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Nutritional Requirements for Growth of *Agaricus brasiliensis*

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ABSTRACT. The nutritional requirements of *A. brasiliensis* in culture media were assessed by supplementing a basal medium (g L^{-1}): (glucose, 10, KH_2PO_4, 1, $MgSO_4.7H_2O$, 0.5, $[NH_4]_2SO_4$, 1, pH 5.5) with $CaCl_2$, trace elements ($FeSO_4.7H_2O$; $MnCl_2.4H_2O$; $ZnSO_4.7H_2O$; $CuSO_4.5H_2O$), casein, yeast extract, peptone, B-vitamins or amino acids. Evaluations were based on the mycelial growth in solid or liquid culture (mm day^{-1} or mg day^{-1}) and visual analysis of the colony. The addition of $CaCl_2$ and trace elements was very important for the major mycelial growth of the fungi. The addition of casein and inositol to the medium did not have a significant effect on growth. The best growth result in solid medium was obtained with the basal medium plus the addition of yeast extract and peptone. In relation to the other nutrient sources, the mycelial growth in the presence of amino acids darkened the medium after two weeks. The addition of B-vitamins to the basal medium lead to slower mycelial growth; however, growth was more visually dense when compared to other nutritional sources. B-vitamins added separately did not have the same result, suggesting that the fungus requires two or more vitamins at the same time for better mycelial growth.

Key words: mushroom, trace elements, B-vitamins, amino acids.

RESUMO. Requerimentos nutricionais para o crescimento micelial de *Agaricus brasiliensis*. Os requerimentos nutricionais de *A. brasiliensis* foram avaliados, com a suplementação de um meio basal (g L^{-1}): (glicose, 10, KH_2PO_4, 1, $MgSO_4.7H_2O$, 0.5, $[NH_4]_2SO_4$, 1, pH 5.5) com $CaCl_2$, micronutrientes ($FeSO_4.7H_2O$; $MnCl_2.4H_2O$; $ZnSO_4.7H_2O$; $CuSO_4.5H_2O$), caseína, extrato de levedura, peptona, vitaminas do complexo B ou aminoácidos. O crescimento micelial foi avaliado em meio sólido e líquido, considerando velocidade de crescimento e produção de massa micelial (mm dia^{-1} ou mg dia^{-1}) e análise visual da colônia. A adição de $CaCl_2$ e micronutrientes foi muito importante para o melhor crescimento micelial do fungo, enquanto que a adição de caseina e inositol não apresentou efeito significativo sobre o crescimento. O melhor crescimento em meio sólido foi obtido quando o meio basal foi suplementado com extrato de levedura e peptona. Quando o fungo foi cultivado no meio basal suplementado com aminoácidos, observou-se um escurecimento do meio após duas semanas de cultivo. A adição de vitaminas proporcionou um crescimento micelial mais lento no meio sólido, entretanto, mais denso em relação ao meio suplementado com outros nutrientes. Quando as vitaminas do complexo B foram adicionadas separadamente não se observou o mesmo resultado, o que sugere que o fungo requer duas ou mais vitaminas no meio para melhorar o crescimento micelial.

Palavras-chave: cogumelo, elementos-traço, vitaminas do complexo B, aminoácidos.

Introduction

Agaricus brasiliensis [*Agaricus blazei* Murrill] (WASSER et al., 2002) is a mushroom which grows naturally in the mountainous areas near the city of Piedade, São Paulo State, Brazil. It is known as the almond portobello, sun mushroom or Royal Sun Agaricus (SOUZA DIAS et al., 2004), princess mushroom and Himematsutake (ITOH et al., 1994). Several studies have demonstrated different polysaccharides and other substances from *A. brasiliensis* with anti-tumor and anti-mutagenic activity (DELMANTO et al., 2001; FUJIMIYA et al., 1999; ITOH et al., 1994; MIZUNO et al., 1990; TAKAKU et al., 2001; ZOU, 2005). Consequently, many enterprises in Brazil have promoted the production of this mushroom, aiming to export it to Japan, its principal consumer.

An essential step for successful commercial cultivation is to determine the nutritional factors that are necessary for better mycelial growth, fruiting and the enzymes involved in the process. The fruit body induction is not a well understood process (KÜES; LIU, 2000; MAGAE et al., 2005). Fruit body induction is influenced by different

factors, including the genetic make-up of the strain, environmental parameters and the nutrition of the growth medium (KÜES; LIU, 2000). Several basidiomycetes require one or more vitamins for vegetative growth and fruiting body development (CHANG; MILES, 2004). Shin et al. (1997) reported that thiamin was the active constituent in yeast extract added to the culture medium for fruit body formation. Saponin, a natural surfactant, and derivatives showed a hormone-like effect on the morphogenesis of *Pleurotus ostreatus* (MAGAE, 1999; MAGAE et al., 2005). Additionally, enzymes are connected to the regulation of fruiting body formation (KÜES; LIU, 2000).

In order to use agro-industrial residues in the production of the compost for cultivation of *A. brasiliensis* it is important to assess the nutritional factors which are necessary for better and faster mycelium growth and those that influence the formation of the fruiting bodies. Thus, it is vital to study the composting process used for compost production and the nutritional factors produced by the microorganisms present in the process. Preparatory to large scale composting and production trials using agro-industrial wastes this study evaluates the effect of different nutrients on the growth of *A. brasiliensis* in liquid and solid culture medium.

Material and methods

Microorganism and culture medium

Agaricus brasiliensis (strain CS6) was cultured on PDA prior to use in the study. A basal culture medium (BM) was used (SHIN et al., 1997) in all experiments (g L^{-1}): [glucose, 10, KH_2PO_4, 1, $MgSO_4.7H_2O$, 0.5, $[NH_4]_2SO_4$, 1). The nutritional requirements were determined by amending BM with $CaCl_2$ (0.5 g L^{-1}), trace elements (mg L^{-1}) ($FeSO_4.7H_2O$, 10, $MnCl_2.4H_2O$, 7, $ZnSO_4.7H_2O$, 4, $CuSO_4.5H_2O$, 1) (Synth, São Paulo, São Paulo State, Brazil) casein (0.3 g L^{-1}), yeast extract (0.1%), peptone (PEP; 0.1%) (Vetec, Rio de Janeiro, Rio de Janeiro State, Brazil), inositol (0.03 g L^{-1}), B-vitamins (VIT; 0.01%) or amino acids (0.01%) (Sigma, St Louis, MO, USA). The following vitamins (D-Pantothenic acid- B_5, 4-aminobenzoic acid- PABA/B_x, biotin- B_7, cyanocobalamin- B_{12}, riboflavin- B_2, pyridoxine- B_6, thiamine- B_1) and amino acids (arginine, histidine, isoleucine, leucine, phenylalanine, proline, threonine, tryptophan, tyrosine) were evaluated. In a subsequent experiment B-vitamins were tested separately using BM and compared with BM added with all B-vitamins together.

The assessment of $CaCl_2$, trace elements, casein and inositol were made both in solid and liquid BM culture medium, but the assessment of yeast extract, peptone, vitamins and amino acids were made only in solidified BM by measuring the mycelial growth in mm day^{-1} and visual analysis of the colony.

The solidified culture medium was inoculated in the center of the Petri dish with a 6-mm agar disc containing mycelium of *A. brasiliensis* isolate CS6. The plates were arranged in a completely randomized design with five replications per treatment. The cultures were incubated at 25°C for 7 days and the radius of each colony was measured using a ruler. A visual assessment of each colony was made at the end of the experiment. Data were converted to mycelial growth in mm day^{-1}.

The liquid culture medium (50 mL of media per flask) was inoculated with three 6-mm mycelial discs, excised from the actively growing edges of 10-day old cultures of *A. brasiliensis*, isolate CS6. Five replications per treatment were incubated at room temperature for 14 days and gently shaken by hand daily. After the incubation period, the media were filtered through analytical filter paper (Whatman) and the filter paper and its mycelial residue were dried under forced ventilation at 65°C for 24 hours or until the weight was constant.

Additionally, a CBM (complete basal medium with 0.1% yeast extract and 0.1% peptone added to BM) was used to evaluate the effect on growth by the supplementation with trace elements, casein and inositol. The pH was adjusted with HCl 0,1 M to 5.5 for all media. The nutritional requirements for *A. brasiliensis* were evaluated both in liquid and solid culture medium by assessing the mycelial growth in mg day^{-1} and mm day^{-1}, respectively.

Data were analyzed using SISVAR-UFLA (FERREIRA, 2000) and means were separated using Tukey's honestly significant difference test at 5% level.

Results and discussion

Effects of adding calcium to the basal medium

The addition of calcium to the culture medium was significantly ($p < 0.05$) important for the development of *A. brasiliensis*. The mycelium production was 9.2 mg day^{-1} when calcium chloride was added to the basal medium and 6.3 mg day^{-1} in the non-supplemented BM. Calcium has an important role in the maintenance of the integrity of membranes and a possible function in enzymatic activity, especially in the functioning of the microtubules and microfilaments (LANDECKER, 1996). In the literature normally does not include calcium in the culture media formulations because it

is considered a trace element that usually comes as an impurity in the reagent (CARLILE et al., 2001).

Effect of micronutrients to the mycelial growth of *A. brasiliensis*

The addition of trace elements to the basal culture medium significantly ($p < 0.05$) increased the daily mycelial production of *A. brasiliensis* (Figures 1 and 2). The average mycelial growth per day on solid medium was the same for both BM with trace elements and CBM (Figure 2). Both CBM and CBM with trace elements had significantly ($p < 0.05$) greater growth per day than the BM. The mycelial growth on solid CBM was always more dense and vigorous than on the solid BM, even when supplemented with trace elements. In liquid medium, BM with trace elements, CBM and CBM with trace elements produced significantly ($p < 0.05$) greater quantity of mycelia than BM alone.

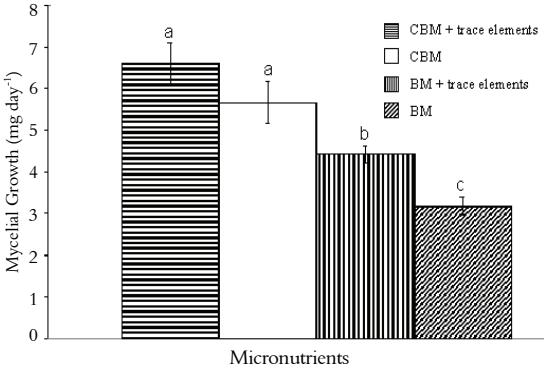

Figure 1. Mycelial growth (mg day^{-1}) of *Agaricus brasiliensis*, isolate CS6, in liquid basal (BM) or complete basal medium (CBM) with and without the addition of trace elements. Data represent the average ± SEM, n = 5. Means followed by the same letter are not significantly different (Tukey's test; $p \leq 0.05$).

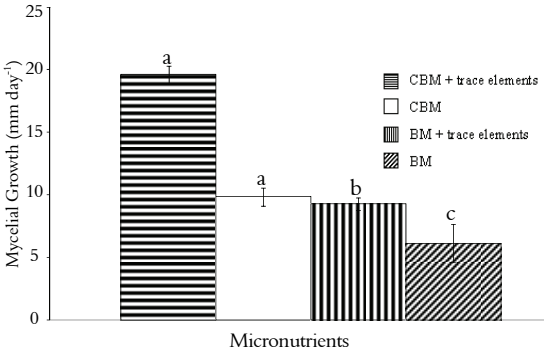

Figure 2. Mycelial growth (mm day^{-1}) of *Agaricus brasiliensis* isolate CS6, in solid basal medium (BM) or complete basal medium (CBM) with and without the addition of trace elements. Data represent the average ± SEM, n = 5. Means followed by the same letter are not significantly different (Tukey's test; $p \leq 0.05$).

However, the addition of trace elements to the CBM did not increase significantly ($p < 0.05$) the daily output (Figure 1). This probably happened due to the supplementation with yeast extract and peptone containing trace elements. We could conclude that there was always a tendency of better growth when trace elements were added in culture media either in solid or liquid form.

Not all fungi require the addition of trace elements to the culture media. However, they are essential to metabolism, acting as co-factors in the enzymatic activities. In those cases, it is assumed that these elements are supplied by reagents normally used for preparing media because these reagents are generally contaminated with sufficient quantities of trace elements (CARLILE et al., 2001). In our study, the basal culture medium alone was not sufficient, requiring the addition of trace elements. This requirement is also common in other cellulolytic fungi species, necessitating the addition of trace elements together with the major nutrients ordinarily used (LYND et al., 2002). The trace elements used in the basal medium to cultivate different basidiomycetes by Inglis et al. (2000) were similar to this study. Shin et al. (1997) also used the same trace elements to improve the growth of *Lentinula edodes*. Zou (2005) reported that Zn supplementation of 300 mg L^{-1} increased polysaccharide production in fermentation medium and anti-tumor activity of *A. brasiliensis*. Thus, increased concentrations of other trace elements may improve its biosorption and medicinal properties (MALINOWSKA et al., 2009).

Effect of adding casein and inositol, peptone, yeast extract, B-vitamins and amino acids

The addition of casein and inositol to the BM or CBM did not significantly ($p < 0.05$) affect the mycelial growth of *A. brasiliensis* in liquid culture (Figure 3). According to Eguchi et al. (1995), the addition of casamino acids and inositol is important for the formation of the fruiting body of *A. brasiliensis*. In eukaryotes, inositol is used for the production of phosphatidylinositol and derivatives, which are associated with the mechanisms of signal transduction of the cytoplasmic membrane (LEHNINGER et al., 1993). It is possible that *A. brasiliensis* is able to synthesize inositol or that the experimental conditions used here were not sensitive enough to measure its effect quantitatively or it may be important only for the fruiting body formation.

Additives to the basal medium affected significantly ($p < 0.05$) (Table 1) the rate of mycelial growth (mm day^{-1}). The addition of both peptone and yeast enabled the fastest growth. The addition of

yeast alone or in combination with peptone provided a significant source of nutrients for the growth of *A. brasiliensis.*

A rapid mycelial growth did not always mean that the overall growth was desirable. Treatments which permitted a relatively rapid growth such as BM with amino acids did not always show vigorous growth. When the growth was rapid, but less dense, this growth suggested that the nutritional conditions were not adequate. Additionally, where there was a less dense mycelium, e.g., in BM with amino acids, the culture medium darkened, indicating inadequate conditions for the metabolism of the fungus. In contrast, the BM with B-vitamins had a slower mycelial growth, but the growth was denser when compared to the BM with amino acids. This suggests that the addition of vitamins affected growth more than the addition of amino acids. Furthermore, when complex B-vitamins were added individually, this growth was not the same (Figure 4), suggesting that this fungus requires a combination of two or more vitamins for better mycelial growth.

Supplements are important for fungal growth. Besides carbon and nitrogen, these supplements are sources of amino acids and vitamins. According to Lynd et al. (2002), peptones and yeast extract are additional components, not usually necessary for agar media. Mantovani et al. (2007) indicated that the addition of nitrogen is very important in media or substrates poor in nitrogen. These authors concluded that nitrogen from urea enhanced *A. brasiliensis* mycelial growth better than ammonium sulfate. Fan et al. (2007) also reported that yeast extract, as a nitrogen source, was superior to peptone and inorganic nitrogen sources (such as KNO_3, $NH_4)_2SO_4$ and urea) for exopolysaccharide production by *A. brasiliensis*.

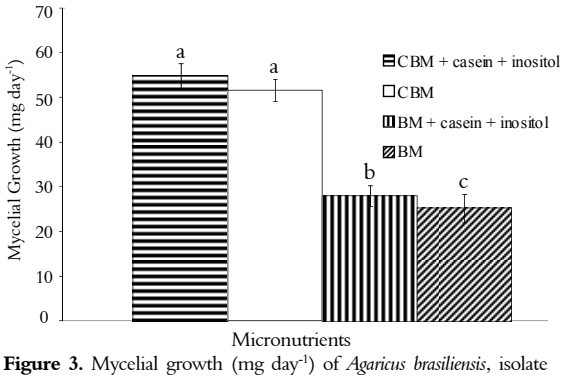

Figure 3. Mycelial growth (mg day^{-1}) of *Agaricus brasiliensis*, isolate CS6, in solid basal medium (BM) or complete basal medium (CBM) with and without the addition of casein and inositol. Data represent the average ± SEM, n = 5. Means followed by the same letter are not significantly different (Tukey's test; $p \leq 0.05$).

Conclusion

The addition of $CaCl_2$ and trace elements was very important for the major mycelial growth of the fungi. Despite the slower growth of *A. brasiliensis* on chemically defined medium this fungus did develop without the addition of complex sources of nutrients, thus permitting studies on the specific nutritional requirements of this fungus and enzyme activity.

Table 1. Mycelial growth (mm day^{-1}) of *Agaricus brasiliensis*, isolate CS6, in solid basal medium (BM) supplemented with different nutrients.

Treatments	Means[1] (mm day^{-1})
BM + peptone 0.1% + yeast 0.1%	3.4562 a
BM + yeast 0.1%	2.7937 ab
BM + amino acids 0.01%	2.2375 bc
BM + peptone 0.1%	2.0750 bc
BM	1.4812 c
BM + B-vitamins 0.01%	1.4187 c

[1]Means followed by a different letter are significantly different at the 5% level according to Tukey's honestly significant difference test.

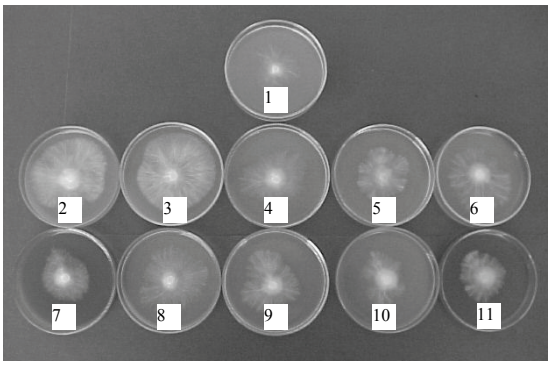

Figure 4. Mycelial growth of *Agaricus brasiliensis* CS6 in BM supplemented with different B-vitamin at 0.01%. 1: BM (control); 2: BM + YE 0.1%; 3: BM + PEP 0.1% + YE 0.1%; 4: BM + D-pantothenic acid; 5: BM + PABA; 6: BM + biotin; 7: BM + cyanocobalamin; 8: BM + riboflavin; 9: BM + pyridoxine; 10: BM + thiamine; 11: BM + all B vitamins.

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