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Challenges in the *in vitro* establishment of *Guadua glomerata* Munro, a bamboo native from the Amazon

Desafios no estabelecimento *in vitro* de *Guadua glomerata* Munro, um bambu nativo da Amazônia

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Abstract

This study aimed to establish *in vitro* protocols using nodal segments and inflorescences of *Guadua glomerata*, a bamboo native to the Amazon. To reduce contamination of nodal segments introduced *in vitro*, the culture medium was supplemented with different concentrations of the biocide PPM®. For inflorescences introduced *in vitro*, the surface disinfestation methodology was not effective, which made it impossible to analyze the influence of 6-Benzylaminopurine (BAP). Fungi from contaminated flasks were identified as possible fungi of the genera *Trichoderma* sp., *Colletotrichum* sp., and *Arthrobotrys* sp. in nodal segments introduced *in vitro*, while *Fusarium* spp. was identified in inflorescences introduced *in vitro*. We conclude that it is necessary to test different disinfestation methodologies for the *in vitro* establishment of *G. glomerata*. Furthermore, the identification of the fungi found in our study opens the possibility of using specific fungicides in future disinfestation attempts and thus improving the effectiveness of the process.

Keywords: Biotechnology; Plant tissue culture; Micropropagation; Bambusoideae.

Resumo

Este estudo objetivou o estabelecimento *in vitro* utilizando segmentos nodais e inflorescências de *Guadua glomerata*, um bambu nativo da Amazônia. Para a redução da contaminação dos segmentos nodais introduzidos *in vitro*, o meio de cultura foi suplementado com diferentes concentrações do biocida PPM®. Para as inflorescências introduzidas *in vitro*, a metodologia de

desinfestação superficial não apresentou eficácia, o que impossibilitou analisar a influência da 6-Benzilaminopurina (BAP). Fungos provenientes dos frascos contaminados foram identificados como possíveis fungos dos gêneros *Trichoderma* sp., *Colletotrichum* sp., e *Arthrobotrys* sp em segmentos nodais introduzidos *in vitro*, enquanto *Fusarium* spp. foi identificado nas inflorescências introduzidas *in vitro*. Concluímos que é necessário testar diferentes metodologias de desinfestação para o estabelecimento *in vitro* de *G. glomerata*. Além disso, a identificação dos fungos encontrados em nosso estudo abre a possibilidade de utilizar fungicidas específicos em futuras tentativas de desinfestação e assim aprimorar a eficácia do processo.

Palavras-chave: Biotecnologia; Cultura de tecidos vegetais; Micropropagação; Bambusoideae.

1. Introduction

In vitro propagation is a biotechnological tool that has enabled large-scale seedling production. This technique allows the clonal production of plants from small fragments of the plant (known as explants), which are inoculated in a culture medium under aseptic conditions and controlled environmental conditions, such as light, temperature, relative humidity and CO_2. In addition, *in vitro* propagation enables the production of uniform, season-independent and disease-free seedlings, and can be applied to several plant species (Mehbub et al., 2022).

In vitro propagation is carried out in a few steps, initially with the choice of plant material, *in vitro* establishment, *in vitro* multiplication, *in vitro* or *ex vitro* rooting and acclimatization of seedlings, where the aim at the end of the process is to obtain complete and healthy seedlings (George et al., 2008; Bridgen et al., 2018).

In vitro establishment is a crucial step to obtain an effective *in vitro* propagation protocol. At this stage, it is necessary to use chemical agents at varying concentrations and times to disinfect the explants and eliminate microorganisms which can present limitations for aseptic cultivation (Esposito-Polesi, 2020). The main agents used for this purpose are washing the explants and immersion in sodium hypochlorite, alcohol, antibiotics, and fungicides (Liang et al., 2019; Abdalla et al., 2022). Additionally, Plant Preservative Mixture (PPM®) is a biocide that has been used to reduce contamination caused by bacteria and fungi (Kushnarenko et al., 2022; Grimaldi and Bastos, 2023).

Various tissue culture techniques have been applied for the propagation of various species, among them bamboo, which has economic and environmental

importance. Bamboos, belonging to the family Poaceae and the subfamily Bambusoideae, are classified as woody bamboos (tribes Arundinarieae and Bambuseae) and herbaceous bamboos (tribe Olyreae) (Soreng et al., 2022). Additionally, bamboo has various uses and applications, such as in the food, pharmaceutical, paper and pulp industries, crafts, furniture manufacturing, landscaping, construction, and its environmental importance for the recovery of deforested areas and soil conservation (Nirmala et al., 2018; Azevedo-Junior et al., 2019; Cardoso et al., 2019; Ahmad et al., 2023).

Species of bamboo from the genus *Guadua* Kunth are among those that have economic and environmental importance. Some studies involving *in vitro* propagation of species from this genus, such as *G. longifimbriata*, *G. angustifolia*, *G. chacoensis*, *G. magna*, and *G. latifolia*, already have well-established protocols (Costa et al., 2017; Ornellas et al., 2019; Nogueira et al., 2019; Leão et al., 2020). However, there are still no reports in the literature on the *in vitro* propagation of the species *G. glomerata*.

G. glomerata, popularly known as taboquinha, is a bamboo native to the Amazon with confirmed occurrence in the states of Acre, Amazonas, Amapá, Pará, Rondônia, and Roraima, occurring in areas such as Riparian or Gallery Forest, Igapó Forest, Terra Firme Forest, and Várzea Forest (Shirasuna et al., 2020). In addition, *G. glomerata* is characterized by having a climbing stem, a height of 8-12 m, and a stem diameter of 0.67-1.86 cm (-4.5 cm) (Shirasuna et al., 2020). Thus, studying the propagation of *G. glomerata* enables its use and application in programs for the recovery of deforested areas and, consequently, the conservation of this species.Therefore, the objective of this study is to use different explants of *G. glomerata* aiming at the *in vitro* establishment of this bamboo native to the Amazon.

2. Material and methods

2.1. Research development location

The research was carried out at the Biotechnology Laboratory of the Federal Rural University of the Amazon (UFRA), located in the state of Pará on the Belém campus. The laboratory is currently conducting research focused on the *in vitro*

propagation of endangered native species of the Amazon and ornamental and agricultural plants.

2.2. Plant material

The collection of plant material of *G. glomerata* was carried out at the Universidade Federal do Pará (UFPA), located in the metropolitan region of Belém/PA (Figure 1). Nodal segments and inflorescence of the mother plant with approximately 3 cm in length were collected and stored in antioxidant solution (citric acid 1 g L^{-1}) and taken to the Biotechnology laboratory at UFRA.

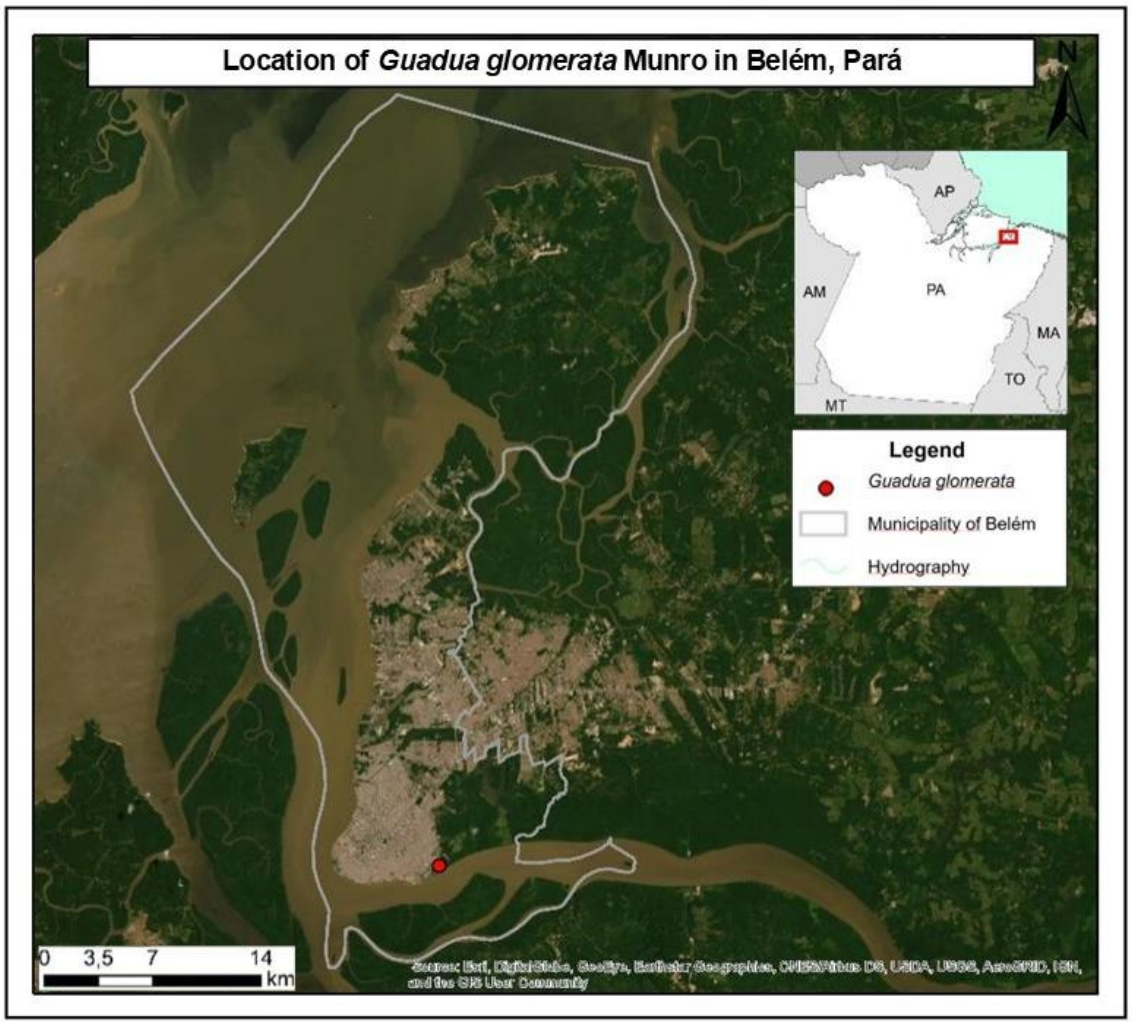

Figure 1. Location of *Guadua glomerata* at the Universidade Federal do Pará, in Belém, Pará. Source: Author (2021).

2.3. Disinfestation and *in vitro* introduction of nodal segments

The nodal segments were initially disinfected with running water and neutral detergent. Then, the nodal segments were immersed for 20 hours in a solution with 12.5% Bendazol® fungicide. Subsequently, in a laminar flow chamber, the explants were disinfected by immersion in 70% ethanol for 1 minute, followed by immersion in 2.0-2.5% commercial sodium hypochlorite solution with 3 drops of Tween 80® for 20 minutes. Then, the explants were washed three times with distilled and autoclaved water.

After disinfestation, the explants were inoculated into tubes containing 10 mL of MS culture medium (Murashige and Skoog, 1962) supplemented with 30 g L^{-1} of sucrose, 0.5 g L^{-1} of the antibiotic streptomycin sulfate, different concentrations of PPM® (0; 2.5; 5 and 7.5 mg L^{-1}) and 2 g L^{-1} of Phytagel® after adjusting the pH to 5.8. Subsequently, the culture medium was autoclaved at 121°C for 20 minutes. The tubes were capped and sealed with plastic film in the laminar flow chamber and subsequently kept in B.O.D with a photoperiod of 16-h at a light intensity of 55 μ mol m^{-2} s^{-1} and a temperature of 25 ±2 °C. The experiment was conducted in a completely randomized design, containing four treatments with five repetitions with two tubes per repetition.

2.4. Disinfestation and *in vitro* introduction of inflorescences

The inflorescences were initially disinfected with running water and neutral detergent. Then, the inflorescences were immersed for 20 hours in a solution with fungicide Bendazol® 0.1% + Nativo® 0.4%. Subsequently, in a laminar flow chamber, the inflorescences were disinfected by immersion in 70% ethanol for 1 minute (Figure 2A), followed by immersion in 2.0-2.5% commercial sodium hypochlorite solution with 3 drops of Tween 80® for 20 minutes. Then, the inflorescences were immersed in a streptomycin sulfate solution(1 g L^{-1}) under agitation for 60 min and washed three times with distilled and autoclaved water.

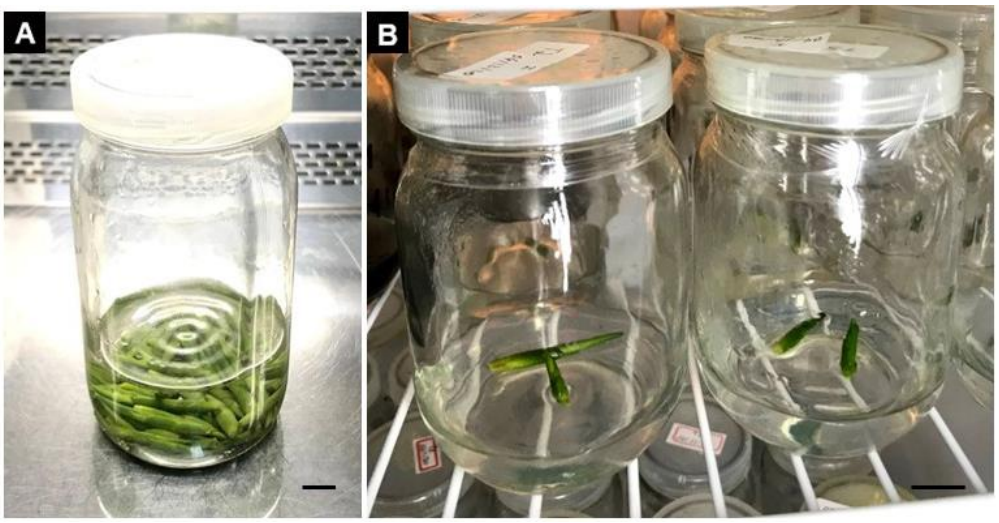

Figure 2. *Guadua glomerata* inflorescences immersed in 70% ethanol (A) and incubated in B.O.D. (B). Bars: 1 cm. Source: Author (2021).

Two inflorescences were inoculated into flasks containing 30 mL of MS culture medium (Murashige and Skoog, 1962) supplemented with 30 g L^{-1} of sucrose and 2 g L^{-1} of Phytagel® after adjusting the pH to 5.8. The treatments consisted of the addition of different concentrations of 6-Benzylaminopurine (BAP) in the culture medium (0; 2.5; 5.0 and 7.5 mg L^{-1}). Subsequently, the culture medium was autoclaved at 121°C for 20 minutes. The tubes were capped and sealed with plastic film in the laminar flow chamber and subsequently kept in B.O.D with a photoperiod of 16-h at a light intensity of 55 μmol m^{-2} s^{-1} and a temperature of 25 ±2 °C (Figure 2B). The experiment was conducted in a completely randomized design, containing 4 treatments with 6 replicates of flasks.

2.5. Statistical analysis of data

The experimental design was completely randomized. The data were analyzed using analysis of variance (ANOVA) ($P < 0.05$), followed by Tukey's test using R software (R Core Team 2018).

3. Results and discussion

After 12 days of installation, the evaluation of the experiment using nodal segments was performed. In this experiment, we observed a high percentage of fungal and bacterial contamination in all treatments tested (Table 1). In the

treatments with the highest concentrations of PPM® (T3 and T4) in culture medium, there was a lower percentage of contamination compared to the treatment without exogenous addition of PPM® in the culture medium (T1). However, the explants that did not present contamination subsequently presented oxidation.

Table 1. Percentage of contamination in culture flasks with nodal segments of *G. glomerata* after 12 days introduced *in vitro*.

Treatments	PPM® (mL L^{-1})	Contamination (%)	Microorganism
T1	**0.0**	100 a	Fungi and bacteria
T2	**2.5**	90 ab	Fungi and bacteria
T3	**5.0**	80 b	Fungi and bacteria
T4	**7.5**	80 b	Fungi and bacteria

Note: Means followed by different letters within the column differ statistically by Tukey's test ($p < 0.05$). CV = coefficient of variation. (n = 5; CV of contamination = 7.0%).

PPM® is a biocide that contains active ingredients that penetrate the cell wall of fungi and bacteria, inhibiting the activity of key enzymes in citric acid metabolism and the electron transport chain, and thereby neutralizing and preventing the growth of microorganisms (Compton and Koch, 2001; Plant Cell Technology, 2021). The use of PPM® at a concentration of 2.0 mL L^{-1} in culture medium was effective in reducing microorganisms in the *in vitro* establishment of the species *G. angustifolia* (Jiménez et al., 2006) and *G. latifolia* (Leão et al., 2020). In our study, the use of PPM® was not effective when added to culture medium, presenting high contamination rates. On the other hand, the explants that presented oxidation may be associated with the high concentration of the biocide PPM®, which may have promoted cell degradation and caused the death of the explants.

A study by Thomas et al. (2017) highlights several endophytic bacteria resistant to the use of PPM® in the *in vitro* introduction of papaya explants. The high contamination rate in our study suggests the presence of endophytic microorganisms resistant to PPM®, which may be a limiting factor for the *in vitro* establishment of *G. glomerata* (Figure 3). Endophytic microorganisms present in explants are considered a major restriction to *in vitro* establishment, as they are more difficult to remove by superficial disinfestation of the explants (Abdalla et al.,

2022). Additionally, when chemical agents are added to the culture medium and contamination is persistent, the identification of microorganisms may be an efficient method for the use of specific chemical agents.

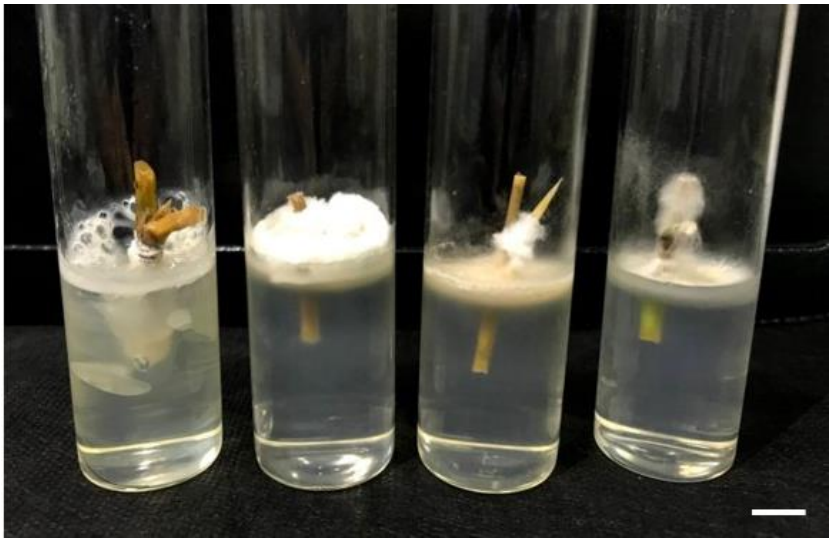

Figure 3. Fungal and bacterial contamination in nodal segments of *Guadua glomerata* introduced *in vitro*. Bar: 1 cm. Source: Author (2021).

Contamination is a limiting factor in *in vitro* propagation, as these microorganisms prevent the development of the explant *in vitro* due to the release of toxic compounds into the culture medium, causing the death of the explant (Smith, 2000). The fungi found in the vials in our study were possibly identified as *Trichoderma* sp. (Figure 4A), *Colletotrichum* sp. (Figure 4B), and *Arthrobotrys* sp. (Figure 4C). With the identification of the possible fungi that occurred in our study, specific fungicides can be used to control and reduce contamination in the *in vitro* establishment.

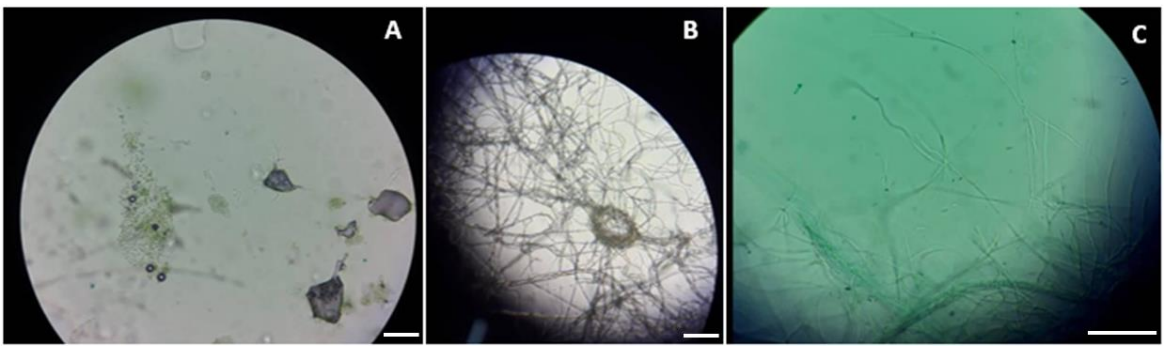

Figure 4. Identification of possible fungi present in culture flasks with nodal segments of *Guadua glomerata* introduced *in vitro*. *Trichoderma* sp. (A), *Arthrobotrys* sp. (B) and *Colletotrichum* sp. (C).

Bar: 200 μm. Source: Author (2021).

After seven days of installation, the evaluation of the experiment using *G. glomerata* inflorescences was performed. In this experiment, 100% fungal and bacterial contamination occurred in all treatments used. In this sense, some fungi were identified, possibly being *Fusarium* spp. (Figures 5A and 5B). In addition, the high percentage of microbial contamination in our study limited the observation of the effect of different concentrations of the cytokinin BAP supplemented in culture medium when the inflorescences were introduced *in vitro*.

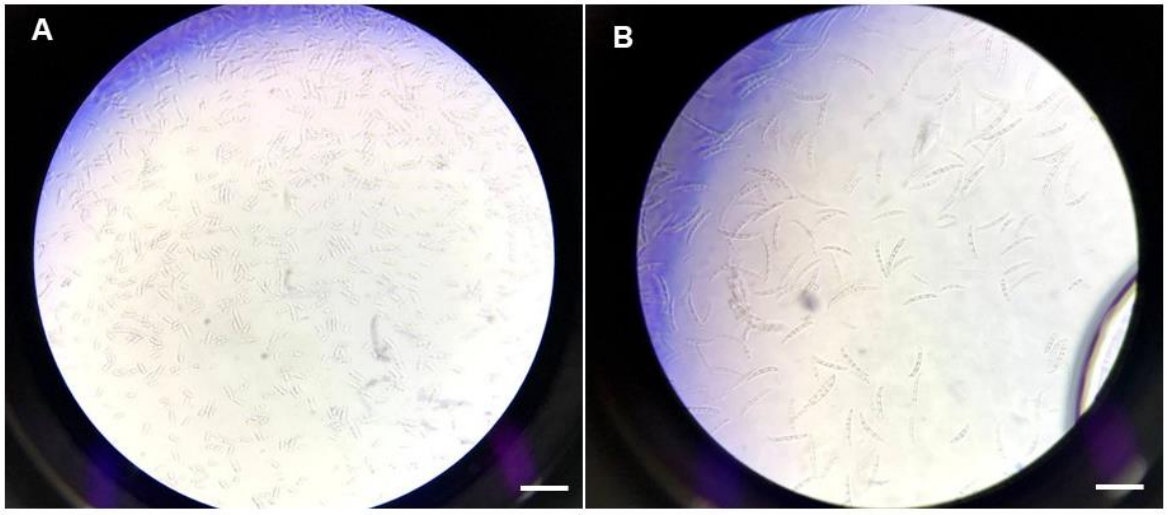

Figure 5. Possible species of *Fusarium* spp. identified in culture flasks with *Guadua glomerata* inflorescences introduced *in vitro* (A and B). Bar: 200 μm. Source: Author (2021).

According to Andrade et al. (2014), fungal contamination can be caused by several factors, such as the source of the material, since plants in the field, where there is no control of phytosanitary conditions, tend to presente a higher percentage of contaminating microorganisms. For a disinfection protocol to be effective, it is necessary to eliminate microorganisms, which are often resistant to the process. Consequently, this technique requires different disinfestation tests to overcome bottlenecks aimed at its establishment.

5. Conclusion

The disinfestation methodologies tested in our study were not effective for

the *in vitro* establishment of *G. glomerata*, highlighting the need for adjustments in the concentrations and exposure times to the disinfectant agents. These adjustments are essential to optimize this crucial stage of *in vitro* propagation, ensuring the reduction of microbial contamination without causing phytotoxicity in the explants. In addition, the identification of fungi, such as *Trichoderma* sp., *Arthrobotrys* sp., *Colletotrichum* sp. and *Fusarium* spp., suggests the future possibility of using specific fungicides, which can significantly improve the disinfestation process of *G. glomerata*.

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