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ORIGINAL PAPER

# Cytological studies of *Agaricus brasiliensis*

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**Abstract** *Agaricus brasiliensis* is a medicinal mushroom native to Brazil. It was first identified as *Agaricus blazei* and its scientific name continues to be debated. We examined the cytology of different Brazilian commercial strains of *A. brasiliensis* and the nuclear behavior of strain CS1 during basidiospore development using fluorescent microscopy. All strains have multinucleate hyphae and no significant differences in nuclei numbers were observed between them. Basidia from *A. brasiliensis* strain CS1 are typically tetrasporics and produce binucleate basidiospores, demonstrating that a postmeiotic mitosis occurs during basidiospore development. This result suggests that *A. brasiliensis* is primarily a heterothallic species.

**Keywords** Heterothallism · *Agaricus blazei* · *Agaricus subrufescens* · Nuclear behavior · Nuclei number · Basidiospore number · Life cycle

## Introduction

*Agaricus brasiliensis* ss. Heinemann is mushroom native to Brazil and has attracted the attention of many scientists in the world because of its medicinal properties (Kawagishi et al. 1989; Mizuno et al. 1990; Mizuno 1995; Ito et al. 1997; Kaneno et al. 2004; Mizuno and Kawakami 2006; Fan et al. 2007). This widely cultivated mushroom in Brazil was first identified as *Agaricus blazei* Murrill (Heinemann 1993), but Wasser et al. (2002) concluded that this Brazilian cultivated species was different from *A. blazei* identified by Murril in 1945. Thus, Wasser et al. (2002) proposed the name *A. brasiliensis* for the Brazilian species. However, Kerrigan (2005) proposed that the Brazilian *A. blazei* strains and the North American *A. subrufescens* were the same species. This controversy still remains (Wasser et al. 2005; Kerrigan 2007; Wasser 2007), but many scientists around the world have adopted Wasser's identification, and we will use the name *Agaricus brasiliensis* in the present work.

Kerrigan (2005) reported a reproductive micromorphology and genetic behavior of an *A. subrufescens* strain. However, no detailed cytological information is available from the Brazilian strains identified as *A. brasiliensis* or *A. blazei* ss. Heinemann. This study examines the cytology of different Brazilian commercial strains of *A. brasiliensis*.

## Materials and methods

### Strains and medium

This study was carried out in the Laboratory of Cytogenetics, Department of Biology, Federal University of Lavras, Brazil. Different strains (CS1, CS2, CS5 and CS7; Department of Biology, Federal University of Lavras) were used in experiments to determine nuclei number in the hyphal cells. These strains were obtained from different growers and locations in Brazil and were confirmed to show genetic diversity by RAPD molecular markers (Tomizawa 2007). For fluorescence microscopy

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experiments only strain CS1 was used. All strains were cultivated on CBM medium prepared by a modification of the media described by Shin et al. (1997) (containing per liter: glucose 10 g; peptone 1 g; yeast extract 1 g; $KH_2PO_4$ 1 g; $MgSO_4$. $7H_2O$ 0.5 g; $(NH_4)_2SO_4$ 1 g and agar 13 g).

### Nuclei in mycelium and cell sizes using light microscopy with Giemsa staining

The mycelium for Giemsa staining was obtained and prepared based on Roane (1952) and Robinow (1975) with modifications. The mycelium was grown in CBM solid medium covered with cellophane squares (2 × 2 cm). The mycelium was first fixed in methanol and then treated with HCl 1 M at 60°C for 10 min. Next, the material was first washed in water and then in 0.05 M phosphate buffer pH 6.8 before staining with 3% (w/v) Giemsa's stain for 2 h. Ten slides were prepared from each strain and 15 hyphal segments from each slide were examined for nuclei counts and cell measurements.

### Basidia and basidiospores cytology (light microscopy with diamidino phenylindole (DAPI) staining

Fruit bodies were harvested when the gills were exposed or unexposed to obtain samples of gill tissue for observation of basidiospore formation and nuclei. No stain was used for observation on basidiospore formation. Gill sections were placed on slides and covered with immersion oil and observed at 100× amplification. For nuclei staining, gills were obtained from basidiocarps after veil rupture, cut in small pieces, fixed in methanol for 3 min, hydrolysed with 1 M HCl for 10 min at 60°C, washed twice in distilled water and then stored in McIlvaine/$MgCl_2$ buffer. The gills were mounted in 40 µl Sybr Green 1 µl/ml. An Olympus BX60 microscope was used (40x amplification) for fluorescent microscopy (460–490 nm wavelengths). Photos were taken with a Nikon CoolPix 950 digital camera.

### Statistical analysis

Data for nuclei counts and cell measurements were analysed using SISVAR-UFLA (Ferreira 2000) and means separated according to Tukey's Honest Significant Difference test at the 5% level.

## Results and discussion

### Nuclei in mycelium and cell measurements

All *Agaricus brasiliensis* strains had multinucleate hyphae with an average of 5.8 nuclei per cell (Table 1, Fig. 1). No significant differences ($P < 0.05$) were observed between strainsfor the number of nuclei per cell (Table 1). Cells with more than six nuclei were frequently observed (Fig. 2); however, this was dependent on nuclei division as observed in Fig. 1. A multinucleate mycelium confirms that *Agaricus brasiliensis* has a nuclear organization different from typical dikaryotic basidiomycetes. *Agaricus* presents a genus with a nuclear behavior quite different from other dikaryotic basidiomycetes. *Agaricus bisporus* has fertile multinucleate mycelium (Raper et al. 1972; Kamzolkina et al. 2006), while *A. bitorquis* presents a homokaryotic multinucleate phase and a heterokaryotic binucleate phase (Raper 1976).

**Table 1** Nuclei number and cell measurements in *Agaricus brasiliensis* strain CS1 mycelium

| Strains | Nuclei per cell | Cell length (µm) | Cell diameter (µm) |
|---|---|---|---|
| CS1 | 5.67 a | 68.32 b | 4.77 b |
| CS2 | 5.34 a | 49.20 b | 4.38 b |
| CS5 | 5.48 a | 60.78 b | 5.45 a |
| CS7 | 6.12 a | 56.96 b | 5.33 a |

A mean followed by a different letter in the same column is significantly different at $P < 0.05$) according to Tukey's honest significant difference test. Each value represents a mean of 10 slides with 15 hyphal segments observed

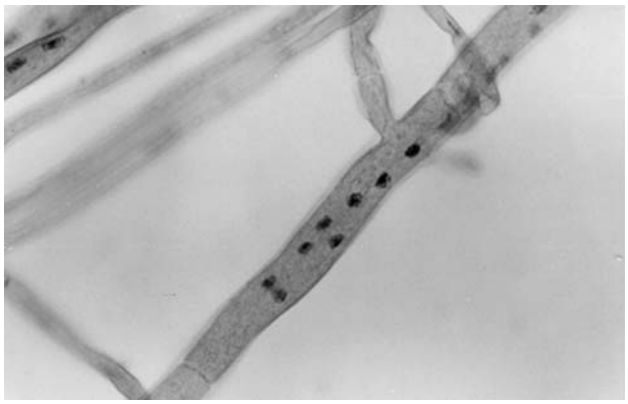

**Fig. 1** Multinucleate cell in vegetative mycelium of *Agaricus brasiliensis* CS1

Clamp connections were rarely observed in the fertile mycelium of *A. brasiliensis* (Fig. 3). Clamp connections are common in typical dikaryotic basidiomycetes and are an important morphological feature to distinguish between monokaryons and heterokaryons, but they are not common in some *Agaricus* species such as *A. bisporus* and *A. bitorquis* (Raper et al. 1972; Raper 1976).

A wide variation in the diameter and length of *A. brasilisensis* hyphal cells was observed (Figs. 4 and 5). Strains CS1 and CS2 had lower values for hyphal cell diameter while CS5 and CS7 had greater cell diameter

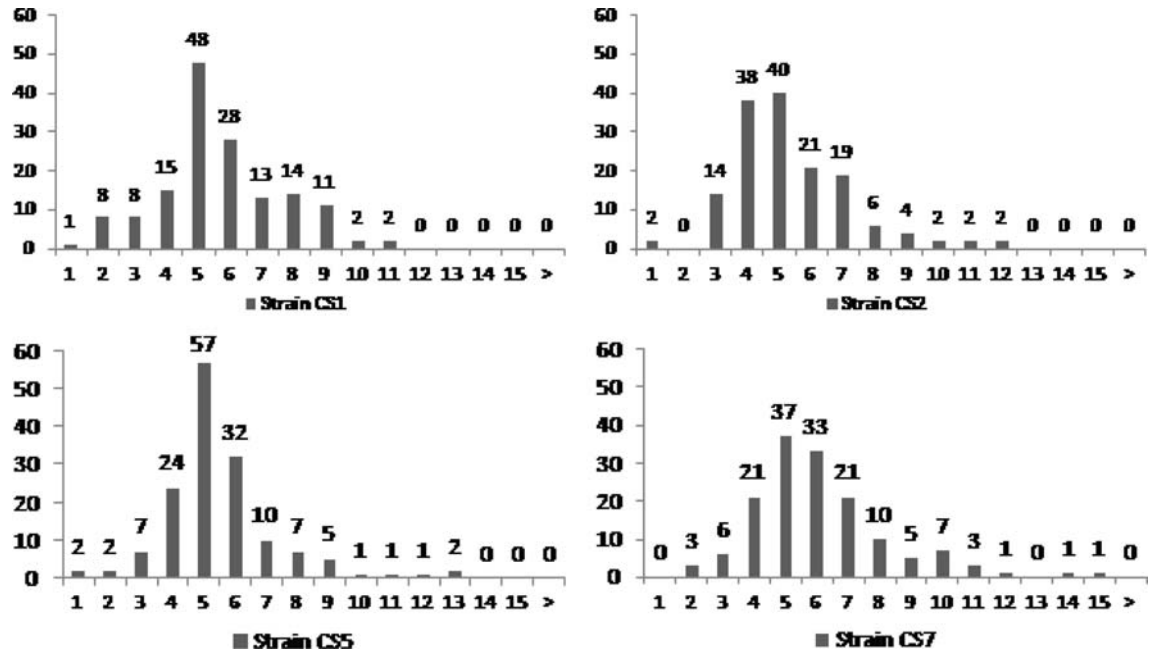


**Fig. 2** Histogram of frequency distribution of nuclei number per cell in vegetative mycelium of four strains of *Agaricus brasiliensis*. X-axis: nuclei number; Y-axis: frequency

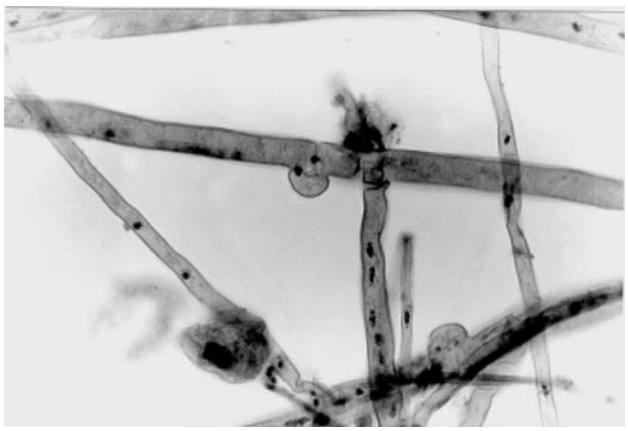

**Fig. 3** Clamp connections in vegetative mycelium of *Agaricus brasiliensis* CS1

(Table 1). It is interesting to note that strain CS2 had the lowest values for nuclear number, cell length and cell diameter. Tomizawa et al. (2007) analysed different isolates of *A. brasiliensis*, including CS1, CS2, CS5 and CS7, using RAPD markers and concluded that CS2 demonstrated the greater genetic divergence in relation to other isolates.

## Basidia and basidiospore cytology

Basidia from *A. brasiliensis* were typically tetrasporics (Fig. 6), although some trisporic basidia were observed. According to Kerrigan (2005) some strains of *A. subrufescens*, which he considered the same species as *A. brasiliensis*, tended to produce substantial numbers of bi- and trisporic basidia, but that tendency was not observed with our strain CS1, which was used for basidia cytology studies.

*Agaricus bisporus* var. *bisporus* normally produces bisporic basidia that are primarily secondary homothallic (Raper et al. 1972), but Callac et al. (1993) discovered a novel and tetrasporic variety of *A. bisporus* (var. *burnettii*) which is primarily heterothallic. Similarly, *A. bitorquis* is a tetrasporic species and behaves as typically heterothallic (Raper 1976). Saksena et al. (1976) suggested that a considerable variation in spore number per basidium exists between strains of *A. bisporus*. On the other hand, environmental conditions may affect spore numbers. Kerrigan and Ross (1987) reported that low temperatures influence basidial development, concluding that basidial spore number is not fixed in *Agaricus* but might change according to environmental conditions. Therefore, basidia with two or three spores will produce basidiospores receiving more than one postmeiotic nuclei and would produce a fertile heterokaryon if the nuclei are sexually compatible. Kerrigan and Ross (1987) suggested the term amphithallism as more appropriate to describe such a life cycle and Kerrigan (2005) considered *A. subrufescens* as an amphithallic species. A more detailed study with the Brazilian strains under various environmental conditions is necessary to delineate the percentage of trisporic and bisporic basidia in *A. brasiliensis*.

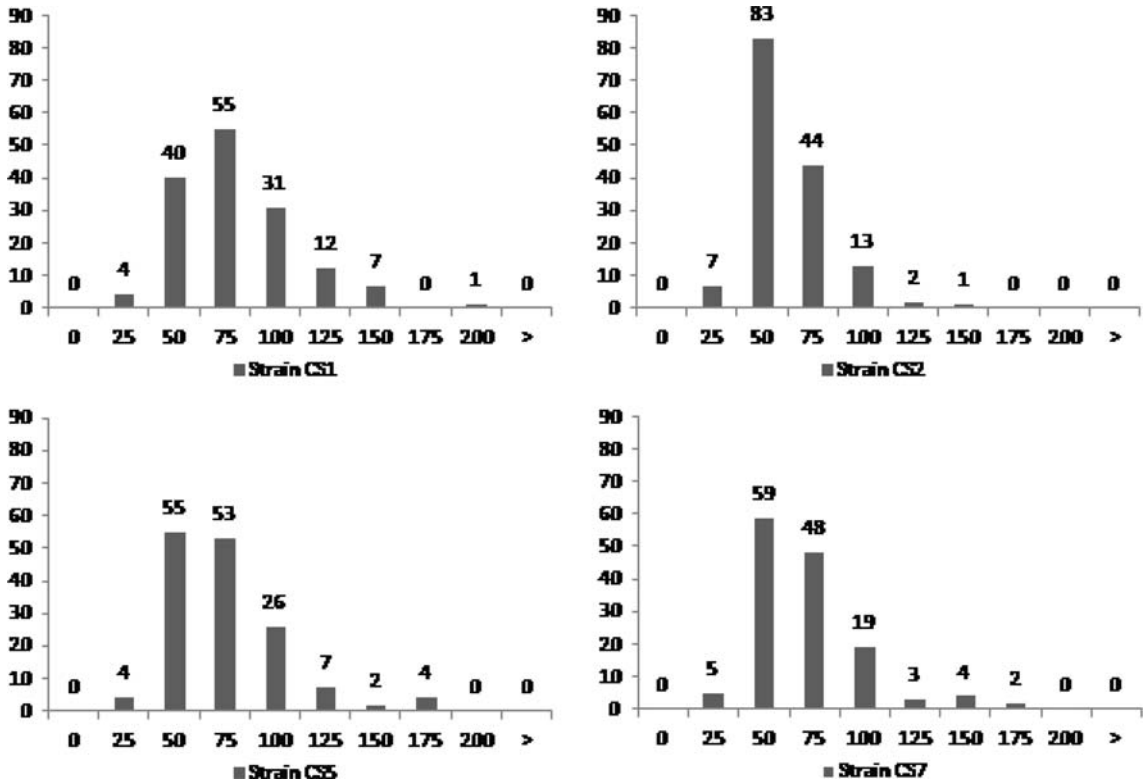


**Fig. 4** Histogram of frequency distribution of cell length (μm) in vegetative mycelium of four strains of *Agaricus brasiliensis*. X-axis: length; Y-axis: frequency

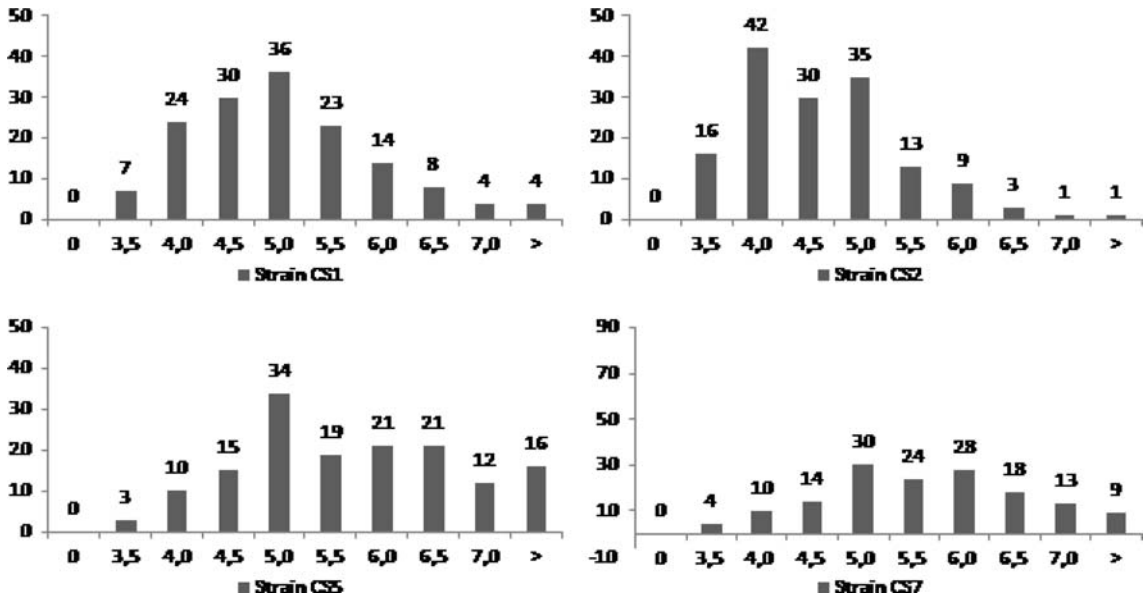


**Fig. 5** Histogram of frequency distribution of cell diameter (μm) in vegetative mycelium of four strains of *Agaricus brasiliensis*. X-axis: diameter; Y-axis: frequency

A postmeiotic mitosis occurs during basidiospore development in *A. brasiliensis*. We observed basidia with one, two and four nuclei, but not basidia with eight nuclei (Fig. 7), suggesting that the postmeiotic mitosis occurs in the basidiospores. If we had observed basidia with eight nuclei we would conclude that the postmeiotic mitosis

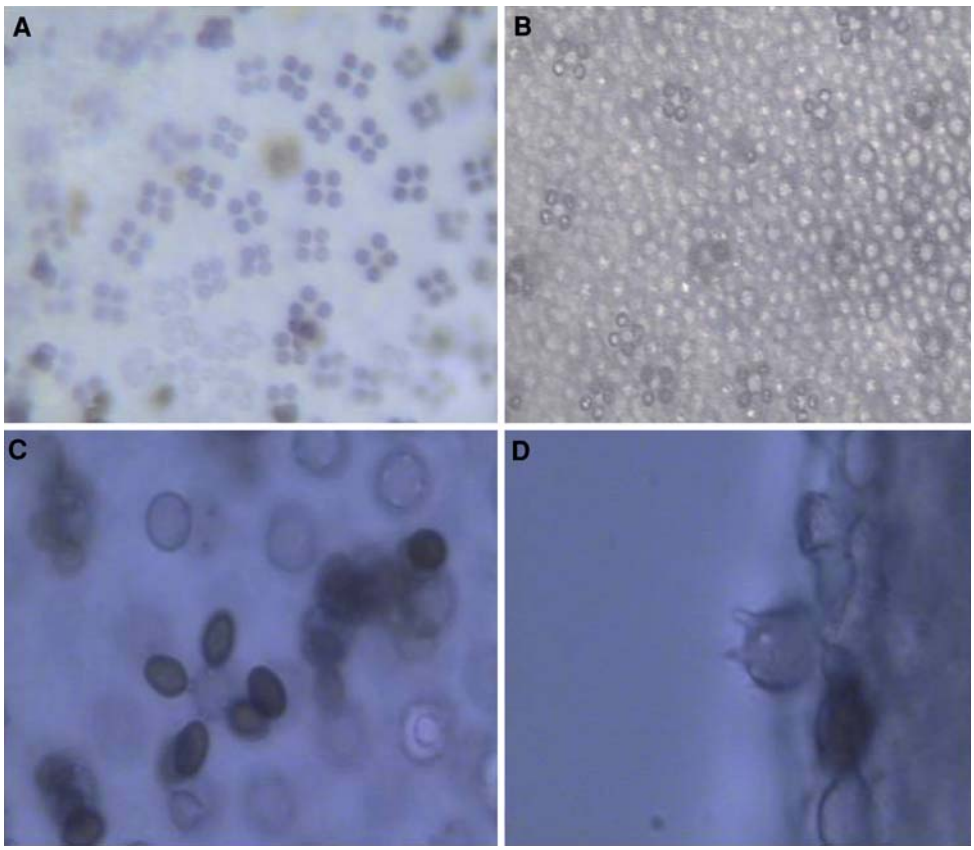


**Fig. 6** Basidia development in *A. brasiliensis* strain CS1. (**a**) Tetrasporic pattern; (**b**) Developing basidiospores; (**c**) Mature basidiospores; (**d**) Basidium after basidiospore discharge

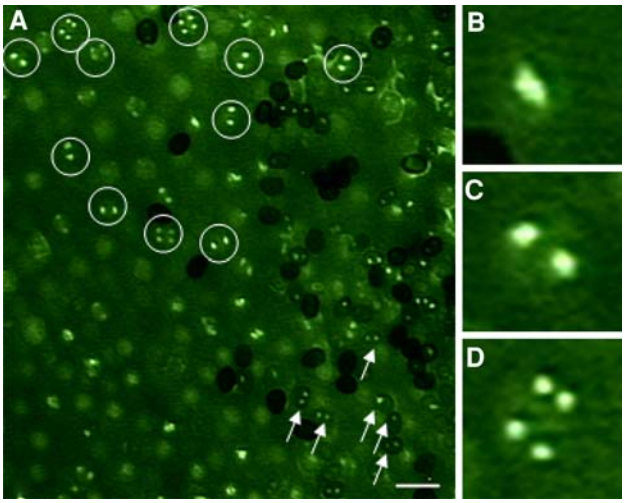


**Fig. 7** Fluorescence micrographs of basidia and basidiospores of *A. brasiliensis* strain CS1. (**a**) Gill surface in different developmental stage showing basidia with two or four nuclei (circles) and basidiospores with one or two nuclei (arrows). (**b**) Basidium in detail with one nucleus; (**c**) Basidium in detail with two nuclei; (**d**) Basidium in detail with four nuclei

occurs in the basidia but this was not observed. However, we observed basidiospores with one or two nuclei. A single nucleus indicates that each basidiospore receives only a post meiotic nucleus. And basidiospores with two nuclei indicate that after the basidium receives a postmeiotic nucleus a postmeiotic mitosis occurs in the basidiospore.

The observation of basidiospores with one and two nuclei (Figs. 7 and 8), disagrees with the pattern of postmeiotic mitosis occurring in the basidium as described by Tommerup et al. (1991). Therefore, there are two alternative patterns, known as pattern C and pattern D (Duncan and Galbraith 1972). In pattern C postmeiotic mitosis takes place within the basidiospores but a nucleus moves back into the basidium and the mature basidiospores are uninucleate. In pattern D postmeiotic mitosis takes place in the basidiospores but both nuclei remain in the basidiospores so that they are binucleate at maturity. We attempted to observe mature basidiospores after they were discharged but we were not successful, because they were too dark at maturity and did not fluoresce in the conditions tested. Nevertheless, we can deduce that the basidiospores of *A. brasiliensis* are normally homokaryotic and even if they are present in the pattern D they are functionally equivalent to uninucleate basidiospores (Horton 2006).

Considering the nuclear behavior and the predominance of tetrasporic basidia of strain CS1 we would tentatively suggest that *A. brasiliensis* is primarily a heterothallic species.

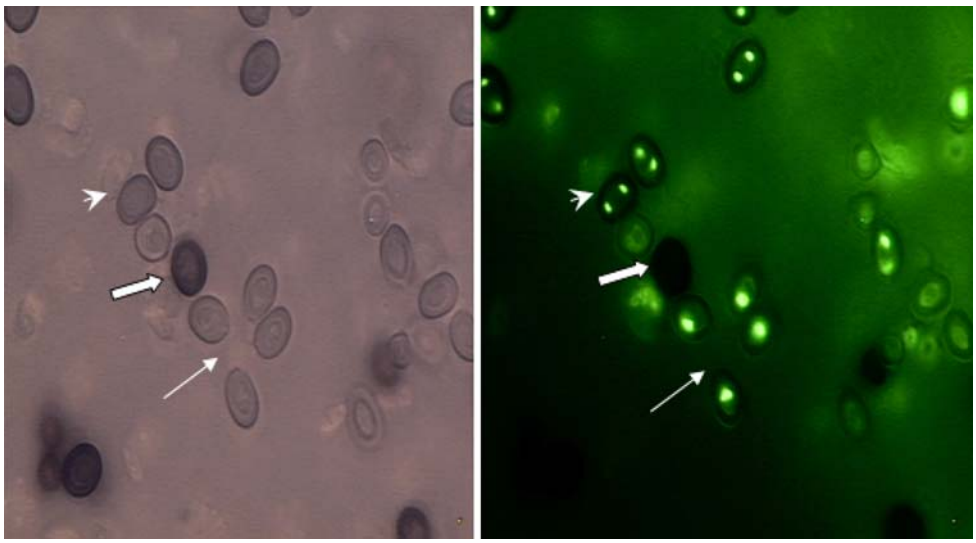

**Fig. 8** Basidia of *Agaricus brasiliensis*, strain CS1, showing four basidiospores with one larger nucleus each (thin arrow) and basidiopores binucleate (arrowhead). Dark spores did not fluoresce (thick arrow)

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