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Carbon-to-nitrogen ratios for *Agaricus brasiliensis* on the axenic method

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ABSTRACT. Cultivation techniques for *Agaricus brasiliensis* (composting method) and substratum formulations are the same ones used for *Agaricus bisporus*. Most of the carbon-to-nitrogen (C:N) ratios reported for *A. brasiliensis* are similar to those used for *A. bisporus* on the composting method and there are few studies about the variation of C:N ratios for *A. brasilienses* on the axenic method. The objective of this study was to verify the mycelial growth of *A. brasiliensis* on different C:N ratios using regional by-products as substrate formulation on the axenic method. Studied C:N ratios of substrate (mixture of soybean and cassava fibers) ranged from 11:1 to 248:1, with nitrogen content ranging from 4.25 to 0.20%, respectively. It was concluded that substrate with only soybean fiber generates higher mycelial growth than any formulation with cassava fiber; the highest mycelial growth on substrate is with C:N ratio of 11:1 (N = 4.25%); the intermediate growth is with C:N ratio range from 15:1 to 50:1 (N from 3.31 to 0.98%); and the lowest growth is with C:N ratio of 100:1 or higher (N ≤ 0.50%).

Key words: *Agaricus blazei*, mycelial growth, substrate, cultivation.

RESUMO. Relação carbono/nitrogênio do substrato pelo método de cultivo axênico para *Agaricus brasiliensis*. As técnicas de cultivo do *Agaricus brasiliensis* (método de compostagem) e formulação de substrato são as mesmas utilizadas para o *Agaricus bisporus*. A maioria das relações carbono/nitrogênio (C/N) relatadas para *A. brasiliensis* são similares às usadas para *A. bisporus* no método de compostagem. Há poucos estudos sobre a variação da relação C/N para *A. brasilienses* para o método axênico. O objetivo deste trabalho foi verificar o efeito de diferentes relações C/N no crescimento micelial de *Agaricus brasiliensis* utilizando subprodutos regionais como substrato pelo método axênico. As relações C/N no substrato (misturas de fibra de soja e de mandioca) estudadas variaram de 11 a 248 com consequentes concentrações de nitrogênio de 4,25 a 0,20%, respectivamente. Concluiu-se que os substratos somente com fibra de soja propiciam maior crescimento micelial que qualquer mistura com fibra de mandioca; o crescimento micelial é maior no substrato com relação C/N de 11 (N = 4,25%), intermediário na faixa de relação C/N entre 15 e 50 (N = 3,31 e 0,98%) e menor na relação C/N de 100 ou superior (N ≤ 0,50%).

Palavras-chave: *Agaricus blazei*, crescimento micelial, substrato, cultivo.

Introduction

Agaricus brasiliensis Wasser et al. (*A. blazei* Murrill *sensu* Heinemann), (WASSER et al., 2002), also denoted *A. subrufescens* (KERRIGAN, 2005), is a fungus native to Brazil (BRAGA et al., 1998; COLAUTO et al., 2002; DIAS et al., 2004; EIRA, 2003; WASSER et al., 2002) and it has been reported many therapeutic properties (KIM et al., 2005; KIMURA et al., 2004; MIZUNO, 2002; OSHIMAN et al., 2002; RODRIGUES et al., 2003; SOUZA-PACCOLA et al., 2004). It has been produced in Brazil for commercial purposes since 1990 and 90% of its current production is exported to Japan (EIRA, 2003). The *A. brasiliensis* cultivation techniques (composting method) and substratum formulations are the same ones used for *Agaricus bisporus* (BRAGA et al., 1998; SÁNCHEZ, 2004). However, because *A. brasiliensis* is a fungus from tropical origin, the cultivation techniques need to be adapted to its own characteristics (EIRA, 2003; MANTOVANI et al., 2007). One of the most important aspects for a good mycelial growth is the substratum source, which directly affects the bioconversion ability of raw material and consequently the productivity. It is important as well to develop a substratum formulation from available local organic sources, fully taking advantage of regional by-products (EIRA et al., 2005), and also reducing transportation costs (DIAS et al., 2004).

Brazil is one of the biggest producers of soybean (*Glycine max*) and cassava (*Manihot esculenta*). In 2006, the national production of soybean and cassava was, respectively, more than 52 and 26 million tons (IBGE, 2007). Soybean and cassava fibers resulting from industrialization process are generally used as animal feed, burned or dumped on the soil, increasing environmental damages associated to agricultural and industrial activities in Brazil (LEONEL et al., 1999). These by-products could be a sustainable alternate method to reduce environmental pollution and aggregate value as components of a substratum for *A. brasiliensis*.

Soybean and cassava fibers present different nitrogen (N) content. The excess or lack of N content in the substrate may be a limiting factor to fungus growth (CARLILE; WATKINSON, 1995). According to Rajarathnam and Bano (1989) there is a reduction of substratum degradation when N is excessively added. There is also a great variation of N content and carbon-to-nitrogen (C:N) ratios in substrate from agricultural or agro industrial by-products. A C:N ratio of 20:1 is related as suitable for most fungi (CHANG; MILES, 1989). For *Agaricus bisporus* C:N ratio variation is from 15:1 to 17:1 (MACCANNA, 1984), for *Pleurotus fabellatus* is from 30:1 to 117:1 (SRIVASTAVA; BANO, 1970), for *Pleurotus tuber-regium* is from 18:1 to 36:1 (WU et al., 2004), for *Pleurotus ostreatus* is around 85:1 (RAJARATHNAM; BANO, 1989), for *Ganoderma lucidum* is from 70:1 to 80:1 (HSIEH; YANG, 2004) and for *Lentinula edodes* is around 25:1 (OEI, 1996). Although research is extensive and advanced for several of these fungi, there is no studies of nitrogen sources or optimal range of C:N ratios use on axenic method for *A. brasiliensis*. Most of C:N ratios reported for *A. brasiliensis* are similar to those used for *A. bisporus* on composting method (ANDRADE et al., 2007; BRAGA et al., 1998; DONINI et al., 2006; EIRA, 2003; EIRA et al., 2005; KOPYTOWSKI FILHO; MINHONI, 2003). However Mantovani et al. (2007) have reported a study about variation of C:N ratios for *A. brasilienses* with mineral nitrogen sources on axenic method.

The axenic method is considered expensive because the substratum has to be sterilized but it was not found any report comparing the feasibility of axenic and composting method for *A. brasiliensis* cultivation. Most disadvantages on composting method are considered advantages on the axenic method. It is because the composting method is often empiric and presents handling difficulties like process standardization and control in relation to temperature, moisture and micro biota (STRAATSMA et al., 2000); reduction of raw material during composting process (HERNÁNDEZ et al., 2003); need of ammonium neutralization after pasteurization (VAN GRIENSVEN, 1988) and damages by *Sciaridae* larvae (EIRA et al., 2005; MENZEL et al., 2003). Thus other alternate methods, which are more efficient, inexpensive, standardized and simplified for preparing the substratum formulation, should be studied in order to optimize *A. brasiliensis* production in Paraná State, Brazil.

Considering the limited knowledge of the *A. brasiliensis* cultivation techniques, generally adapted from *A. bisporus* cultivation technique (BRAGA et al., 1998), the objective of this study was to verify the mycelial growth of *A. brasiliensis* on different C:N ratios using regional by-products as substratum formulation on the axenic method.

Material and methods

This experiment was carried out in the Molecular Biology Laboratory of the Paranaense University (UNIPAR), Campus I of Umuarama, Parana State, Brazil. *Agaricus brasiliensis* ABL97/11 strain from the Molecular Biology Laboratory Fungus Collection of UNIPAR was used. The fungus was kept at 20°C on solid malt extract medium (24 g L^{-1}), cut, and transferred to 90 mm Petri dishes with the solid malt extract medium - MEA- (45 g L^{-1}), and incubated in the dark at 28°C. After that, three dishes were selected to inoculate all experimental treatments.

Soybean and cassava fibers were used as raw materials for substratum formulation (culture media). Dried soybean fiber was provided by Solae do Brazil. Cassava fiber was obtained from tubercles that were peeled, washed and grounded in a mixer. The obtained mass was transferred to a fabric filter and washed until the washing water was completely clear. The resulting cassava fiber was dried with air circulation at 45°C for 10h. Both fibers were grounded in a mixer, sieved until granules were smaller than 355 μm, and kept in a freezer at -70°C.

Both fibers were analyzed for moisture content at 105°C, total nitrogen content (Kjeldahl's method), and ash content at 550°C (LARA et al., 1976). All determinations were performed in triplicate. C:N ratios were calculated according to Gerrits (1988), considering the carbon content to be 50% of the organic matter (total dried mass minus ash content multiplied by 0.5). From these data, substrate used in the treatments were compound of 30 g of fiber mixture and 15 g of agar per liter of distilled water in order to achieve each C:N ratio desired. Substratum made of

malt extract agar (45 g L^{-1}) and only agar (15 g L^{-1}) was used as positive and negative controls, respectively. Each substratum was sterilized in autoclave at 121°C for 20 min. After that pH was adjusted to 6 using NaOH or HCl solutions which had previously been sterilized by filtration (0.22 μm pore size filter). All treatments were quadrupled.

Substrate containing fiber mixtures and positive and negative controls were inoculated with five millimeter diameter cylinders taken from the peripherical area of the fungal growth on MEA. Inoculum was placed in the middle of each Petri dish (treatment) and special care was taken to keep the cylinder mycelium in direct contact with each substrate. All steps of the experiment were carried out under aseptic conditions in a laminar flow chamber. Inoculated dishes were randomly arranged for mycelia growth in a chamber with air circulation and controlled temperature at 28.0°C ± 0.1°C.

For each replication, the mycelial growth was verified by calculating the average of four different measurements of the diameter (in mm) 17 days after inoculation. Four replications were made to calculate the mycelial growth diameter average for each treatment. Obtained data were evaluated using variance analysis and significant differences were determined by Tukey's test with a significance level of $p < 0.05$. Levels of mycelial density among the treatments were compared visually and registered at the end of the experiment.

Results and discussion

The analysis results of the soybean fiber, N and ash dry matter contents were 4.25 and 3.41%, respectively; for cassava fiber, 0.20 and 0.75%; and for MEA medium, 2.90 and 4.07%. Substratum formulations with these regional by-products were prepared based on these results (Table 1). Based on that MEA culture medium was calculated with C:N ratio of 17:1 and agar medium was considered inert to all treatments and with a estimated C:N ratio of 500:1.

Table 1. Carbon and nitrogen content (dry matter) and carbon-to-nitrogen (C:N) ratio of the substratum formulations with soybean and cassava fibers (30 g L^{-1}) and agar (15 g L^{-1}).

Treatment	Soybean fiber (g L^{-1})	Cassava fiber (g L^{-1})	Total fiber (g L^{-1})	Carbon* (%)	Nitrogen (%)	C:N
T1	30.00	0.00	30.00	48.30	4.25	11:1
T2	23.00	7.00	30.00	48.61	3.31	15:1
T3	16.80	13.20	30.00	48.88	2.47	20:1
T4	10.80	19.20	30.00	49.15	1.66	30:1
T5	7.70	22.30	30.00	49.28	1.24	40:1
T6	5.80	24.20	30.00	49.37	0.98	50:1
T7	2.20	27.80	30.00	49.53	0.50	100:1
T8	0.97	29.03	30.00	49.58	0.33	150:1
T9	0.00	30.00	30.00	49.63	0.20	248:1

*Carbon (%) = calculated according to Gerrits (1988).

A. brasiliensis presented three significantly ($p < 0.05$) different growing groups in function of C:N ratio and N content (Fig. 1). The maximum mycelial growth was obtained on the substratum formulation with soybean fiber only (C:N = 11:1; N = 4.25%), however, this fungus, apparently, can grow in higher N contents, as reported by Mantovani et al. (2007), which were not tested in this study because of the natural N limitation presented in the fibers used. In substratum formulations with C:N ratios that ranged from 15:1 to 100:1 (N from 3.31 to 0.50%) mycelial growth was constant, with no significant ($p < 0.05$) difference. However there was a lower mycelial growth in substratum formulations with C:N ratios higher than 100:1 (N < 0.50%). In the substrate with C:N ratio of 15:1 (about 23% of cassava and 77% of soybean fiber; Fig. 1) there was a significant ($p < 0.05$) reduction of the mycelial growth when compared to the substrate with C:N ratio of 11:1 (100% of soybean fiber). This may not be related just to N content difference. We suppose that cassava fiber may contain fungus growth inhibiting compounds even after autoclaving. On the other hand, the higher mycelial growth in the substratum formulation with soybean fiber only may be related to polysaccharides composition, mainly arabinogalactanes, whereas cassava fiber contains mostly starch and cellulose (LEONEL et al., 1999). Besides, soybean fiber is more porous and has higher water absorption capacity, approximately 10 g of water for each gram of fiber, whereas cassava fiber has 4 g of water for each gram of fiber (LINDE; MACHADO, 2001), providing a better enzymatic access and higher nutrient absorption to mycelial growth. Thus, the soybean fiber structure and the composition of protein and polysaccharides, associated to a higher N content, provided a more appropriate substrate to the development of *A. brasiliensis*.

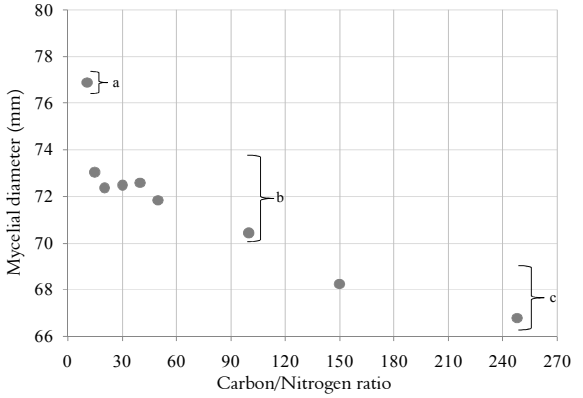

Figure 1. Average mycelial diameter of *Agaricus brasiliensis* ABL97/11 after 17 days of growth on substrate consisting of mixtures of soybean and cassava fiber with different carbon/nitrogen ratios and nitrogen content. Different letters represent significant differences ($p < 0.05$) in the mycelial growth.

The *A. brasiliensis* mycelial gowth ability to the higher N content in the substratum (C:N = 11:1; Figure 1) was not expected and it was higher than to other fungi like *Agaricus bisporus* (BRAGA et al., 1998; MACCANNA, 1984; VAN GRIENSVEN, 1988; ZHEN et al., 1995), *Lentinula edodes* (OEI, 1996; ROSSI et al., 2003), *Ganoderma lucidum* (HSIEH; YANG, 2004), *Pleurotus ostreatus* (RAJARATHNAM; BANO, 1989) and *Pleurotus tuber-regium* (WU et al., 2004). According to Maccanna (1984) it is recommended to *Agaricus bisporus*, when compost is used as substratum, a C:N ratio from 15:1 to 17:1, against a C:N ratio of 11:1 (N = 4.25%) to *A. brasiliensis* found in this study on axenic method. This capacity to use substrate containing high N contents is an advantage to *A. brasiliensis*, since high N contents can inhibit mycelial growth of other basidiomycete by repressing lignin degradation (KAMRA; ZADRAZIL, 1986). According to Macaya-Lizano (1988) substrate with high N contents and, consequently, low C:N ratios, did not provide the total substratum colonization and did not allow appropriate basidiocarp production for *Pleurotus* spp. Rajarathnam and Bano (1989) reported similar data, in which high N contents caused the increase of toxic substances in the culture medium, limiting the mycelial development and, consequently, basidiocarp production. However, *A. brasiliensis* was capable of keeping the vegetative growth with a high N contents (C:N = 11:1, N 4.25%; Figure 1), probably due to lignocellulolytic activity maintenance or yet the use of alternate enzymatic routes to keep carbon and nitrogen availability, suggesting that it is not a limitation for basidiocarp production. Zadrazil (1980) reported a relationship among basidiocarp protein content and its nutritional necessity and nitrogen availability in the substrate. This higher N metabolization capacity of *A. brasiliensis*, mainly when compared to *A. bisporus*, can be supported by the higher protein content in the basidiocarp, approximately 40% in dry matter (BRAGA et al., 1998). Other mushrooms, for instance, present inferior amounts of proteins in dry matter: *Pleurotus ostreatus* (30.4%), *Agaricus bisporus* (23.9%), *Volvariella volvacea* (21.2%), *Flammulina velutipes* (17.6%) and *Lentinula edodes* (13.4%) (MILES; CHANG, 1997).

Eira et al. (2005) reported that *A. brasiliensis* production, on composting cultivation method, with C:N ratio of 17:1, was lower due to the increase of the incidence of *Sciaridae* larvae. In axenic cultivation, it may occur only after the casing layer phase. Therefore if *A. brasiliensis*, in axenic cultivation, efficiently convert substrate with low C:N ratio (high N content) into basidiocarps, the use of this method for commercial purposes may become economically viable.

It was observed that the mycelial growth showed two distinct types of mycelia according to the level of mycelial density (Table 2). For C:N ratios from 11:1 to 50:1 (N from 4.25 to 0.98%), the mycelial growth was denser and similar to the one obtained in the MEA culture media (C:N = 17:1; N 2.90%; Table 2). For C:N ratios from 100:1 to 248:1 (N from 0.50 to 0.20%), the mycelium presented more disperse ramification (Table 2), which is typical in substrate with nutritional limitation, according to Carlile and Watkinson (1995). Comparing the mycelial growth in the substrate with C:N ratio of 100:1 to C:N ratio of 50:1, they were found equal ($p < 0.05$; Figure 1). However a more disperse mycelial ramification on C:N ration of 100:1 because of nutritional limitation to the mycelial growth showed the opposite (Table 2). The negative control (C:N = 500:1, estimated), with agar only, showed a mycelial growth with very disperse ramifications which indicates a complete draining of nutrients that were provided by the cylinder with MEA culture media (Table 2).

Table 2. Mycelial density of *Agaricus brasiliensis* ABL 97/11 after 17 days of growth on different carbon:nitrogen (C:N) ratios resultant from mixtures of soybean and cassava fibers. The greater number of asterisks, the higher mycelial density among treatments.

Treatment	C:N ratio	Nitrogen (%)	Level of mycelial density
T1	11	4.25	⋆ ⋆ ⋆
T2	15	3.31	⋆ ⋆ ⋆
T3	20	2.47	⋆ ⋆ ⋆
T4	30	1.66	⋆ ⋆ ⋆
T5	40	1.24	⋆ ⋆ ⋆
T6	50	0.98	⋆ ⋆ ⋆
T7	100	0.50	⋆ ⋆
T8	150	0.33	⋆ ⋆
T9	248	0.20	⋆ ⋆
T10	17	2.90	⋆ ⋆ ⋆
T11	500	0.10	⋆

Note: T10 = Positive control; T11 = Negative control (estimated values of C:N ratio and N).

Conclusion

Substrate with soybean fiber alone provide higher mycelial growth than any mixture with cassava fiber; the highest mycelial growth on substrate is with C:N ratio of 11:1 (N = 4.25%); the intermediate growth is with C:N ratio range from 15:1 to 50:1 (N from 3.31% to 0.98%); and the lower growth is with C:N ratio of 100:1 or higher ($N \leq 0.50\%$).

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