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In vitro culture of zygotic embryos of *Butia eriospatha*

Cultivo *in vitro* de embriões zigóticos de *Butia eriospatha*

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Abstract

The economic use of *Butia eriospatha* is justified by its potential but is limited by its slow germination. The *in vitro* culture of zygotic embryos can solve this problem as well as contribute to the conservation and to insert this species in the productive context. The objectives of this study were to evaluate the procedures for surface disinfestation, the effect of gibberellic acid (GA_3) on *in vitro* germination, and the effect of 3 culture media on the increase in fresh mass of zygotic embryos of *Butia eriospatha*. In the first assay, disinfestation was tested by immersing only the seeds, by immersing the seeds followed by isolation of the embryos, and by immersing only the embryos in sodium hypochlorite (NaOCl). After 30 days of *in vitro* culture, microorganism contamination was evaluated. In the second assay, *in vitro* germination was tested using different concentrations (0, 2, 4, 6, and 8 mg L^{-1}) of GA_3. In the third assay, 3 culture media (Murashige and Skoog [MS], Woody Plant Medium [WPM], and Y3) were evaluated. Soaking embryos in NaOCl, with or without seed immersion, produced satisfactory control of microorganisms, unlike the disinfestations tests conducted only on the seed, which were not efficient. *In vitro* germination of embryos increased with the GA_3 concentration. Embryos cultured in Y3 and MS culture media showed a higher increase in fresh mass than those cultured in WPM. To control microorganisms, disinfestation consisted of immersing the seed in 2% NaOCl for 15 min followed by immersing the embryo directly in 1% NaOCl for 10 min. A GA_3 concentration of 8 mg·L^{-1} was required to optimize the germination. After 4 weeks of the *in vitro* culture, 80% of the embryos germinated. Thus, the use of MS or Y3 culture media is recommended to promote the *in vitro* growth of *Butia eriospatha* zygotic embryos.

Key words: Gibberellic acid, sodium hypochlorite, tissue culture, culture media

Resumo

A exploração econômica do butiazeiro (*Butia eriospatha*) é justificada por suas potencialidades, porém apresenta uma germinação lenta, problema que o cultivo *in vitro* de embriões zigóticos pode eliminar, além de poder contribuir para a sua conservação e para inserir a espécie no contexto produtivo. Os objetivos foram avaliar metodologias de desinfestação superficial, o efeito do Ácido Giberélico (GA_3) sobre a germinação *in vitro* e do meio de cultura sobre o ganho de massa fresca de embriões zigóticos de *Butia eriospatha*. No primeiro ensaio foram aplicadas metodologias de desinfestação que incluíram imersão apenas das sementes, ou das sementes e, depois, dos embriões isolados ou, ainda, apenas dos embriões em hipoclorito de sódio (NaOCl), sendo avaliada, após 30 dias de cultivo *in vitro*, a contaminação por microrganismos. No segundo ensaio, diferentes concentrações (0, 2, 4, 6 e 8 mg L^{-1}) de GA_3 foram testadas em relação à germinação *in vitro*. Três meios de cultura (MS, WMP e Y3) foram avaliados no terceiro ensaio. A imersão dos embriões em NaOCl associada ou não à imersão das

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sementes promoveu um controle satisfatório dos microrganismos; ao contrário, desinfestações efetuadas apenas nas sementes não foram eficientes. A germinação dos embriões aumentou com a concentração de GA_3. Embriões cultivados nos meios de cultura Y3 e MS apresentaram maiores ganhos de massa fresca que aqueles cultivados em WPM. Para efetuar o controle de microrganismos deve-se realizar a desinfestação em NaOCl a 1% por 10 minutos, diretamente no embrião, após a imersão da semente a 2% durante 15 minutos. A concentração 8 $mg \cdot L^{-1}$ de GA_3 é necessária para otimizar a germinação *in vitro* de *Butia eriospatha*, sendo obtidos 80% de germinação após quatro semanas de cultivo. Recomenda-se o emprego dos meios de cultura MS ou Y3 para promover o crescimento *in vitro* de embriões zigóticos de *Butia eriospatha*.
Palavras-chave: Ácido giberélico, hipoclorito de sódio, cultura de tecidos, meios de cultura

Introduction

Butia eriospatha is native to southern South America, and in Brazil, it is found growing naturally mainly in the state of Rio Grande do Sul (SOARES; LONGHI, 2011), where it is in danger of becoming extinct (SEMA, 2007). Natural regeneration of *Butia eriospatha* is difficult in fields that support systematic cattle farming, because the cattle feed on new plants, which increases the threat of extinction for the species (LORENZI, 2010).

This species has many features and qualities that support its economic exploration. Its fruits are globose, juicy, and small (mean diameter 1.7 to 1.9 cm), and they are classified as small fruits. Mature fruits can be consumed in their natural state or after processing for juices, liquors, wines, ice creams, and jams (SCHWARTZ et al., 2010; AMARANTE; MEGGUER, 2008). Its leaves are used to make straw hats, baskets, and textile fibers and to cover huts. The leaves are also considered excellent fillers for mattresses and upholstery. In addition, *Butia eriospatha* is used as an ornamental plant in squares, parks, and gardens (LORENZI, 2010).

Butia eriospatha germinates 8 months after sowing due to tegumentar dormancy, like in other palm trees species (COSTA; MARCHI, 2008; STURIÃO et al., 2012). The removal of fruit tissues that coat the seeds and immersion in water, among other treatments, can accelerate embryo emergence (LORENZI, 2010). Therefore, the culture of zygotic embryos may enable the propagation of plants in a shorter time (PINHEIRO, 1986). No reports were found in the literature regarding the *in vitro* culture of zygotic embryos of *Butia eriospatha* and the procedures of surface disinfestation, the effect of gibberellins, or better nutritive media for *in vitro* germination.

One of the main difficulties in the *in vitro* culture of forest species is obtaining aseptic cultures. The presence of microorganisms is commonly associated with explants, and surface disinfestation procedures used successfully in herbaceous species are relatively inefficient in forest species. Disinfestation procedures typically include the use of distilled water, sodium hypochlorite, calcium hypochlorite, mercuric chloride, and other substances (GAMBORG; PHILLIPS, 1995). In *Butia capitata*, the disinfestation of zygotic embryos was successful by immersion in a 0.25% solution of chlorine for 10 min (RIBEIRO et al., 2011).

Gibberellins (GAs) like gibberellic acid (GA_3) promote seed germination in many species of plants by stimulating the growth of the embryo and inducing the production of hydrolases that weaken the structures around the embryo (LOPES et al., 2009). Much research has focused on the physiological differences between dormant and nondormant seeds. Abscisic acid has been shown to play a role in the induction and maintenance of seed dormancy, whereas GAs are associated with dormancy breaking and germination (KUCERA; COHN; LEUBNER-METZGER, 2005). Among the hundreds of types GAs, GA_1, GA_3, GA_4, and GA_{4+7} have been shown to be effective in breaking seed dormancy and promoting germination (CHEN et al.,

2007; CHEN; KUO; CHIEN, 2008). GA_4 and GA_7 probably are more active for seed germination in the medicinal Asian plant *Phellodendron amurense* var. *wilsonii* (Rutaceae), a deciduous tree (CHEN; KUO; CHIEN, 2008). However, the role of GA_3 in the promotion of germination cannot be excluded, since a higher concentration of endogenous GA_3 was observed in seeds that were ready to germinate than in those that were not ready to germinate (CHEN; KUO; CHIEN, 2008). In *Givotia rottleriformis* Griff., a commercially valuable tree belonging to the family Euphorbiaceae, the highest germination frequency (78.3%) was achieved from mature zygotic embryo axes isolated from acid-scarified fresh seeds cultured on Murashige and Skoog (MS) (1962) medium (half-strength major salts) with 28.9 µM GA_3 (SAMUEL et al., 2009).

The composition of the nutritive medium used to sustain embryos is a key to successful culture. In *Cocos nucifera* L., zygotic embryos were successfully cultivated in Y3 (EEUWENS, 1976) liquid nutritive medium without activated charcoal (LÉDO et al., 2007). In a previous study (TRIQUES et al., 1997), *Cocos nucifera* zygotic embryos were obtained in tissue culture medium composed of MS (1962) with activated charcoal.

No reports about the *in vitro* culture of zygotic embryos of *Butia eriospatha* have been found in the literature, and thus the goals of this paper were to establish a methodology for surface disinfestation, evaluate the effect of GA_3 on *in vitro* germination, and define the best culture medium for the growth of *Butia eriospatha* zygotic embryos.

Material and Methods

Seeds were collected at the end of March 2007 in the Vale dos Butiazais (420 m altitude; latitude 28°01′40″ S and longitude 54°21′00″ W), Giruá, Rio Grande do Sul. At the time, the seeds were fully ripe and were found distributed around mother plants on the soil surface. After removing the woody endocarp that covers the seed with a nutcracker, the nuts were stored for 15 days in flasks capped with aluminum foil at 22°C until the start of the experiments. To remove the *B. eriospatha* embryo, the seeds were soaked for 24 h in sterile water at 22°C.

Embryos were isolated in a laminar flow chamber using a scalpel and tweezers and were kept soaking in the sterile water until the start of the treatments to avoid tissue dehydration. Zygotic embryo cultures were kept in a growth room at 25 ± 3°C, with a photoperiod of 16 h and irradiance (20 mmol m^{-2} s^{-1}) provided by cool light fluorescent lamps.

To promote embryo surface disinfestation, the following treatments were evaluated: T1, soaking the seed in 2% (v/v) sodium hypochlorite (NaOCl) for 15 min followed by soaking the embryo in 1% (v/v) NaOCl for 10 min; T2, soaking only the embryo in 2% (v/v) NaOCl for 15 min; and T3, soaking only the seed in 2% (v/v) NaOCl for 15 min. Subsequently, embryos were triple rinsed in sterile water and inoculated in 150 mL glass tubes containing 30 mL of MS (MURASHIGE; SKOOG, 1962) culture medium supplemented with 100 mg L^{-1} myo-inositol, 30 g L^{-1} sucrose, and 5 g L^{-1} agar, adjusting pH to 5.7. The experimental design was completely randomized, with 4 replicates of 6 explants.

Another experiment was carried out using the most effective surface disinfestation treatment to evaluate the effect of different concentrations of GA_3 (0, 2, 4, 6, and 8 mg L^{-1}) on the germination of *Butia eriospatha* zygotic embryos. This growth regulator was added to the MS culture medium before autoclaving. Conditions were the same as those described previously. A completely randomized block design with 5 replicates, each containing 5 explants, was used.

In both experiments, the accumulated values of the assessments performed 28 days after explant inoculation were considered for the variables bacterial contamination, fungal contamination, and *in vitro* germination and expressed in percentages. The embryo was considered germinated when there was protrusion of first leaf sheath.

The effect of 3 different culture media, Y3 (EEUWENS, 1976), MS (MURASHIGE; SKOOG, 1962), and Woody Plant Medium (WPM) (LLOYD; McCOWN, 1980), on the growth of *Butia eriospatha in vitro* zygotic embryos was also investigated. After emergence, the embryos were cultured for 2 weeks in MS medium with 0.3% of activated charcoal added to eliminate GA_3 effect. The embryos were measured for initial fresh mass (mg) and divided into categories. They were inoculated in 150 mL glass tubes with 30 mL of culture medium containing 30 g L^{-1} sucrose and 5 g L^{-1} agar, adjusting pH to 5.7. The culture was incubated under the conditions described previously. Four weeks later, the increase in fresh mass (mg) of embryos was determined. A completely randomized design was used, in a factorial scheme (3 × 3), with culture media and categories of the initial mass of embryos (>35 mg, 23 to 35 mg, <23 mg) as main factors, with 5 replicates of 4 embryos each.

After testing data normality using the Kolmogorov–Smirnov test and variance homogeneity using the Bartlett test, transformations were performed, wherever necessary, using the function $\sqrt{x+0.5}$ and data were submitted to an analysis of variance. Mean values were compared using Tukey's test ($P < 0.05$) or polynomial regression analysis. The results presented are the original mean values obtained. The statistical program SISVAR (FERREIRA, 2006) was used for data processing.

Results and Discussion

Soaking embryos in 2% or 1% NaOCl, with or without seed immersion, produced satisfactory control of microorganisms, unlike disinfestations carried out on the seed only, which were not efficient (Table 1).

Table 1 Percentage of bacterial and fungal contamination and *in vitro* germination of *Butia eriospatha* zygotic embryos after different treatments of surface disinfestation in MS culture medium.

Treatment[1]	Contamination (%)		*In vitro* germination (%)
	bacterial	fungal	
seed + embryo[1]	2.56a[2]	0.00a	62.62a
embryo	0.00a	0.00a	5.76b
seed	42.77b	39.37b	0.00c
CV (%)	49.11	20.05	37.63

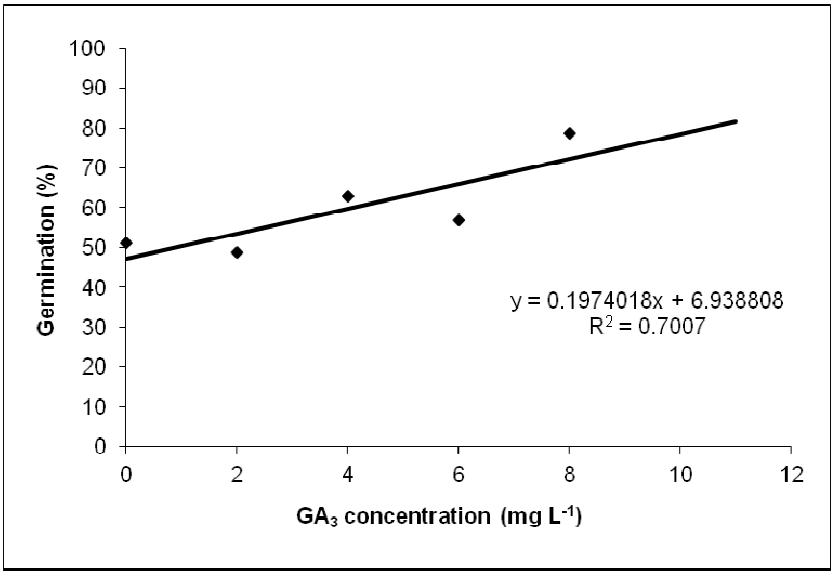
[1]seed + embryo: soaking of seed in 2% NaOCl solution for 15 min followed by soaking of embryo in 1% NaOCl for 10 min; embryo: soaking of embryo only in 2% NaOCl for 15 min; seed: soaking of seed only in 2% NaOCl for 15 min. [2]Means followed by the same letter within columns are not different (Tukey's test, $P < 0.05$).
Source: Elaboration of the authors.

In vitro germination (Table 1) after disinfestation in treatments where both the seed and the embryo were exposed to NaOCl confirms the results observed regarding contamination and embryo growth, since there was no germination in the treatment that had the greatest microorganism contamination; as time progressed, fungal and bacterial colonization may have increased in both the culture medium and the embryo, probably leading to the release of toxic compounds that affected development or even killed the explants. Consequently, embryos did not germinate. Similarly, in *Euterpe edulis* Mart., the least contamination (14.4%) was observed after immersion in 2% NaOCl for 10 min; however, this concentration was completely lethal to embryos. With immersion in 1.6% NaOCl for 5 min, contamination was relatively high (18.9%), but all of the embryos germinated (YOKOO; RAMOS;

BOVI, 1992). Pereira et al. (2006) also obtained healthy seedlings of 'Murmuru' (*Astrocaryum ulei*) after disinfestation by immersion of embryos in 1% NaOCl for 10 min. In this study, 25–56.5% of embryos germinated in various concentrations of sucrose added to MS medium with salt composition reduced to 75%. Exposing only the embryos to 2% NaOCl for 15 min probably affected their growth, injuring their tissues, since germination was reduced.

The *in vitro* germination of embryos in response to GA_3 concentration adjusted to a positive linear function (Figure 1), meaning that the increase in gibberellin concentration stimulates germination in *Butia eriospatha*. After around 14 days, the embryo elongated, and after 28 days, around 80% of embryos germinated. In *Butia capitata*, 38.8% of the embryos presented the first leaf sheath after 28 days of *in vitro* culture in MS medium without growth regulators, including gibberellins, suggesting that its use could optimize embryo germination (RIBEIRO et al., 2011). Gibberellins are plant hormones that promote growth and stimulate cell elongation and division. They play a key role in seed germination and are involved not only in overcoming dormancy but also in the control of the hydrolysis of reserves, which is necessary for embryo growth (FLOSS, 2006).

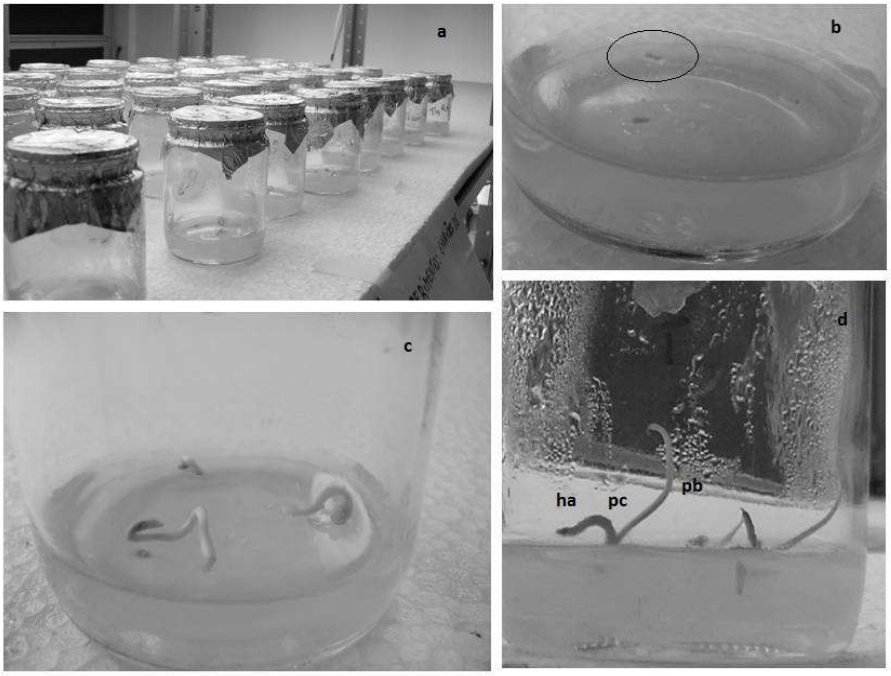
Figure 1 Percentage of *in vitro* germination of *Butia eriospatha* zygotic embryos in Murashige and Skoog nutritive medium as a function of gibberellic acid (GA_3) concentration after 4 weeks of culture.



Source: Elaboration of the authors.

Furthermore, after only 4 weeks of *in vitro* culture (Figure 2), 80% germination was obtained, while under natural conditions, from sowing to emergence of *Butia eriospatha* seedlings, 8 months are required (LORENZI, 2010). In another palm tree, 'Macaúba' (*Acrocomia aculeata*), plants were obtained after a 16 week *in vitro* culture (TABAI; MELO; CROCOMO, 1990).

Figure 2 Culture of zygotic embryos of *Butia eriospatha*: a) overview of experimental units in growing room; b) the beginning of embryo culture; c) elongation of the embryos; d) seedlings after 4 weeks of *in vitro* culture in culture medium Murashige and Skoog (MS) supplemented with 8 mg L^{-1} GA_3. **ha**: haustorium; **pc**: cotyledon petiole; **pb**: the first leaf sheath.



Source: Elaboration of the authors.

In *Cocos nucifera*, 80% germination was obtainable in a 5 week culture with only 1.6 mg L^{-1} GA_3 (PECH-AKÉ et al., 2007). In other palm trees species such as *Aiphanes erosa*, *Arenga microcarpa*, *Hyphaene schatan*, *Sabal palmetto*, and *Phoenicophorium borsigianum*, the best results were obtained with concentrations between 10 and 25 mg L^{-1} GA_3 (ODETOLA, 1987). In *Coffea arabica* L., embryo growth was promoted by using 4–8 mg L^{-1} GA_3 (CARVALHO et al., 1998).

There was no interaction between culture medium and initial size of explants, but both factors showed a significant difference individually. Embryos cultivated in Y3 and MS culture media showed a higher increase in fresh mass (Table 2), and both media produced higher concentrations of NO_3^- + NH_4^+ compared to WPM. This result agrees with the argument that the palm tree family is characterized by a tolerance to or even need for higher concentrations of salts (FERREIRA NETO et al., 2007). In addition, myo-inositol, which is present in Y3 and MS media and absent in WPM, increased the fresh weight of tobacco (*Nicotiana tabacum*) calli; when added to phospholipid molecules, a major component of the plasmatic membrane, myo-inositol has several functions, including acting on different combinations of auxins (TORRES; CALDAS; BUSO, 1998). Auxins act in the internal mechanism that controls stem, leaf, and root growth; induce seed germination; and regulate apical dominance (FLOSS, 2006).

Table 2. Means of increases in fresh mass of *Butia eriospatha* zygotic embryos after 4 weeks of *in vitro* culture in different culture media, as a function of explant size class.

Culture medium	Increase in fresh mass (mg)			Mean
	Initial size class of explant (mg)[1]			
	>35[1]	23–35	<23	
Murashige and Skoog (MS)	19.40	12.20	11.20	14.00a[2]
Y3	19.20	12.20	10.80	13.67a
Woody plant medium (WPM)	5.00	2.20	2.60	3.00b
Mean	14.53A	8.86B	8.20B	10.53
CV (%)	16.30			

[1]Means followed by the same letter (lower case within columns and upper case within rows) are not different (Tukey's test, ($P < 0.05$). [2]Initial fresh mass (mg) of zygotic embryos after 4 weeks of culture in MS culture medium and 2 weeks in MS medium + activated charcoal.
Source: Elaboration of the authors.

Y3 medium, elaborated by Eeuwens in 1976, has been used in the tissue culture of palm trees such as *Cocos nucifera* L. (SILVA, 2002) and in the present study was shown to be suitable for the *in vitro* culture of *Butia eriospatha*. This culture medium has a higher potassium level than MS and WPM and, like this nutrient, is involved with osmotic regulation of plant cells and the adaptation of palm trees in saline soils, which is expected to require higher levels of potassium.

For the germination of *Cocos nucifera* L. embryos in 3 culture media, Silva (2002) obtained better performances with Y3 and MS. With the same species, Rillo et al. (1998) observed better results with Y3. In contrast, in 'Aceroleira' (*Malpighia glabra* L.), WPM was superior to MS (MELO et al., 1999).

There was a significant difference in fresh mass increase in the different embryo categories, indicating that greater explants are more vigorous (Table 2). This result is in agreement with Pierik (1990), who found that it is more difficult to induce growth and regeneration in small structures. In 'Ipeca' (*Psychotria ipecacuanha*), for example, the number of sprouts was directly proportional to the length of nodal segments used as explants (REIS et al., 2004).

Conclusions

Disinfestation in 1% NaOCl for 10 min, directly on the embryo and after immersion of the seed in 2% NaOCl for 15 min, must be carried out to control contaminating microorganisms and promote germination of *Butia eriospatha* embryos.

At a concentration of 8 mg L^{-1} GA_3, the germination process is accelerated, and 80% germinated embryos were obtained after 4 weeks of *in vitro* culture.

The use of MS and Y3 culture media is recommended for the *in vitro* culture of zygotic embryos of *Butia eriospatha*.

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