Obtaining monokaryotic and dikaryotic mycelial cultures of two Amazonian strains of Geastrum (Geastraceae, Basidiomycota)

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ABSTRACT

The high diversity of the genus Geastrum and the difficulty of obtaining mycelial cultures impairs the study of the ecophysiology and the exploration of the biotechnological potential of the taxon. In this study, different culture media were tested to obtain mycelial cultures for G. lloydianum and G. subiculosum collected in the Brazilian Amazon. Data on spore germination, and isolation of monokaryotic cultures and in vitro sexual reproduction are presented, as well as a brief morphological description of the cultures obtained. For both species, Potato Dextrose Agar (PDA) was the most promising of the tested culture media. The highest growth in agar culture ever recorded for this genus is reported (4.9 mm per week for G. lloydianum and 7.5 mm for G. subiculosum). In the PDA culture medium, spores germinated after 35-40 days of incubation and the isolation of monokaryotic cultures of the two species, as well as in vitro sexual crosses, were successfully performed.

KEYWORDS: earthstars; gasteroid fungus; Geastrum lloydianum; Geastrum subiculosum; mycelial growth

INTRODUCTION

Geastrum Pers. is one of the genera of Basidiomycetes popularly known as earth stars, due to the aspect of the exoperium, that forms rays on dehiscence, conferring a stellariform appearance to mature basidioma (Hemmes and Desjardin 2011; Jeppson et al. 2013). The genus is polythetic (Wilson et al. 2011), with approximately 120 species (Zamora et al. 2014), making it the most diversified in the family Geastraceae Corda (Hosaka et al. 2006). Collectively, members of the genus have the ability to colonize a wide variety of environments (Zamora et al. 2013), including sandy soils, and those rich in organic material, as well as acting as a decomposer of wood (Cortez et al. 2011) and having ectomycorrhizal associations (Karun and Sridhar 2014).

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Despite the many studies on Geastrum and the continuing discovery of new species (Hemmes and Desjardin 2011; Leite et al. 2011; Trierveiler-Pereira et al. 2011; Silva et al. 2011, 2013; Zamora et al. 2013, 2014, 2015; Cabral et al. 2014; Crous et al. 2015; Caffot et al. 2016), the monograph by Sunhede (1989) is, by far, the most complete study on this genus, as it not only considers the morphological aspects of the basidiomata, but also the isolation and the characteristics of the mycelial cultures of diverse European species.

Sunhede (1989) reported the slow in vitro growth of Geastrum cultures, describing a range of 0.5 to 3.7 mm per week for some species. Stoytchev et al. (2001), studying G. pouzarii V.J. Staněk, recorded growth of 3 to 4 mm in six weeks. Zamora et al. (2014) in a study of G. argentinum Speg., observed up to 4 mm of growth per week. All these authors considered, it is necessary to improve mycelial growth in this group, mainly due to its potential for antimicrobial, anti-inflammatory, astringent and anti-hemorrhagic activity (Guerra-Dore et al. 2007) in the biotechnological production of bio-active compounds (Liu and Zhang 2004) and in bioremediation (Chittaragi et al. 2013; Sevindik et al. 2017; Kuhar et al. 2016; Santana et al. 2016).

In this study, we tested different mycelial growth media for the Amazon strains of G. lloydianum and G. subiculosum, report spore germination and the successful rearing of monokaryotic and dikaryotic cultures, in vitro sexual reproduction for growth of 2 mm fragment from each culture, G. subiculosum and 2 mm of G. subiculosum, were individually isolated from sections removed from the pseudoparenchymatous layer of a fresh basidial exoperidium and fragments of approximately 1 × 1 mm were inoculated onto a 90 mm diameter Petri dish containing 15 mL of medium (PDA, Difco4), and then incubated at 25 °C, in the absence of light in a Biological Oxygen Demand (BOD) chamber. After one week, 2 × 2 mm agar blocks containing mycelium were harvested and placed on new plates of the same volume and content to give pure cultures.

**Evaluation of the mycelial culture medium**

Mycelial growth of G. lloydianum and G. subiculosum was tested in three solid culture media: Potato Agar Dextrose (PDA, Difco4), Malt Extract Pепtone Agar (MEPA) [3% Malt Extract (Becton Dickinson); 0.3% soy peptone (Acumedia); 1.5% agar (Becton Dickinson)], and Sabouraud Dextrose Agar (SDA) Becton Dickinson”. Culture media were autoclaved at 121 °C for 15 minutes and 15 mL poured into 90 mm diameter Petri dishes. A 2 × 2 mm fragment from each culture was transferred to the center of a Petri dish and maintained at 25 °C in the absence of light in the BOD chamber.

The experiment was conducted with a completely randomized design, using five replicates per treatment for each species. On experimental day 42, diameter of colonies and mycelial mass dry weight were measured, following Vargas-Isla and Ishikawa (2008). Mean colony masses and diameters were submitted to analysis of variance (two-way ANOVA) followed by a Tukey test, if significant, with level of significance set at p < 0.05. Statistical analyzes were performed with the ASSISTAT program (7.7 beta).

**Obtaining a monokaryotic mycelium**

Basidiospores of G. lloydianum and G. subiculosum were obtained from suspensions of spores in sterile distilled water, plus 20 μL of Tween 80 (Sigma-Aldrich”) stirred lightly on a Vortex® agitator. A 50-μL aliquot was then spread onto a Petri-dish surface, to test which culture provided the best dikaryotic growth medium for each species, and maintained at 25 °C, in the absence of light in a BOD chamber.

Germinated basidiospores were selected under an optical microscope and transferred to new Petri dishes of volume and content equal to those described above, then maintained under the same conditions and analyzed for growth and absence of connections over a five week period. For each species, the ten monokaryotic cultures showing the highest mycelial growth rates were selected, multiplied with transfer to new Petri dishes and used for crossing tests.

**Mycelial crossing**

Crosses were determined by pairing the ten monokaryotic cultures of each species. Fragments measuring 2 × 2 mm of the various monokaryotic cultures were inoculated in pairs, separated by distances of 2 mm from each other in Petri dishes with 15 mL of the culture medium that provided the best growth rates in the dikaryotic cultures. These were maintained at five pairs per plate with three replicates and kept at 25 °C in a BOD chamber in the absence of light and analyzed over a five-week period for the formation of clamp connections.
After confirming clamp connection formation in cultures of both species, the three cultures with the highest growth for each species were selected and the original crossing-derived dikaryotic mycelium grown-on in new Petri dishes to multiply the stock. After growth, five 2 × 2 mm fragments were inoculated into 125 mL capacity Erlenmeyer flasks, with 50 mL of the liquid of the culture medium (agar absent) which supported the best dikaryotic culture growth and, in triplicate, maintained in a BOD at 25 °C in the dark.

After five weeks growth, mycelia were separated from the medium by vacuum-pump filtration, washed three times in sterile distilled water and subjected to DNA extraction following Raeder and Broda (1985).

Amplification of the rDNA internal transcriber-spacer region (ITS) was performed with previously described primers and protocols (Gardes and Bruns 1993). PCR product purification was undertaken with ExoSAP-IT (Affymetrix Inc.), and sequencing was performed with the BigDye™ Terminator Cycle Sequencing Ready Reaction Kit GFX (Amersham Pharmacia®), using the same primers.

Sequences of *G. hariotii* Lloyd, *G. pectinatum* Pers., *G. triplex* Jungh. and *G. parvistriatum* J.C. Zamora & Calonge were added in order to test the effectiveness of crosses for species separation. *Myriostoma coliforme* (Dicks.) Corda was used as an outgroup. Sequences were first aligned with Clustal X 2.1 (Larkin et al. 2007), and then BioEdit (Hall 1999). An ITS-based distance matrix was calculated using the Kimura-2 parameter replacement model with MEGA (Tamura et al. 2013) to assess between species divergence.

Maximum parsimony (MP) phylogenetic analyses with ITS concatenation were performed. For this, PAUP (Swofford 1998) was used, and the trees assembled using a heuristic search for branch exchange using the TBR algorithm. The initial tree was obtained by stepwise addition of 100 repeated random sequences and 1000 bootstrap repititions. The resulting tree was edited with FigTree (http://tree.bio.Ed.ac.uk/software/figtree/).

**RESULTS**

**Taxonomy**

*Geastrum lloydianum* Rick, Brotéria 5: 26 (1906). Figure 1a-b.
≡*Geaster lloydianum* Rick (1906) [MB#528074]
≡*Geastrum saccatum* var. *lloydianum* (Rick) Rick (1961) [MB#349502]

Basidioma curved expanded, from 14 to 27 mm in height, including the peristomium, 14 to 27 mm in diameter, non-hygroscopic, fibrous papyraceous, cotonous, persistent fleshy mycelial layer, detaching itself in irregular brown sections. Endoprostheses dark grayish brown to dark brown, globose depressent, with apophysis present, sessile and short stipulate.

*Figure 1.* *Geastrum lloydianum:* A) Immature and expanded basidioma; B) Basidiospores. *Geastrum subiculosum:* C) Immature and expanded basidioma; D) Basidiospores. Scale bar: A; C = 1 cm; B; D = 3 µm. This figure is in color in the electronic version.
peristomium conical grooved, not delimited and concolorate with endoperidium. Basidiospores globose to subglobose, measuring 3.6 to 5.4 μm in diameter, slightly warty, apicule vermilion in presence of KOH.

Material examined: Brazil. Amazonas, Manaus. INPA Campus III, 18 II 2014. Santana, MDF. INPA 259923.

**Geastrum subiculosum** Cooke & Massee, Grevillea 15: 97 (1887). Figure 1c-d.

Immature basidioma subglobose to obovoid, with surface smooth to slightly wrinkled on the subicule, white yellowish to yellowish gray fading to beige. Basidioma expanded with persistent fleshy mycelial layer of yellowish brown to light yellow, with revolute rays cream. Endoperidium dark grayish brown, peristome mamiform, fibrillous, non-delimited, concolor with endoperidium saculiform expansion. Basidiospores small, globose to subglobose, measuring 2.4 to 3.3 μm in diameter, slightly warty, hyaline.

Material examined: Brazil. Amazonas, Manaus. INPA Campus III, 22 III 2014. Santana, MDF. INPA 259933.

**Evaluation of culture medium in dikaryotic growth**

Despite slow growth, cultures of the two species developed in all tested media (Figure 2). PDA and MEPA produced the best colony diameter values for *G. lloydianum*, with grow rates of some 4.5 and 4.9 mm per week, respectively. For *G. subiculosum*, PDA was the best medium, with growth of around 7.5 mm per week. For *G. lloydianum*, no significant statistical difference was observed in colony biomass between the culture media. However, for *G. subiculosum*, PDA and DAS produced the best results (Table 1).

### Table 1. Evaluation of growth in different culture media by diameter (mm) and biomass (mg) for *Geastrum lloydianum* and *G. subiculosum* over 42 days of incubation at 25 °C in the dark.

| Species              | Mycelial diameter (mm) | Colony biomass (mg) |
|----------------------|------------------------|---------------------|
|                      | PDA        | DAS      | MEPA     | PDA        | DAS      | MEPA     |
| *Geastrum lloydianum*| 22.3bA     | 11.7bb   | 24.7aA   | 0.07bA     | 0.05bA   | 0.04aA   |
| *G. subiculosum*     | 37.7aA     | 30.7aB   | 26.3aC   | 0.25aA     | 0.11aB   | 0.07aC   |
| CV (%)               | 10.27      |          | 20.02    |            |          |          |

BDA = Potato Dextrose Agar; DAS = Dextrose Agar Sabouraud; MEPA = Malt Extract Peptone Agar; VC = Coefficient of Variation. Letters with different suffixes indicate significantly different values among groups (ANOVA, p < 0.05). Small letters refer to comparison between isolates and capital letters, to comparison among treatments.

During the first week of cultivation, *G. lloydianum* and *G. subiculosum* hyphae showed little or no clamp branching of more distant connections. At 42 days, the cultures showed uneven margins, larger hyphae, numerous clamp connections, and the presence of chlamydospores in terminal and interim positions on the hyphae.

**Obtaining monokaryotic mycelia**

The germination period of basidiospores of *G. lloydianum* and *G. subiculosum* was similar, ranging from 35 to 40 days after incubation. Under these conditions, it was possible to isolate 84 monokaryotic mycelia of *G. lloydianum* and 96 of *G. subiculosum*.

Cultures grew, on average, 1.2 mm per week, but differed in mycelial morphology. Monokaryotic cultures of *G. lloydianum* on PDA were thicker, with a denser mycelium and a slightly irregular border, while *G. subiculosum* hyphae were thinner, less dense, with a thin appearance and irregular growth.

The slow development of the two species made it difficult to analyze *in vitro* pairings. However, the presence of multiple clamp connections in the resulting mycelium indicated dikaryotic mycelium establishment for all pairs (Figure 3), thus indicating sexual crossing (Taylor et al. 2000; Taylor et al. 2006). The dikaryotic mycelia resulting from *in vitro* crossings were grouped with the dikaryotic mycelia isolated from the respective basidiomas (Figure 4), showing that the method can likely be employed with other species of the group.

**DISCUSSION**

The slow *in vitro* growth rates of some *Geastrum* species was first reported by Sunhede (1989), who noted a growth rate variation of 0.5 to 3.7 mm per week. Stoytchev et al. (2001) reported growth of 3 to 4 mm after six weeks for *G. pouzarii* V.J. Staněk, and Zamora et al. (2014) observed growth of 4 mm per week for *G. argentinum* Spog. All these species were grown in a malt extract medium. In this same culture medium, *G. lloydianum* and *G. subiculosum* showed higher growth speeds of about 4.6 and 5.2 mm per week, respectively.
Both our species showed best results with PDA. This has also been observed for other Geastrum species (G. schweinitzii (Berk. & M.A. Curtis) Zeller, G. triplex and G. echinulatum B.D.B. Silva & Baseia (M.D.F. Santana, unpublished data). Although the values are still low, means for mycelial growth in PDA were the most promising recorded so far for the genus.

In vitro germination of sexual spores is the key first step in monokaryotic culture isolation, and can even be used to determine the fungal reproduction system (Anderson et al. 1980; Carvalho et al. 1997; Capelari and Fungaro 2003), a field of research still little explored for gasteroid fungi. However, the basidiospores of only a few species of Geastrum have germinated under laboratory conditions, the main examples to date being the few cases described by Sunhede (1989) and the report by Stoytchev et al. (2001) for G. pouzarii.

Even if germination rates are low (less than 1% in this study, for both species) these are the first records of basidiospore germination for both G. subiculosum and G. lloydianum. Basidiospore germination for gasteroid fungi, such as Pisolithus Alb. & Schwein, for example, ranges from 0.001 to 0.38% (Bulmer 1964; Silvério 2013). This may be the possible cause of the lack of success with previous reported attempts, such as by Kuhar and Papinutti (2009) for G. episcopale Kuhar & Papin, synonym for G. violaceum Rick.

![Figure 3. In vitro crossover for two species of Geastrum. A-C) G. subiculosum; D-F) G. lloydianum, where A) and D) is the monokaryotic mycelium 35 days after incubation; B) is the monokaryotic and dikaryotic mycelium resulting from crossover; E) is the dikaryotic mycelium resulting from crossover; C) and F) show connection clamps indicating success at crossover. MO = monokaryotic mycelium; DI = dikaryotic mycelium. This figure is in color in the electronic version.](image)

![Figure 4. Molecular similarity between basidioma-derived dikaryotic mycelia (square) and dikaryotic mycelia derived from in vitro crossing (triangle) for Amazonian strains of Geastrum lloydianum and G. subiculosum. The tree was constructed based on the rDNA ITS region sequences (ITS1, 5.8S and ITS2).](image)
Low germination rates under laboratory conditions were historically reported in the literature for gasteroid fungi (Bulmer 1964; Fries 1978, 1987; Carvalho et al. 1997; Silvério 2013). For *Rhizopogon roseolus* (Corda) Th. Fr., the first germination may take two to three weeks (Kawai et al. 2008) and for *Pisolithus arhizus* (Scop.) Rauschert., eight weeks after incubation (Carvalho et al. 1997).

**CONCLUSIONS**

PDA culture medium was the most promising for mycelial growth of Amazonian strains of *Geastrum lloydianum* and *G. subiculorum*. This culture medium was also effective for the germination of basidiospores and for obtaining monokaryotic cultures. It is necessary to improve the production of *Geastrum* mycelial cultures, as well as to apply the methodology used in here to other species, in order to broaden the evaluation of its applicability for ecophysiological and biotechnological studies of *Geastrum*.

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