementada com RB, SMS e MIX durante o ciclo de cultivo. SD: serragem; WS: palha de trigo; RB: Farelo de arroz; SMS: substrato exaurido de cogumelo shiitake; Mix: RB+SMS.

No final do ciclo de produção, ocorreu uma inversão na concentração de MnP produzida pelos substratos, onde os substratos com WS apresentaram resultados superiores a SD (Tabela 3, contraste *a;* Figura 1). Nota-se que no início e durante o cultivo, a MnP produzida pelo WS+SMS, era inferior aos demais substratos. Os substratos com WS possuíam fontes de C disponíveis para consumo imediato do fungo (Tabela 3, Figura 3), não ocorrendo a necessidade do micélio produzir elevadas concentrações de MnP. No final do ciclo de produção, provavelmente a disponibilidade de compostos de C simples diminuiu, sinalizando a necessidade de aumentar a síntese de MnP para degradar lignina.

Tabela 3. Contrastes ortogonais das variáveis bioquímicas de *P. ostreatus* produzidos em SD e WS com diferentes suplementos.

		Contrastes ortogonais				
		a) SD (RB + SMS + MIX) *versus* WS (RB + SMS + MIX)	b) SD (RB + SMS) *versus* SD + MIX	c) SD + RB *versus* SD + SMS	d) WS (RB+ SMS) *versus* WS + MIX	e) WS + RB *versus* WS + SMS
Dias	Variável	---------- *Pr>F* ----------				
0	MnP	ns	ns	ns	ns	ns
	PT	<.0001	ns	0.0398	ns	ns
	AT	0.0004	ns	ns	ns	0.0060
7	MnP	0.0076	0.0136	ns	ns	ns
	PT	<.0001	ns	0.0379	ns	ns
	AT	0.0013	ns	0.0172	ns	0.0007
14	MnP	ns	ns	ns	ns	ns
	PT	<.0001	ns	ns	ns	0.0025
	AT	ns	ns	ns	0.0462	ns
40	MnP	ns	ns	ns	ns	ns
	PT	<.0001	ns	ns	0.0494	ns
	AT	ns	0.0073	0.0404	ns	0.0005
60	MnP	ns	ns	ns	ns	ns
	PT	<.0001	ns	0.0017	<.0001	0.0005
	AT	0.0006	0.0280	<.0001	<.0001	<.0001
80	MnP	0.0150	0.0242	ns	ns	0.0193
	PT	<.0001	0.0072	0.0175	ns	0.0001
	AT	<.0001	0.0179	0.0080	ns	0.0132

SD: serragem; WS: palha de trigo; RB: Farelo de arroz; SMS: substrato exaurido de cogumelo shiitake; Mix: RB+SMS; MnP: Manganês peroxidase; PT: Proteínas totais e AT: Açúcares totais. ^{ns}: não significativo $p<0.05$.

As concentrações de PT foram influenciadas ($p<0.05$) pelos substratos durante o período de cultivo (Tabela 3). A análise das PT neste estudo é baseada na interação das macromoléculas de proteínas que contém aminoácidos de cadeias laterais básicas ou aromáticas (Zaia et al., 1998). Deste modo, nosso objetivo foi quantificar as demais enzimas hidrolíticas e oxidativas, uma vez que a maior parte das enzimas produzidas pelo fungo são de natureza proteica, podendo contribuir com a concentração de PT no substrato (Kadari, 1999; Singh et al., 2008; Knop et al., 2015).

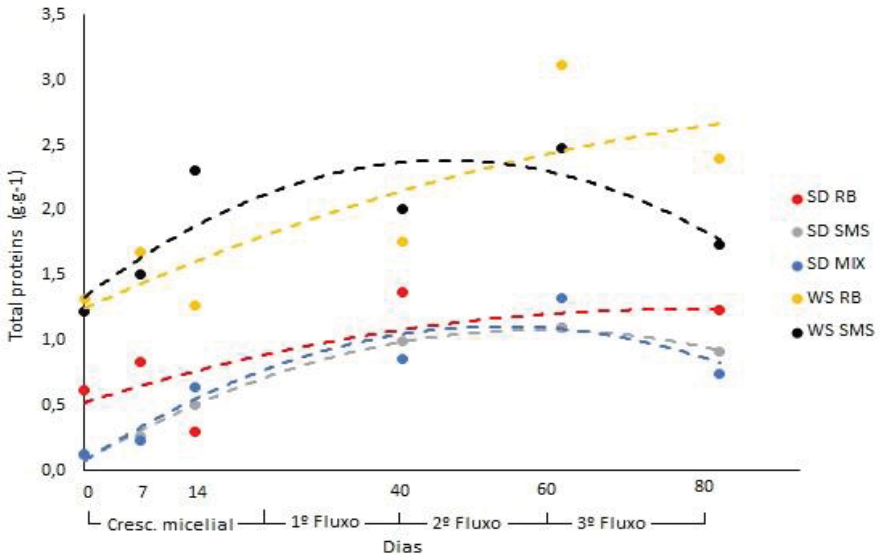

Figura 2. Teores de proteínas totais produzidas por *Pleurotus ostreatus* cultivado em SD e WS suplementada com RB, SMS e MIX durante o ciclo de cultivo. SD: serragem; WS: palha de trigo; RB: Farelo de arroz; SMS: substrato exaurido de cogumelo shiitake.

O teor de PT ao longo do cultivo foi superior nos substratos com WS na composição (Figura 2). A relação C/N de cada substrato foi responsável pelas diferenças nos teores de PT apresentados, pois os substratos formulados com WS tinham menor relação C/N (57-52/1) que os substratos com SD (86-75/1) (Tabela 1). A baixa relação C/N é explicada pelo teor intrínseco de N da matéria-prima WS, independente da suplementação (Tabela 2). Existem diversos parâmetros que afetam a produção de enzimas, no entanto o N é um dos principais (Singh et al., 2008). Além da síntese de proteínas, o N é importante na constituição dos ácidos nucleicos, purinas, pirimidinas e polissacarídeos

constituintes da parede celular de muitos fungos (Drozdowski et al., 2010; Abdullah et al., 2015).

Além da concentração de PT ser superior diretamente pelo N da WS, possivelmente essa diferença seja proveniente da síntese das demais enzimas extracelulares envolvidas no processo de degradação da celulose, hemicelulose e lignina, como por exemplo: as hidrolíticas (endoglucanases (EC 3.2.1.4), cellobiohidrolases (EC 3.2.1.91) e β-glicosidases (EC 3.2.1.21)), que produzem hidrolases responsáveis pela degradação de polissacarídeos; e ligninolíticas oxidativas (lignina peroxidases (EC 1.11.1.14) (LiP), manganes peroxidases (EC 1.11.1.13) (MnP) e laccases (EC 1.10.3.2)), que degradam a lignina e compostos aromáticos. Deste modo, nossos resultados corroboram com estudos anteriores (Schuttmann et al., 2014; Knop et al., 2015), demonstrando a importância da adição de N, através da suplementação, na indução da atividade ligninolítica.

Durante o ciclo de cultivo, as concentrações AT aumentaram expressivamente (Figura 3) e foram influenciadas ($p<0.05$) pelos substratos (Tabela 3). A análise de açúcares totais (AT), pelo método fenol-sulfúrico, baseia-se na determinação de açúcares simples, polissacarídeos e seus derivados incluindo os metil-ésteres com grupos redutores livres (Silva et al., 2003). Desta maneira, o aumento dos AT pode ser atribuído à maior eficiência enzimática do *P. ostreatus* na degradação das frações lignocelulíticas dos substratos em açúcares de cadeias mais curtas. Resultados semelhantes no aumento de açúcares também foram obtidos por *P. ostreatus* quando cultivados em casca de cacau (Alemawor et al., 2009). Alguns polissacarídeos e monossacarídeos como por exemplo xilo-oligossacarídeos, glicose, xilose, arabinose, celobiose, manose e maltose foram observados devido à biodegradação de resíduos de hemicelulose por *P. ostreatus* e *P. tailandia* (De-Menezes et al., 2009). Desta forma, o aumento concentração de AT significa um aumento na energia disponível do substrato, concordando com Alemawor et al., 2009.

Cabe ressaltar as diferenças avaliadas nas concentrações de AT aos 60 dias de cultivo (Figura 3, Tabela 3 contraste *a*) onde as concentrações de AT foram superiores nos substratos à base de WS. Possivelmente a eficiência

enzimática na conversão dos compostos lignocelulíticos em subprodutos energéticos, como AT, seja responsável pela diferença encontrada, concordando com Alemawor et al., 2009.

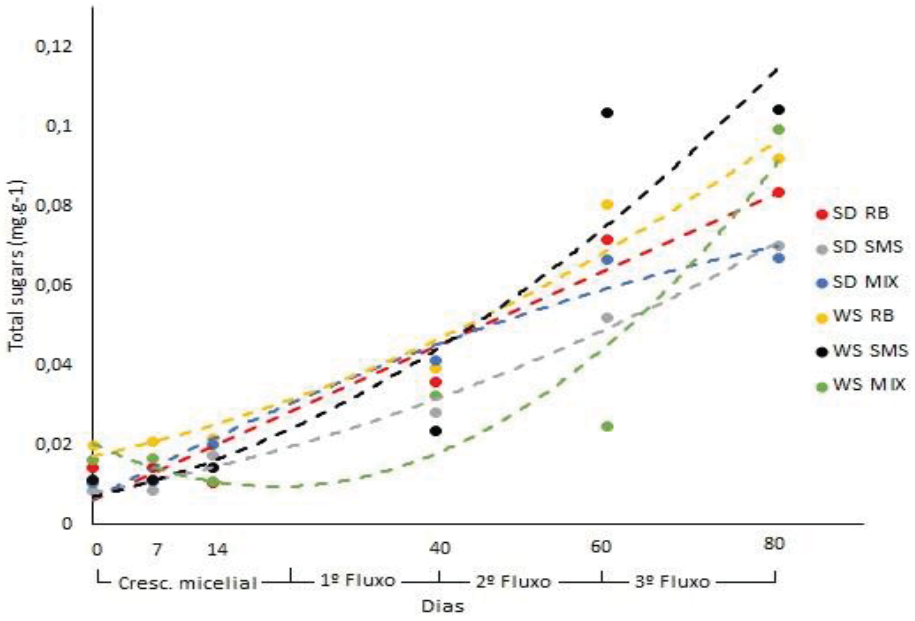

Figura 3. Teores de açúcares totais produzidos por *Pleurotus ostreatus* cultivado em SD e WS suplementada com RB, SMS e MIX durante o ciclo de cultivo. SD: serragem; WS: palha de trigo; RB: Farelo de arroz; SMS: substrato exaurido de cogumelo shiitake; Mix: RB+SMS.

Na tabela 4 são apresentadas as equações da análise de regressão das variáveis bioquímicas de *P. ostreatus*. A atividade enzimática oscila de acordo com o metabolismo do fungo e com o período do ciclo de cultivo, podendo-se observar para algumas variáveis um ajuste baixo (R^2) da equação quadrática.

Tabela 4. Equações da análise de regressão e R^2 das variáveis bioquímicas de *P. ostreatus* produzidas em SD e WS com diferentes suplementos.

	MnP		Total Proteins		Total Sugars	
Substrates	**Equations**	**R^2**	**Equations**	**R^2**	**Equations**	**R^2**
SD + RB	$y = -0.00081750x^2 + 0.15803x + 2.66772$	0.60	$y= -0.00022440x^2 + 0.02853x + 0.42924$	0.48	$y= 4.773298E\text{-}7x^2 + 0.00007711x + 0.00069871$	0.95
SD + SMS	$y = -0.00047970x^2 + 0.14801x + 3.47881$	0.48	$y= -0.00027874x^2 + 0.03237x + 0.03539$	0.94	$y= 5.462391E\text{-}7x^2 +0.00004662x + 0.00082304$	0.97
SD + MIX	ns*		$y= -0.00034248x^2 + 0.03597x + 0.05841$	0.71	$y= -6.29825E\text{-}7x^2 +0.00014398x + 0.00041148$	0.94
WS + RB	$y = -0.00090140x^2 + 0.16156x + 1.98655$	0.54	$y= -0.00009362x^2 + 0.02469x + 1.20754$	0.59	$y= 6.824848E\text{-}7x^2 +0.00006008x + 0.00178$	0.95
WS + SMS	$y =0.00060819x^2 + 0.11997x + 1.38360$	0.80	$y= -0.00048229x^2 + 0.04360x + 1.29216$	0.42	$y= 0.00000118x^2 +0.00006006x + 0.00068636$	0.87
WS + MIX	$y =-0.00070050x^2 + 0.20990x + 1.51252$	0.76	ns		$y= 0.00000265x^2 +-0.00011018x + 0.00218$	0.86

*Não signifcativo. SD: serragem; WS: palha de trigo; RB: Farelo de arroz; SMS: substrato exaurido de cogumelo shiitake; Mix: RB+SMS.

O crescimento micelial médio e o período de colonização não foram influenciados ($p<0.05$) pelos diferentes substratos (Tabela 5). Esperava-se que eles fossem afetados diante das características bioquímicas encontradas em cada substrato e também pelas diferentes relações C/N dos substratos (Tabela 1). Assim, pode-se supor que a suplementação com RB e SMS, forneceu um suprimento nutricional de açúcares e amidos (carboidratos facilmente disponíveis) adequado, justificando o crescimento micelial semelhante em ambos os substratos.

Da mesma maneira, o comprimento e largura média dos píleos (cm); número de píleos e produtividade no 1º fluxo também não foram afetados ($p<0.05$) pelos substratos (Tabela 5). As características de qualidade (Número de píleos, comprimento e largura dos píleos) também são influenciadas por outros fatores além da composição do substrato, como por exemplo a espécie e linhagem do fungo e as condições de cultivo (temperatura, umidade relativa etc.), como já relatados por alguns autores (Zhang et al., 1998; Khlood e Ahmad, 2004). Possivelmente seja o caso do *P. ostreatus* deste estudo, indicando que o comprimento, largura e número de píleos desta linhagem não é influenciada pelos substratos testados e sim pelos demais fatores citados.

Além destas características, a produtividade no 1º fluxo não apresentou diferença nos substratos (Tabela 6). Sabe-se que a síntese de C está intimamente ligada ao desenvolvimento do corpo de frutificação. Por exemplo, foi notada uma mudança no metabolismo de *A. bisporus* durante esta fase, estando intimamente ligada a um aumento da taxa de degradação de celulose e hemicelulose (Wood e Goodenough, 1977). Neste caso, a produção de laccase e celulase foram sugeridas como enzimas envolvidas neste processo (Wells et al., 1987; Claydon et al., 1988). No presente trabalho, concomitantemente a produtividade no 1º fluxo, a concentração de AT foi semelhante em todos os substratos (Tabela 3, Figura 3). Supõe-se desta forma que as enzimas produzidas nos substratos proporcionaram uma síntese de açúcares semelhante para a formação dos corpos de frutificação de *P. ostreatus*, não alterando a produtividade no 1º fluxo.

Tabela 5. Contrastes ortogonais das variáveis de produção de *P. ostreatus* produzidos em SD e WS com diferentes suplementos. SD: serragem; WS: palha de trigo; RB: Farelo de arroz; SMS: substrato exaurido de cogumelo shiitake; Mix: RB+SMS.

Variable	Orthogonal contrasts				
	a) SD (RB + SMS + MIX) *versus* WS (RB + SMS + MIX)	b) SD (RB + SMS) *versus* SD + MIX	c) SD + RB *versus* SD + SMS	d) WS (RB+ SMS) *versus* WS + MIX	e) WS + RB *versus* WS + SMS
	---------- *Pr>F* ----------				
Mycelial growth rate (cm²/day)	*ns*	*ns*	*ns*	*ns*	*ns*
Mycelial time colonization (days)	*ns*	*ns*	*ns*	*ns*	*ns*
Number of clusters	0.0149	*ns*	*ns*	*ns*	*ns*
Number of pileus	*ns*	*ns*	*ns*	*ns*	*ns*
Cap length (cm)	*ns*	*ns*	*ns*	*ns*	*ns*
Cap width (cm)	*ns*	*ns*	*ns*	*ns*	*ns*
First flush (%)	*ns*	*ns*	*ns*	*ns*	*ns*
Second flush (%)	*ns*	*ns*	0.0065	0.0061	*ns*
Third flush (%)	0.0005	*ns*	*ns*	*ns*	*ns*
Average clusters weight (g)	*ns*	*ns*	0.0018	*ns*	*ns*
Productivity (%)	<.0001	*ns*	0.0041	*ns*	*ns*
Biological efficiency (%)	<.0001	0.0103	0.0008	*ns*	0.0002

[ns]: não significativo p<0.05.

Tabela 6. Médias das variáveis de *P. ostreatus* produzidos em SD e WS com diferentes suplementos (± erro padrão). SD: serragem; WS: palha de trigo; RB: Farelo de arroz; SMS: substrato exaurido de cogumelo shiitake; Mix: RB+SMS

Substrates	Mycelial growth rate (cm²/day)	Colonization period (days)	Productivity per flush (%)			Total productivity (%)	EB (%)	Clusters number	Pileus number	Cap length (cm)	Cap width (cm)	Average clusters weight (g)
			1º Flush	2º Flush	3º Flush							
SD + RB	5.7±1.13	17.5±1.44	11.2±0.88	7.2±0.71	3,6±0.62	22.1±1.10	77.4±2.82	8.1±0.32	18.4±1.10	4.2±0.18	3.6±0.17	58.2±6.35
SD + SMS	5.3±1.20	19.1±1.74	10.1±0.85	4.5±0.56	2,6±0.34	17.5±0.54	57.0±1.77	8.4±.0.21	14.3±01.24	3.6±0.15	3.2±0.15	40.1±1.91
SD + MIX	5.7±1.23	17.8±1.75	12.0±0.85	5,1±0.55	3,1±0.30	20.0±0.75	53.8±1.81	10.2±0.35	17.0±1.23	3.5±0.17	3,0±0.16	40.6±3.18
WS + RB	5.8±1.10	17.1±1.31	12.8±0.90	7.1±0.72	4,3±0.64	24.4±1.49	92.3±5.62	10.5±0.36	14.4±1.27	3.9±0.16	3.4±0.16	45.6±2.82
WS + SMS	6.1±1.14	16.3±1.37	13.2±0.75	8.0±0.76	5,1±0.50	26.3±1.19	115.3±5.1	10.8±0.56	17.8±1.26	3.9±0.17	3.5±0.15	49.6±3.71
WS + MIX	5.4±1.15	18.4±1.47	11.9±0.75	5.5±0.82	4,0±0.52	22.0±1.27	99.9±5.51	10.1±0.54	19.8±1.31	3.8±0.19	3,3±0.17	51.1±4.36
Pr > F	0.0643	0.0812	0.0978	0.0011	0.0111	<.0001	<.0001	0.0392	0.0534	0.1703	0.1963	0.0146

Com relação a EB de cada matéria-prima, diversos relatos na literatura demonstram que a SD normalmente apresenta valores inferiores de EB comparando com WS, corroborando com os resultados apresentados (Tabela 6). Por exemplo, a EB de *Pleurotus* spp. em vários tipos de SD variou de 4% a 74%, enquanto em substratos à base de WS foi de 50% a 97% (Philippoussis et al., 2001; Obodai et al., 2003; Salmones et al., 2005; Zhang e Fadel, 2002).

Além disso, os substratos com WS proporcionaram maior número de cachos, maior produtividade total (PR) e produtividade no 3º fluxo, além de melhor eficiência biológica (BE) (Tabela 5, contraste *a*). O substrato WS+SMS, que apresentou a maior produtividade e eficiência biológica, teve durante todo o ciclo de cultivo a menor síntese de MnP e maiores concentrações de AT e PT. A baixa atividade de MnP deste substrato, no início do cultivo, deve-se a composição química do WS+SMS que possivelmente proporcionou maior síntese das outras enzimas oxidativas responsáveis pela degradação de compostos lignocelulósicos, podendo ser verificado pelo aumento do teor de PT (Figuras 2). Desta maneira, uma eventual taxa elevada de MnP no início do ciclo de cultivo, necessariamente não propicia ganhos expressivos em produção de *P. ostreatus.* Ao passo que ao final do ciclo de cultivo, possivelmente o complexo enzimático produzido (enzimas hidrolíticas como celulases e endoglucanases) suportam o incremento nos teores de AT (Figura 3) nesse mesmo período, justificando a superioridade de produtividade e eficiência biológica desse substrato (Tabela 6).

Tais resultados reforçam a importância da dinâmica enzimática envolvida no processo de produção de *P. ostreatus*. Estudos futuros que contemplem as principais enzimas oxidativas e hidrolíticas, em ambas as fases (vegetativo e reprodutivo) do cultivo são de extrema importância para elucidação dos principais componentes da produtividade e eficiência biológica de *Pleurotus*.

De fato, a suplementação dos substratos para a produção de *Pleurotus* potencializa a EB e a qualidade dos cogumelos (Sánchez, 2010; Gaitán-Hernández et al., 2014; Liang et al., 2016; Pardo- Giménez et al., 2016; Xie et al. al., 2017). No caso da utilização de SMS de shiitake, algumas causas podem ser levantadas. Primeiramente, o SMS é um material que já foi colonizado pelo

micélio de shiitake, deixando a celulose, hemicelulose e lignina degradadas, facilitando a utilização destes componentes pelo micélio de *P. ostreatus*. Além disso, o SMS contém N orgânico que é facilmente absorvido e demonstrou substituir parcialmente ou integralmente o uso de farelos, como no substrato WS + SMS. Além do mais, segundo Wang et al. (2015), o SMS contém fatores de crescimento (vitaminas e minerais), provenientes do cultivo anterior de shitake, que podem ter aumentado a PR e EB.

5.4 CONCLUSÃO

A formulação do substrato com palha de trigo apresentou resultados produtivos superiores de *P. ostreatus* em comparação com a serragem de eucalipto.

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6. GENERAL CONCLUSIONS

It is possible produce *Pleurotus ostreatus var florida* 'MB', *Pleurotus ostreatus* 'SB', *Pleurotus sajor-caju* and *Pleurotus djamor* using *Cynodon dactylon* and SMS from *Lentinula edodes* as substrate formulation. The C/N ratio of 45/1 provided an average productivity of 20.5% and average biological efficiency of 84%.

The doses of Mn among 0 and 80 mg increased MnP enzymatic activity proportionally. However, they did not increase the productivity and biological efficiency of *Pleurotus ostreatus.*

Supplementation with 15% SMS provided productivity and biological efficiency of 25% and 107% in *P. ostreatus*, respectively, where under these conditions the most consumed nutrients were Ca and K.

When comparing different raw materials, the substrate formulation with wheat straw is superior in terms of production of *P. ostreatus* compared to sawdust. Regarding the supplementation of these raw materials, the SMS presents high results in terms of production in wheat straw, while rice bran is superior for the sawdust raw material.

7. FINAL CONSIDERATIONS

The results obtained in this research encourage us to make some considerations and suggestions for future work that may contribute to the improvement of the productive system of *Pleurotus* spp. as a whole, including:

The producers' approach of using any raw material or the one with greater availability in the region for the composition of the substrates, without considering its chemical and bromatological compositions and the recommendations of the literature, be revised. The results obtained by this work showed that productivity and biological efficiency depend on the composition of the raw material of the substrates and perhaps the use of raw materials whose chemical composition is unknown can compromise the production parameters.

The evaluation of other substrate preparation methods, such as composting + pasteurization, in order to introduce SMS during the stages of substrates' production process. In addition, researches that evaluates the feasibility of storage and logistics of using SMS is essential for the future use of this material in the commercial production of substrates. As one of the possible ways to increase productivity and biological efficiency of *Pleurotus* production, it is suggested that future research be carried out seeking a deeper understanding of the chemical composition of the raw materials used and the possibility of applying calcium and potassium in the cultivation substrate.

It is also suggested to carry out a screening of the enzymatic profile involved in the vegetative phase, for example evaluation of lignin peroxidases and laccases, as well as during the reproductive phase endoglucanases and cellulases are evaluated, since they are involved in the degradation processes of lignocellulosic compounds and play important roles in mushroom production. Finally, given the different behavior of the species and strains cultivated in the country, he suggests that studies should be developed that contemplate their nutritional and biochemical behaviors, seeking to maximize the enzymatic efficiency of the degradation of lignocellulosic compounds.

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