

TECHNICAL ARTICLE

Germination of spores of Amazonian ferns *Polypodium aureum* in different culture media

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Abstract

The greatest diversity of ferns occurs in the tropics, where approximately three in four of the species are found. In the Brazilian flora, the ferns have megadiversity, being an important plant group. The objective was to evaluate the spores germination of ferns *Polypodium aureum* in different types of culture medium. The sporangia were removed from fertile fronds and sterilized with sodium hypochlorite 0.57% during 30 minutes. After sterilization, the sporangia were seeded in culture media agar, MS, SH, White and B5. The experimental design was completely randomized being composed of 5 treatments (agar media, B5, SH, White and MS) and 10 replications. The ratings occurred every 3 days over a period of 45 days, counting the number of spores germinated. After this period, the germination percentage, germination time on medium, germination speed index and timing of germination were calculated. The germination percentage and the index of synchronization have not changed in function of different culture media. The medium White presented the highest average for the germination speed index whereas the agar media, MS, SH and B5 had the same germination speeds. The percentage of mortality was higher in MS medium, the White medium presented the lowest percentage of mortality, and the media composed by agar, SH and B5 showed intermediate values. The lowest average germination time occurred at the media SH and B5, since the environment composed by agar showed higher average germination time. The media MS and White showed similar results to the agar, SH and B5. Based on the obtained results, it can be concluded that the White medium is the most effective on the germination of spores of *Polypodium aureum*.

Keywords: Sporangia, synchronization, mortality, Pteridophytes.

Resumo

Germinação de esporos de samambaia amazônica *Polypodium aureum* em diferentes meios de cultura

A maior diversidade de samambaias ocorre nos trópicos onde aproximadamente três quartos das espécies são encontradas. Na flora brasileira, as pteridófitas possuem uma megadiversidade, sendo importante grupo vegetal. O objetivo foi estudar a germinação dos esporos de samambaias *Polypodium aureum* em diferentes tipos de meio de cultura. Os esporângios foram removidos das frondes férteis e esterilizados com hipoclorito de sódio 0,57%. Após a esterilização, os esporângios foram semeados nos meios de cultura Ágar, MS, SH, White e B5. O delineamento experimental utilizado foi o inteiramente casualizado, composto por 5 tratamentos (meios Ágar, B5, SH, White e MS) e 10 repetições. As avaliações ocorreram a cada 3 dias durante 45 dias, contando-se o número de esporos germinados. Foi calculada a porcentagem de germinação, tempo médio de germinação, índice de velocidade de germinação e sincronização da germinação. A porcentagem de germinação e o índice de sincronização não alteraram em função dos diferentes meios de cultura. O meio White apresentou a maior média para o índice de velocidade de germinação já os meios ágar, MS, SH e B5 tiveram velocidades de germinação iguais. A mortalidade foi mais alta no meio MS, o meio White apresentou a menor mortalidade, e os meio compostos por ágar, SH e B5 apresentaram valores intermediários. O menor tempo médio de germinação ocorreu nos meios SH e B5, já o meio ágar apresentou maior tempo médio de germinação. Os meios MS e White apresentaram resultados semelhantes ao meio ágar, SH e B5. Com base nos resultados obtidos, conclui-se que o meio White é o mais efetivo na germinação dos esporos de *P. aureum*.

Palavras-chaves: Esporângios, sincronização, mortalidade, Pteridófitas.

Introduction

The Amazonian ferns, *Polypodium aureum* Lowe, are native from Brazil, from Polypodiaceae family, are perennial, vigorous herbaceous vegetations, and with rhizomes. It has large rhizomes and follicles, with large

and crooked fronds (compound leaves). These species of ferns can be grown in pots containing good soil fertility and enriched with organic matter, are a little colder tolerant (Lorenzi et al., 2008). This ornamental species is mainly marketed and the information on its propagation is still very restricted.

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Received September 29, 2018 | Accepted Jul 24, 2019

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<https://doi.org/10.1590/2447-536X.v25i3.1455>

The ferns of the species *P. aureum* can reproduce in two ways, the first way is by cuttings and the second way is by germination of spores. The cutting is a technique for vegetative propagation, which consists of stem and root segments planting of the plant matrix, giving origin to a new plant, from the rooting of the same. This method is widely used in the creations of seedlings of plants, especially trees and ornamental plants (Hartmann et al., 2002).

The in vitro cultivation of spores of *P. aureum*, is a very efficient method, because it allows the understanding of the life cycle of these plants and decreased the time of cultivation of the same, however, to obtain a culture without any kind of contamination, it is necessary that the spores be sterilized using sodium hypochlorite. (Pence, 2000).

In some countries, spores of *P. aureum* and other species of the same genus are being studied for medicinal purposes. (House et al, 1991; Das and Einstein, 2007). Hires (1940) made the first studies on in vitro culture with ferns, inoculating spores of *P. aureum* in solid nutrient medium; for the realization of the in vitro culture, different culture media can be used, but the *P. aureum* L., prefer culture media with low concentrations of nutrients.

It is only through the understanding of causal factors for genetic diversity that the speciation and evolution of pteridophytes can be understood and these factors are best observed through the sexual propagation obtained by spores (Lloyds, 1974).

The method presented here, is important because generates genetic variability, and by being an ornamental plant, individuals with characteristics of higher commercial value can be generated, adding value to the plant which may turn the activity more profitable. Despite the wide popular use, *P. aureum* presents few scientific studies. Therefore, this work had as objective to study the germination and survival of spores of *P. aureum*, in different culture media.

Material and Methods

Material collection, general method of germination and sterilization

Fertile fronds of *Polypodium aureum* cultivated for commercialization were collected in a region that climate is AW according to the Koppen classification (22°07'04" S; 51°22'04" W and altitude of 430 m). The fronds were collected during the months of August and September 2016.

The Sporangia were removed from the fronds using a brush with stiff bristles, placed approximately 30 mg of Sporangia within a 3 mL syringe to facilitate the disinfection of the sporangia, then the sporangia were sterilized in a solution of sodium hypochlorite 0.57% during 30 min, which were obtained from a commercial solution diluted to 20%. After disinfection, the sporangia were washed

three times in distilled water and autoclaved and quickly inserted into culture media, in sterile environment (Randi and Crozier, 1991).

The experimental design was completely randomized being composed of 5 treatments, being composed by media of agar, B5 (Gamborg et al., 1968), SH (Schenk and Hildebrandt, 1972), White (White, 1951) and MS (Murashige and Skoog, 1962) and 10 replications for each culture medium. The nutrient solutions were previously sterilized in an autoclave, for 20 minutes at 120 °C and were deposited in the covered petri dish. Each 30 mg of spores were homogeneously dispersed in 5 Petri plates. After being seeded in culture media, the petri dishes were covered and sealed with a film of polypropylene.

The spores' germination occurred in a germination chamber, simulating local environmental conditions, with a photoperiod of 16 hours at a temperature of 25 °C ± 2 °C and white light with photon flux density of approximately 30 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

The ratings occurred every 3 days over a period of 45 days, counting the number of germinated spores. In order to count the number of germinated spores, it was necessary to make three circles, at the petri plates, where there were more spores, the circles were made in the magnification of 2x magnifying binocular stereoscopic microscope, using a marker for transparencies of permanent ink. The spores germinated within the circles were photographed in the increase of 2.5x in magnifying binocular stereomicroscope and counted with the help of Microsoft Paint.

Based on counts of germinated spores, the calculations of speed of germination index (Silva and Nakagawa, 1995), average time of germination (Laboriau, 1983) and index of synchronization (Laboriau and Agudo, 1987) were performed. At the end of 45 days, the counting of spores that germinated and that died was carried out to determine the percentage of dead spores.

Statistical Analysis

The data from experiments following completely randomized, were subjected to the Shapiro-Wilk test to verify the data normality and the Levine test to check the homogeneity of variances between culture medium values. Then, the data were subjected to analysis of variance and when significant the means were compared by the Tukey test at 5% probability.

Results and Discussion

The percentage of germination of spores of *Polypodium aureum* has not changed in function of the different culture media (Figure 1A). All culture media provided 100% germination of spores.

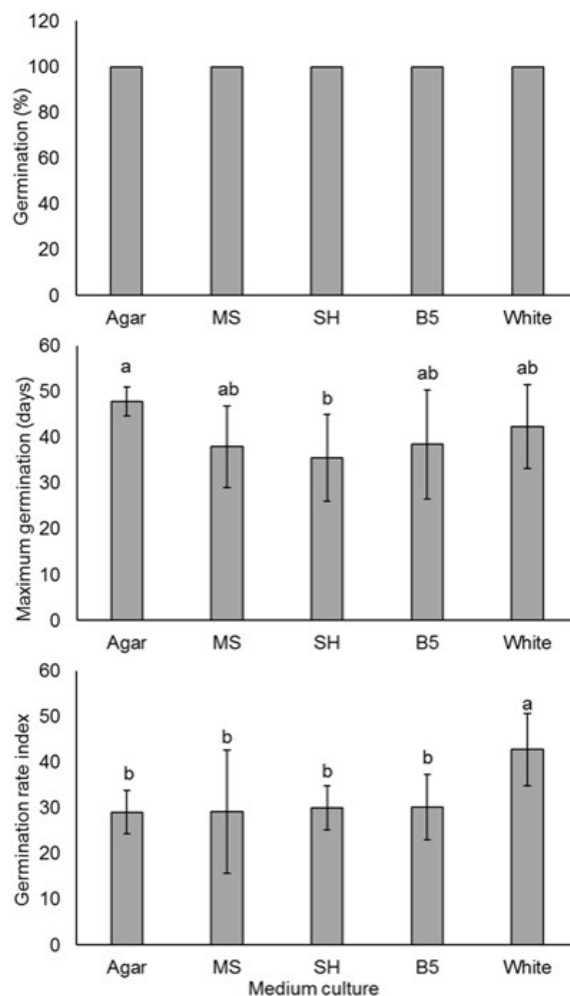


Figure 1. A) Germination of spores (%). B) Number of days to obtain the maximum germination of spores and C) Spore germination Rate Index (IVG) of *P. aureum* (Amazonian fern) in different culture medium (Agar; Murashige and Skoog, 1962; Schenk and Hildebrant, 1972; Gamborg et al., 1968; White, 1951). The bars represent the mean values for each culture medium and the respective error bars indicate the standard deviation. Means followed by the same letters were not significantly different according Tukey test at 5% of confidence.

The viability of spores is directly related to the amount of reserve that the same show (Dyer, 1979) and these organic substances are essential for germination that come from the metabolism of the reserves of the same (Raghavan, 1980). The results of this study indicate that spores of *P. aureum* have some reserve, since they present good germination.

When they come in contact with water or even in a simple saline solution the spores of *P. aureum* are able to germinate, this occurs because the spores have in their interior reserve substances, this substance helps in the germination of spores (Raghavan, 1980 and Mehlreter et al., 2010).

The time required for maximum germination of spores was different in function of the culture media (Figure 1B). The medium composed by agar showed the greatest amount of days so that 100% of the spores germinated since that the SH medium was responsible for 100% germination of spores in only 35.5, and thus, a reduction of approximately 25% is observed. The media MS, B5 and White showed

intermediate results with absolute values of 37.9, 38.4 and 42.3 days, respectively, so that 100% of the spores germinated, the latter, did not present statistical difference (Figure 1B).

To control the growth of tissues and the development pattern of in vitro plants, it is necessary to use nutrient media that provide essential substances, such as macronutrients, micronutrients and vitamins (Pereira et al., 2003). However, these nutrients are not essential to the germination of spores (Soares et al., 2007).

The conditions used in this study favored the reproductive success of *P. aureum* spores. Such conditions influence the germination percentage and speed, influencing the rate of absorption of water and biochemical reactions that determine the whole process. Due to being exposed to these favorable conditions the process of germination of spores occurs with greater efficiency (Marcon et al., 2014).

The culture media influence the diffusion of water

and nutrients for the plants, but if the environment is very consistent they may limit this function (Caldas et al., 1998). According to the same author, the solid media are more efficient, providing an optimal supply of salts for plants.

Some culture media are formulated containing high concentrations of salts, however, culture media with high concentrations of salts hamper the *in vitro* development, causing stress, because the osmotic potential is reduced. Then the water is removed osmotically from the saline solution, increasing the concentrations of salts and consequently becoming less available to plants (Ribeiro, 2001). Excess nutrients cause damage by salinity, leading to an osmotic imbalance, negatively affecting the absorption of water by roots (Schosler et al., 2012). Probably for this reason the MS medium, although containing the highest amount of nutrients, was the one that presented higher spore mortality (Figure 2A).

Media with lower concentrations of nutrients provide a better absorption of water by the spores and consequently the spores can germinate, since water is a requirement to start the germination process (Whittier, 1970).

The SH culture medium had the lowest number of days for maximum spore germination (Figure 1B). According to Spangenberg (2010) the SH medium is a basic culture medium formulated for the culture of tissues, organs, and cells. A basic culture medium is the one that provides essential substances for plants, such as, for example, water, macronutrients and micronutrients, vitamins, among others (Puignau, 1996). According to the same author, the pH of the culture media varies according to the species to be studied and with the culture medium, however all culture media must be corrected for pH= 5.7.

Due to being a basic medium this may have influenced the number of days for obtaining the maximum germination of spores. Before soaking, the spores present in their interior a very negative water potential, so when in contact with water, there is a rapid absorption and a rapid increase in the water potential of the embryo, due to the difference between potential existing between both, then there is a reduction in the water absorption, thus reducing the rate of hydration (Barrs and Weatherley, 1962). This entire process causes the expansion and cell division (Labouriau, 1987; Kermode, 1995).

The different culture media made the speed index of spores germination to be different (Figure 1C). The mean value observed for GVI was 29.93, ranging from 29.05

to 42.72. The medium composed by White presented the highest average (42.72) for IVG, whereas the media Agar, MS, SH and B5 had equal speeds of germination (29.05), their germination occurred more slowly comparing to the White (Figure 1C).

To calculate the germination rate index (IVG), one must calculate the data from the count of germinated plants; these calculations should establish the difference in speed of spores' germination (Biasi et al., 2009).

The culture medium White is composed of macronutrients and micronutrients in its composition. The macronutrients and micronutrients are required for the *in vitro* plants, because they have a heterotrophic metabolism (PIERIK, 1990). The amount of nutrients present in each culture medium influences the speed of spores' germination. Culture media that have smaller quantities of nutrients cause the spores to absorb more water, germinating faster.

A less concentrated culture medium is characterized by presenting a high water potential, this high water potential causes the spores to absorb more water from the environment through reverse osmosis and consequently they germinate more quickly. The water moves toward the regions of low water potential or low free energy, in order to equalize the concentrations. More concentrated culture media, i.e., with a high amount of nutrients, have a low water potential, causing the spores to take more time to moisturize and as a consequence the speed of germination delay (Whittier, 1975).

The amount of nutrients found in different culture media influence on mortality of gametophytes (Figure 2A). The percentage of mortality was higher in MS medium, approximately 37.28%. Whereas the White medium presented the lowest percentage of mortality, approximately 13.07%, in relation to other culture media. The medium composed by Hagar, SH and B5 showed intermediate values with absolute values of 19.2%, 27.99%, 24.07%, respectively.

The White' culture medium (1942) was one of the first basic medium to be formulated for the plants, showing low levels of nitrogen and potassium, even having in its formulation, low concentrations of salts, restricting its use, this medium is still widely used in various situations (Caldas et al., 1998). How it was to observed, the White' medium presented higher IVG (Figure 1C) and lower mortality (Figure 2A).

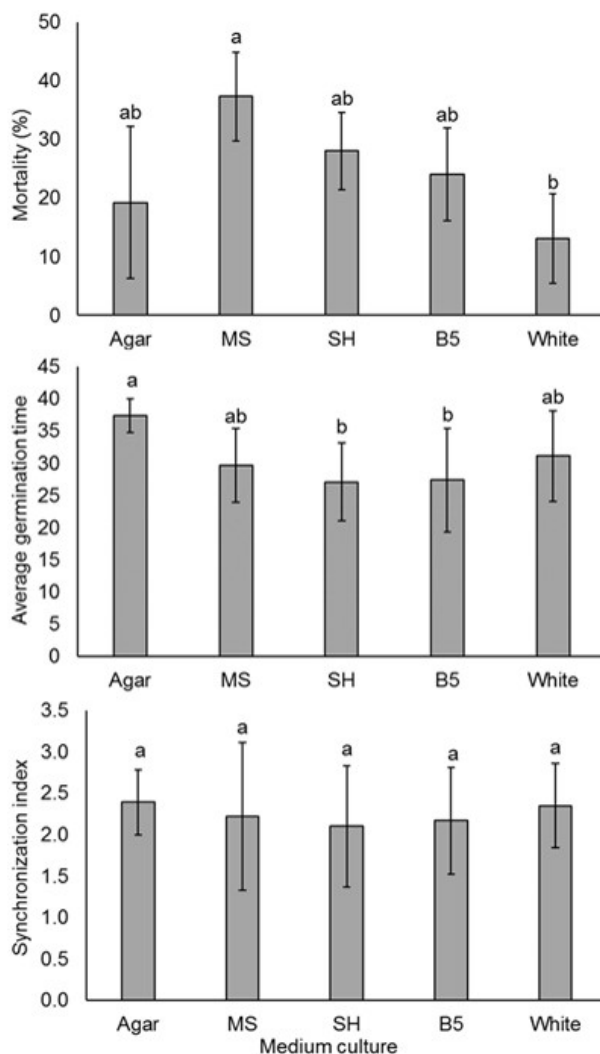


Figure 2. Spore mortality (%); B) Average germination time (TMG) in days and C) Synchronization index of Spore germination (U) of *P. aureum* (Amazonian fern) in different culture medium (Agar; Murashige and Skoog, 1962; Schenk and Hildebrandt, 1972; Gamborg et al., 1968; White, 1951). The bars represent the mean values for each culture medium and the respective error bars indicate the standard deviation. Means followed by the same letters were not significantly different according Tukey test at 5% of confidence.

According to Caldas (1998) the White medium is a basic medium, i.e., provides essential substances for plants, which is characterized by having lower amounts of mineral salts, the cells of some plants prefer this culture medium, because they contain lower amounts of mineral salts, causing a lower percentage of mortality in the White medium.

Higher quantities of nutrients slow the germination process, causing necrosis and the growth of gametophytes is inhibited, this happens due to the high concentration of ammonia present in this environment (Fernández et al., 1997). The excess nutrients cause a nutritional disorder in the plants, these excess nutrients can cause deficiency of other essential elements for the plant, occurring a competition (Audebert and Fofana, 2009).

Due to the fact that the medium contains high levels of minerals, this may have influenced the increase of mortality of spores. Culture media containing large quantities of mineral salts interfere in the water potential of culture medium

decreasing the water potential between the culture medium and the spores surface, this makes the spores not capture water, and increasing the mortality rate (Lopes et al., 2008).

The lowest average germination time occurred at 26 days, at the media SH and B5, since the environment composed by agar showed higher average time of eleven days longer than SH and B5.

The average time of germination is used to evaluate the speed at which a species occupies a given environment (Ferreira et al., 2001). According to Silveira et al. (2013), the average germination time was influenced by the nutrients found in each culture medium, when the spores were placed in the medium with large quantities of nutrients, there was a delay in the germination process, but when placed in media with lower concentrations of nutrients, the spores germinated more quickly, because, they managed to absorb more water and, consequently, initiating the germination process. Thus, this indicates that smaller quantities of

nutrients promote the acceleration of germination of spores of *P. aureum*. Spores that have large quantities of water have a lower average germination time, when compared with spores with lower water contents (Dyer, 1979).

The media composed of agar, MS, SH, B5 and White provided the same timing of germination of spores (Figure 2C). The synchronization indices are interpreted in a simple way, the lower the value the more synchronized the germination is in relation to time and the greater the value is the less synchronized the germination is (Duarte et al., 2015).

The synchronized germination of spores is beneficial, because the spores were able to reach the same stage of development in a short period of time.

When the index of this synchronization has low value means that the system is more orderly, with larger amounts of information, this causes increase of the values of percentage and speed of germination what occurs at the optimum temperature for germination (Labouriau and Osborn, 1987). In some culture media the germination of spores occurred quickly, whereas in other media the germination occurred slowly, but the germination of spores occurred at the same time.

The decrease of the average time of germination and the index of synchronization means that the different concentrations of nutrients influenced differently in water potential of culture media and consequently decreased the time of germination and the spores germinated in less time and in a more synchronized way.

Conclusion

The culture media agar, White (1951), MS (Murishige and Skoog, 1962), through B5 (Gamborg et al., 1968) and SH (Schenk and Hildebrandt, 1972), were efficient, providing 100% of the germination of spores. However, the White medium is the most effective on the germination of spores of *Polypodium aureum* (Amazonian fern), providing a better germination speed and with low mortality rates.

Author Contribution

P.A.S.A. ⁰⁰⁰⁰⁻⁰⁰⁰³⁻¹³⁰⁷⁻⁶⁹¹²: Responsible for obtaining, analysis and interpretation of data. Writing and critically analyzing of manuscript; author of the graduation work. **A.M.A.** ⁰⁰⁰⁰⁻⁰⁰⁰³⁻⁴²²³⁻⁴³⁴²: Assistance in data collection, statistical analysis and critical evaluation of the manuscript. **W.H.S.T.** ⁰⁰⁰⁰⁻⁰⁰⁰²⁻¹⁴⁴⁸⁻⁰⁷²⁵: Advisor who assisted in the designing of the research, analysis and interpretation of data and writing of the manuscript.

Acknowledgments

The authors are thankful to University of Western São Paulo (UNOESTE) for the financial support (CAPI-3336).

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