



## Biological control in the germination of seeds from two species native of the Cerrado region

J. C. Oliveira<sup>a</sup> , J. F. Sales<sup>a\*</sup> , A. Rubio-Neto<sup>b</sup> , C. F. Silva<sup>c</sup> , M. A. Soares<sup>d</sup>   
and F. G. Silva<sup>b</sup>

<sup>a</sup>Laboratório de Sementes, Instituto Federal Goiano – IF Goiano, Campus Rio Verde, Av. Sul Goiana, Km 01, Zona Rural, CEP 75900-000, Rio Verde, GO, Brasil

<sup>b</sup>Laboratório de Cultura de Tecidos Vegetal, Instituto Federal Goiano – IF Goiano, Campus Rio Verde, Av. Sul Goiana, Km 01, Zona Rural, CEP 75900-000, Rio Verde, GO, Brasil

<sup>c</sup>Laboratório de Microbiologia Agrícola, Instituto Federal Goiano – IF Goiano, Campus Rio Verde, Av. Sul Goiana, Km 01, Zona Rural, CEP 75900-000, Rio Verde, GO, Brasil

<sup>d</sup>Instituto de Biociências, Universidade Federal de Mato Grosso – UFMT, Av. Fernando Corrêa da Costa, 2367, Boa Esperança, CEP 78060-900, Cuiabá, MT, Brasil

\*e-mail: [juliana.sales@ifgoiano.edu.br](mailto:juliana.sales@ifgoiano.edu.br)

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### Abstract

Microorganisms have been efficiently used for the biological control of phytopathogens through the production of antimicrobial substances. However, the objectives of this work were: to study the germination of *Butia purpurascens* Glassman and *Butia archeri* Glassman seeds in different substrates, to select and identify the endophytic and rhizospheric bacterial isolates of *B. purpurascens* and *B. archeri*, and to perform antibiosis tests based on the isolated microorganisms of these tree species. No difference was found between the cultivation substrates for the percentages of germination, hard seeds, and fungal contamination in the *B. purpurascens* seeds. The *Bacillus subtilis* isolated showed the best capacity for suppressing the growth of the two deteriorative fungi tested in *B. purpurascens* seeds. No difference was found for inhibition of the growth of *Aspergillus niger* fungus (deteriorative fungus of *B. archeri* seeds) between the microorganisms with *Bacillus* sp. and *Brevibacillus brevis* compared to the control. In the microbiolization of *B. purpurascens* and *B. archeri* seeds performed with microbiological solutions produced from the endophytic and rhizospheric strains of *Bacillus* sp., no differences were observed in the percentages of germination and contamination by fungi. For *B. archeri* seeds, there was contamination by fungi and bacteria after one day of cultivation, primarily in the regions with lesions caused by the extraction and scarification process.

**Keywords:** antibiosis, microbiolization, endophytes, physical dormancy.

## Controle biológico na germinação de sementes de duas espécies nativas da região do Cerrado

### Resumo

Microorganismos específicos tem sido usados eficientemente para o controle biológico de fitopatógenos através da produção de substâncias antimicrobianas. Portanto, os objetivos deste estudo foram: estudar a germinação de sementes de *Butia purpurascens* Glassman e *Butia archeri* Glassman em diferentes substratos; selecionar e identificar isolados bacterianos rizosféricos e endofíticos de *B. purpurascens* e *B. archeri*, e realizar testes de antibiose a partir de microorganismos isolados dessas espécies arbóreas. Não houve diferença entre os substratos de cultivo para as porcentagens de germinação, de sementes duras e de contaminação por fungos nas sementes de *B. purpurascens*. O isolado bacteriano *Bacillus subtilis* apresentou melhor capacidade de supressão do crescimento dos dois fungos deterioradores testados nas sementes de *B. purpurascens*. Não foi encontrada diferença para a inibição do crescimento do fungo *Aspergillus niger* (fungo deteriorador de sementes de *B. archeri*) entre os microorganismos com *Bacillus* sp. e *Brevibacillus brevis* em comparação ao controle. Na microbiolização das sementes de *B. purpurascens* e *B. archeri* realizada com soluções microbiológicas produzidas a partir de cepas endofíticas e rizosféricas de *Bacillus* sp. não foram observadas diferenças na porcentagem de germinação e contaminação por fungos. Para *B. archeri* houve contaminação por fungos e bactérias após um dia de cultivo, principalmente nas regiões de lesão proporcionadas pelo processo de extração e escarificação.

**Palavras-chave:** antibiose, microbiolização, endofíticos, dormência física.

## 1. Introduction

It is estimated that the Brazilian Cerrado contributes 10,000 plant species of the 60,000 phanerogams distributed across the country, marking this region as the richest in terms of flora diversity among the savannas of the world. It includes countless medicinal, ornamental, and fruit-bearing species that are important to the local populations (Gusmão et al., 2006) and is also considered one of the “hotspots” for global biodiversity conservation (Myers et al., 2000).

In this study, we used two plant species of great importance from the Cerrado region, *Butia purpurascens* Glassman and *Butia archeri* Glassman, both belonging to the family Arecaceae, popularly known as purple yatay palm and dwarf jelly palm, respectively. These species have great potential in ornamental, culinary applications owing to the flavor and fragrance of their fruits (Lorenzi et al., 2010), and in the therapeutic treatment of skin diseases (Hoffmann et al., 2014).

Species from the genus *Butia* are classified as critically endangered, with an estimated reduction in the population of over 80% over the next 10 years. As it is native to the southern region of Goiás and the northern region of the Minas Gerais Triangle, it competes for land with soy and sugarcane plantations, further exacerbating the extinction process. Seeds of these species have a high rate of contamination by microorganisms during the pre- and post-emergence processes, resulting in the formation of abnormal seedlings and root rot (Magalhães et al., 2008; Bozza, 2009; IUCN, 2011).

Members of the family Arecaceae reproduce largely through sexual reproduction. Owing to the lengthy duration and low germination efficiency, phytohormones must be applied and/or procedures must be adopted that facilitate the absorption of water, such as mechanical scarification. Although scarification can accelerate and increase the percentage of germination, the resulting wounds become an entry point for harmful microorganisms, which hinder or impede germination, resulting in a significant reduction in the number of viable plants (Rubio Neto et al., 2012, 2014).

The use of specific microorganisms for the biological control of phytopathogens has proven to be efficient against certain fungi and bacteria owing to the production of antimicrobial substances (Rocha et al., 2009; Yuan et al., 2017; Saito et al., 2018; Liu et al., 2018), which leads to an alternative to the chemical control of diseases. These biological control agents can act directly and indirectly by inducing the plant's defense mechanisms in the control of fungal diseases (Benítez et al., 2004).

The interactions between pathogenic microorganisms and their antagonists include antibiosis, competition, parasitism, predation, and induction of the host's defenses (Melo and Azevedo, 1998). Most of the microorganisms involved in biological control operate by means of antibiosis, in which a metabolite produced by one microorganism has a harmful effect on the other, thereby inhibiting its growth. The production of metabolites may result in complete

lysis and dissolution of the cellular structure, regardless of physical contact between the microorganisms (Remuska and Pria, 2007).

The objective of this study was to determine the best germination environment for *B. purpurascens* and *B. archeri*, identifying the deteriorative microorganisms infecting their seeds, and testing the ability of bacteria isolated from these tree species to control these microorganisms.

## 2. Material and Methods

### 2.1. Obtaining the seeds

The study was carried out at the Federal Institute of Education, Science, and Technology of Goiás – Rio Verde Campus, GO, in partnership with the Seeds, Plant Tissue Culture, and Agricultural Microbiology laboratories. The fruits of *Butia purpurascens* and *Butia archeri* were collected, respectively, 17°35'10.51"S, 50°59'13.06"W, 822m and 17°35'10.51"S, 50°59'13.06"W, 822m, in the city of Rio Verde, GO. The two species were intercropped with pasture and their fruits were randomly collected.

The fruits were pulped to obtain the diaspores and, subsequently, the seeds were extracted using a bench vise. The extracted seeds were disinfected in 70% alcohol for 1 minute and 40% sodium hypochlorite for 4 minutes. Next, they were washed in distilled water and autoclaved three times. Mechanical scarification was carried out in the laminar flow, removing the tegument in the hilar region (Rubio Neto et al., 2014). The phytosanitary and germination evaluations of the *B. purpurascens* and *B. archeri* seeds were performed according to the experimental tests described below.

### 2.2. Germination of *B. purpurascens* seeds in different substrates

Three germination substrates were evaluated: rolls of Germitest® paper, glass plates (120 × 150 mm) covered with washed and autoclaved sand, and Germitest® paper. On the plates containing sand, a field capacity of 60% was maintained weekly, while the paper was moistened with distilled autoclaved water in the amount of 2.5 times the weight of this dry substrate.

The seeds were kept in a Mangelsdorf germinator set at 30 °C for 30 days. The experiment used a completely randomized design, with seven repetitions of three germination substrates. The test was evaluated on a daily basis. When contamination by fungi or bacteria was detected, the contaminated seeds were removed from the germination environment and taken to the Laboratory of Agricultural Microbiology for identification and antibiosis tests. After 30 days, we evaluated the percentages of germination, contamination by fungi and bacteria, and hard seeds, the latter of which is characterized by the absence of contamination and germination. Only seeds with a cotyledon petiole of at least 1 cm in length were considered germinated.

Next, the data normality and the homogeneity of the variances were evaluated through the Shapiro–Wilk and

Bartlett tests (5%), respectively, and the averages were compared by the Tukey's test (5%).

### 2.3. Selection of the endophytic and rhizospheric bacterial isolates of *Butia purpurascens* and *Butia archeri*

One hundred and fifty-three bacteria (67 endophytics from roots and 86 rhizospherics) were isolated from *B. purpurascens* and 110 bacteria were isolated from *B. archeri* (22 endophytics and 88 rhizospherics). Next, a qualitative test was performed on a Petri dish, containing PDA culture medium (infusion of 200 g of potatoes, 200 g of dextrose and 15 g of agar), in which four bacterial isolates were inoculated in the center of the plate opposite the fungal isolate. Bacteria that impeded the normal growth of the fungal mycelium were considered antagonistic when compared to the control plate, which was inoculated only with the fungal isolate.

### 2.4. Antibiosis test

To quantify the degree of antagonism of the seed-deteriorating microorganisms, we selected eight bacteria (seven rhizospherics and one endophytic) isolated from the species *B. purpurascens*, and two bacteria (one rhizospheric and one endophytic) from *B. archeri*. The tests were performed according to the dual culture method (Mew and Rosales, 1986).

After the seed-deteriorating fungi were isolated, those that appeared with the greatest frequency were selected. For *B. purpurascens*, the fungal species *Neodeightonia phoenicum* BP91DF and *Penicillium purpurogenum* BP110DF were selected, while *Aspergillus niger* BA163D was isolated from *B. archeri* seeds. The fungi were multiplied in PDA culture medium for a period of seven days. Next, the fungal mycelium was inoculated in the center of the Petri dish. *Neodeightonia phoenicum* BP91DF and *A. niger* BA163DF were distanced 3 cm apart with immediate inoculation of the potential antagonists on opposite sides. The fungus *P. purpurogenum* BP110DF, owing to its slow growth, was inoculated 48 hours in advance, distanced 2 cm apart.

The experiment used a completely randomized design, with three repetitions for each fungus. The plates were incubated at room temperature for 4 to 8 days, depending on the speed of fungal growth. The diameter of the fungus was measured with a caliper, and the zone of fungal growth inhibition owing to the production of suppressive compounds by the bacteria was noted.

### 2.5. Identification of the microorganisms

The bacteria that expressed some capacity for antagonism and the seed-deteriorating fungi that were isolated were submitted for molecular identification. The bacteria were identified based on 16S rDNA, and the fungi were identified through partial sequencing of the internal transcribed spacer (ITS) from the rDNA region taken from one representative of each morphotype group.

Total genomic DNA of the purified bacteria was extracted using the method proposed by Cheng and Jiang (2006). The 16s rDNA gene was amplified using the

primers 27F (5'-AGA GTT TGA TCM TGG CTCAG-3') and 1492R (5'-TAC GGY TAC CTT GTT ACG ACT T-3') (Weisburg et al., 1991). PCR was performed in a total volume of 25 µL, containing 1 µL of DNA extract, 17.45 µL of ultra-pure water, 2.5 µL of PCR 10x buffer, 0.75 µL of MgCl<sub>2</sub>, 2 µL of dNTP, 1 µL of 27F primer, 1 µL of 1492R primer, and 0.3 µL of the Taq polymerase enzyme (Invitrogen®). The amplification conditions were performed as follows: initial denaturation at 94 °C for 2 min; followed by 30 cycles of 94 °C denaturation for 40 sec, annealing at 58 °C for 35 sec, and elongation at 72 °C for 1 min 20 sec; and a final extension at 72 °C for 10 min.

The fungal isolates were grown in a liquid PD medium (400 mL of potato infusion, 20 g of dextrose) at room temperature for seven days. The fungal mycelium was washed in distilled water using a sieve to remove all the culture medium. Next, the sample was dried with a paper towel to remove moisture and placed in the freezer at -20 °C until the analysis.

The genomic DNA of the fungi was extracted using a DNA extraction kit, following the manufacturer's recommendations (Axygen Biosciences, USA). Morphotyping was carried out by analyzing the genetic variability of the ISSR (Inter Simple Sequence Repeat Amplification) and IRAP (Inter Retrotransposon Amplified Polymorphism) molecular markers.

The identification was performed through partial sequencing of the internal transcribed spacer (ITS) of the rDNA region of representatives of each morphotype group. The oligonucleotides ITS 4 (5'-TCC TCC GCT TAT TGA TAT GC-3') and ITS 5 (5'-GGA AGT AAA AGT CGT AAC AAG G-3') (White et al., 1990) were used to amplify the 18S to 28S intergenic region. The reaction mixture of 35 µL contained 22.35 µL of ultra-pure water, 3.5 µL of 10x PCR buffer, 1.5 µL of MgCl<sub>2</sub>, 2.8 µL of dNTP, 1 µL of ITS4 primer, 1 µL of ITS5 primer, and 0.3 µL of the Taq polymerase enzyme. The amplification conditions were performed in an AmpliTherm Thermal Cycler as follows: initial denaturation at 94 °C for 2 min; 35 cycles of 94 °C denaturation for 45 sec, annealing at 50 °C for 45 sec, and elongation at 72 °C for 1 min; and the final extension at 72 °C for 10 min.

The amplification products of the gene 16S (bacteria) and the ITS region (fungi) were purified according to Dun and Blattner (1987). To quantify the DNA, 1 µL of the product was loaded into a 0.8% agarose gel for gel electrophoresis. The sequencing was performed using the Sanger method with a Big Dye Kit on an ABI 3100 (Applied Biosystems). The sequences were compared in the GenBank database (NCBI, 2019) against known sequences through a search for similarity using BLASTN.

### 2.6. Microbiolization of the *Butia purpurascens* and *Butia archeri* seeds

The bacteria were selected according to the best results on the antibiosis test. To prepare the bacterial suspensions, the isolates were cultivated in a nutrient broth medium (3 g meat extract, 5 g peptone) separately for 24 hours.

After this period, the concentration of the inoculation was adjusted to  $OD_{600} = 0.1$  with a sterilized saline solution (0.85 NaCl).

The seeds were submerged in a  $GA_3$  (200 mg L<sup>-1</sup>) solution for 48 hours in a germinator set to 30 °C and then submerged for 30 min in bacterial inoculants. For the *Butia purpurascens* seeds, the following microorganisms were used: control (nutrient broth), the rhizospheric bacteria *Bacillus amyloliquefaciens* BP1RB, *B. amyloliquefaciens* BP70RB, *B. subtilis* BP186RB, and *B. methylotrophicus* BP35EB (endophytic). For *Butia archeri*, seeds taken from the *Bacillus* sp. BA68EB (endophytic) and *Brevibacillus brevis* BA89R (rhizospheric) and the control were used.

Once removed from the inoculum, the seeds were placed on glass plates (120 × 150 mm) containing Germitest® paper to germinate. These were moistened with autoclaved distilled water in the amount of 2.5 times the weight of this paper and kept in a Mangelsdorf germinator set to 30 °C for 30 days.

The experiment used a completely randomized design with five repetitions each. The percentages of germination, contamination by fungi and bacteria, and hard seeds were evaluated. The averages were compared through the Tukey's test (5%).

### 3. Results

#### 3.1. Germination of *Butia purpurascens* seeds in different substrates

There was no difference between the cultivation for the *B. purpurascens* seeds in terms of the percentages of germination, hard seeds, and contamination by fungi (Table 1). A difference between the germination substrates was observed only in the percentage of contamination by bacteria: the glass plate with Germitest® paper yielded the least contamination by bacteria at only 0.8% (Table 1).

We highlight that the contamination occurred mainly near the lesions during seed extraction and scarification, which may have served as a gateway for saprophytic microorganisms (fungi and bacteria), and possibly reduced the germination rates of the species in these substrates.

#### 3.2. Identification of the microorganisms

The grouping of the fungal isolates from seeds of the three evaluated cultivation substrates into morphotypes indicated the presence of 17 groups for *B. purpurascens*, totaling 67 fungi and, for *B. archeri*, 10 groups with 40 fungi. These were distinguished by macroscopic characteristics. After molecular identification of one representative of each

group through the amplification profile of the molecular marker ISSR and/or IRAP of the different strains (data omitted), identical profiles could be detected, enabling taxonomic identification.

At the species level, an identity above 97% of the ITS region was used as a criterion of similarity (O'Brien et al., 2005).

For *B. purpurascens* seeds, this study revealed the association of fungi only from the phylum Ascomycota, from which three classes were identified: Eurotiomycetes (64.2%), Sordariomycetes (20.9%), and Dothideomycetes (14.9%). These were distributed across 10 genera: *Aspergillus* (28.4%); *Penicillium* (25.4%); (Table 2). For *B. archeri*, there was also a predominance of members of the phylum Ascomycota, and 40 taxa from three classes were identified: Eurotiomycetes (82.5%), Sordariomycetes (12.5%), and Dothideomycetes (5%), distributed across seven genera, predominated by *Aspergillus* (67.5%); *Gibberella*, *Penicillium* and *Talaromyces* (7.5%), and *Fusarium* and *Neodeightonia* (5%), as shown in Table 3.

The antagonistic bacteria selected and identified were exclusively of the genus *Bacillus*, and of the species *amyloliquefaciens*, *brevis*, *methylotrophicus*, and *subtilis* (Table 4). Members of this genus are commonly found in environments with soil and water. Some of its species have antifungal activity against plant pathogens and are capable of inhibiting mycelial growth and the germination of conidia, and thus play important roles in the infection cycle of pathogenic agents (Song et al., 2014).

#### 3.3. Antibiosis

For *Butia purpurascens*, the deteriorative fungus *N. phoenicum* BP91DF seeds were inhibited by the rhizospheric bacteria *B. amyloliquefaciens* BP1RB and *B. subtilis* BP186RB (Figure 1 and Table 5).

The fungus *P. purpurogenum* BP110DF was inhibited by the isolates *B. amyloliquefaciens* BP1RB, BP66RB, BP70RB, and BP201RB (rhizospheric); *B. subtilis* BP60RB and BP186RB; and *B. methylotrophicus* BP35EB (endophytic). The rhizospheric bacterial isolates of *B. amyloliquefaciens* BP1RB and *B. subtilis* BP186RB showed the highest capacity for suppressing the growth of the *Neodeightonia phoenicum* BP91DF (Table 5). The action of the bacteria were similar for the fungus *Penicillium purourogenum* BP110DF.

In the evaluation of the results for growth inhibition of the deteriorative fungus of *B. archeri* (*A. niger* BA163DF) seeds, no difference was found between the microorganisms with *Bacillus* sp. BA68EB and *B. brevis* BA89RB when compared to the control (Table 6).

**Table 1.** Percentages of germination, hard seeds, and contamination by fungi and bacteria in *Butia purpurascens* seeds in different substrates.

	Contamination (%)		Germination (%)	Hard seeds (%)
	Fungi	Bacteria		
Petri Dish+Paper	28.5 ± 7.2 a <sup>z</sup>	0.8 ± 0.8 a	50.9 ± 5.1 a	19.9 ± 6.5 a
Petri Dish +Sand	30.9 ± 4.7 a	17.4 ± 6.0 b	36.2 ± 7.3 a	15.5 ± 5.5 a
Paper "Germitest®"	25.8 ± 10.5 a	7.0 ± 3.0 ab	34.9 ± 6.6 a	32.4 ± 6.8 a

<sup>z</sup>Means followed by the same letter in the vertical are not significantly by Tukey test (P > 0.05). ± Average Standard Error.

**Table 2.** Identification of the deteriorative fungi isolates in the *Butia purpurascens* seeds based on the sequencing of the ITS region.

Isolated / Quantity	Identification Blast	N° Access on GenBank	% Identity	Relative Frequency (%)
BP202DF (1)	<i>Aspergillus fumigatus</i>	KC119200	99	1.5
BP209DF (5)	<i>Aspergillus niger</i>	EF175904	99	7.5
BP131DF (3)	<i>Aspergillus parasiticus</i>	HQ340110	99	4.5
BP203DF (10)	<i>Aspergillus terreus</i>	GU966497	99	14.9
BP100DF (9)	<i>Colletotrichum theobromicola</i>	JX010285	99	13.4
BP206DF (1)	<i>Fusarium avenaceum</i>	FJ478097	99	1.5
BP139DF (3)	<i>Fusarium concentricum</i>	HQ379635	99	4.5
BP105DF (1)	<i>Fusarium equiseti</i>	GQ505694	99	1.5
BP91DF (8)	<i>Neodeighthonia phoenicum</i>	HQ443209	99	11.9
BP125DF (3)	<i>Neosartorya fischeri</i>	AF455541	98	4.5
BP94DF (2)	<i>Neosartorya spinosa</i>	EF669973	99	3.0
BP209DF (1)	<i>Paecilomyces variotii</i>	AY753334	97	1,5
BP125DF (7)	<i>Penicillium pinophilum</i>	FJ441618	99	10.4
BP110DF (10)	<i>Penicillium purpurogenum</i>	FN868483	98	14.9
BP104DF (1)	<i>Peyronellaea pomorum</i>	JN003244	99	1.5
BP127DF (1)	<i>Pleosporales</i> sp.	HQ832821	99	1.5
BP138DF (1)	<i>Talaromyces amestolkiae</i>	JX965247	99	1.5

BP = *Butia purpurascens*; D = deteriorative; F = fungi; ( ) = total isolates.

**Table 3.** Identification of the deteriorative fungi isolates in the *Butia archeri* seeds based on the sequencing of the ITS region.

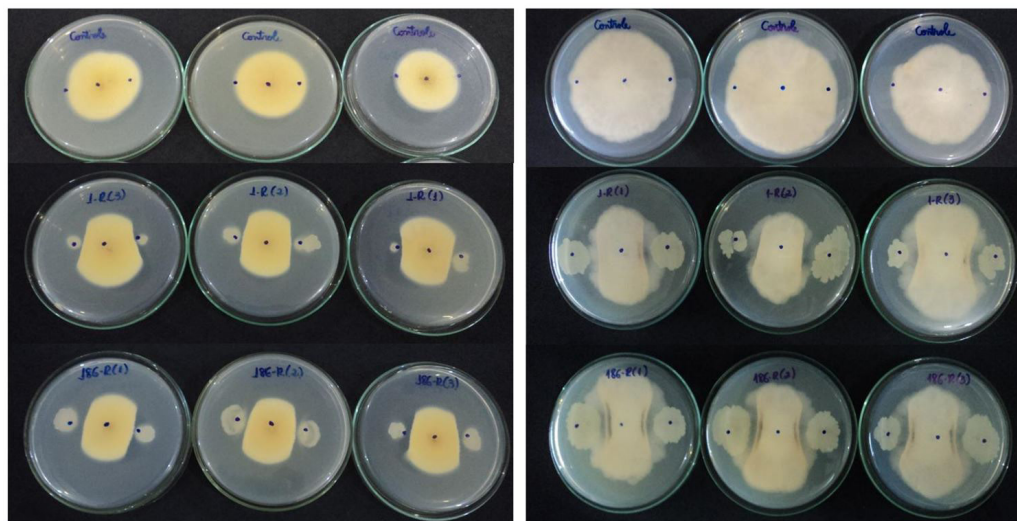
Isolated / Quantity	Identification Blast	N° Access on GenBank	% Identity	Relative Frequency (%)
BA181DF (1)	<i>Aspergillus flavus</i>	EF409804	99	2.5
BA163DF (20)	<i>Aspergillus niger</i>	KF304798	98	50.0
BA142DF (3)	<i>Aspergillus</i> sp.	JF312217	99	7.5
BA149DF (3)	<i>Aspergillus ustus</i>	AY213638	98	7.5
BA141DF (2)	<i>Fusarium proliferatum</i>	EU821492	99	5.0
BA145DF (2)	<i>Gibberella amoniliformis</i>	AB374142	98	5.0
BA139DF (1)	<i>Gibberella intermedia</i>	JQ272470	98	2.5
BA170DF (2)	<i>Neodeighthonia phoenicum</i>	EU673340	98	5.0
BA178DF (3)	<i>Penicillium</i> sp.	JQ912017	98	7.5
BA156DF (3)	<i>Talaromyces</i> sp.	AB808419	99	7.5

BA = *Butia archeri*; D = deteriorative; F = fungi; ( ) = total de isolados.

**Table 4.** Identification of endophytics and rhizosphere bacteria isolated from roots of *Butia purpurascens* based on 16S gene sequencing.

Isolate	Identification Blast	N° Access on GenBank	% Identity
BP35EB	<i>Bacillus methylotrophicus</i>	JF899259	99
BP1RB	<i>Bacillus amyloliquefaciens</i>	KC692174	99
BP60RB	<i>Bacillus subtilis</i>	DQ517306	99
BP66RB	<i>Bacillus amyloliquefaciens</i>	KF483660	99
BP70RB	<i>Bacillus amyloliquefaciens</i>	KC692174	99
BP71RB	<i>Bacillus amyloliquefaciens</i>	KF483660	99
BP186RB	<i>Bacillus subtilis</i>	KF241533	99
BP201RB	<i>Bacillus amyloliquefaciens</i>	KC708068	98
BA68EB	<i>Bacillus</i> sp.	GQ180177	98
BA89EB	<i>Brevibacillus brevis</i>	HM449127	99

BP = *Butia purpurascens*; BA = *Butia archeri*; E = endophytic; R = rhizospheric; B = bacteria.



*Penicillium purpurogenum* BP110DF

*Neodeightonia phoenicum* BP91DF

**Figure 1.** Antibiosis test of isolates of the deteriorative fungi of seeds *Penicillium purpurogenum* BP110DF and *Neodeightonia phoenicum* BP91DF with the bacterias *Bacillus amyloliquefaciens* BP1RB (second line) and *Bacillus subtilis* BP186RB (third line) and controls (first line).

**Table 5.** Antibiosis test of the deteriorative fungi isolates (*Penicillium purpurogenum* and *Neodeightonia phoenicum*) of the *Butia purpurascens* seeds.

Treatment	<i>Penicillium</i>	<i>Neodeightonia</i>
	<i>purpurogenum</i> BP110DF	<i>phoenicum</i> BP91DF
	-----cm-----	
Control	4.5 <sup>z</sup> c	6.7 d
<i>Bacillus amyloliquefaciens</i> BP1RB	2.8 ab	2.9 a
<i>Bacillus subtilis</i> BP60RB	2.5 a	4.4 c
<i>Bacillus amyloliquefaciens</i> BP66RB	2.8 ab	3.5 b
<i>Bacillus amyloliquefaciens</i> BP70RB	2.7 ab	3.4 b
<i>Bacillus amyloliquefaciens</i> BP71RB	3.0 b	3.5 b
<i>Bacillus subtilis</i> BP186RB	2.6 ab	2.5 a
<i>Bacillus amyloliquefaciens</i> BP201RB	2.3 ab	3.6 b
<i>Bacillus methylotrophicus</i> BP35EB	2.8 ab	3.7 b

<sup>z</sup>Means followed by the same letter in the column are not significantly by Tukey test (P > 0.05). ± Average Standard Error.

**Table 6.** Antibiosis test of the deteriorative fungi isolates (*Aspergillus niger*) of the *Butia archeri* seeds.

Treatment	<i>Aspergillus niger</i>
	BA163DF
	-----cm-----
Control	5.95 <sup>z</sup> a
<i>Bacillus</i> sp. BA68EB	5.85 a
<i>Brevibacillus brevis</i> BA89RB	5.1 a

<sup>z</sup>Means followed by the same letter in the column are not significantly by Tukey test (P > 0.05).

3.4. Microbiolization of *Butia purpurascens* and *Butia archeri* seeds

In the microbiolization of *B. purpurascens* and *B. archeri* seeds using endophytic and rhizospheric strains of *Bacillus* sp., no differences were found in the percentages of

germination and contamination by fungi, the latter of which varied between 19.3 to 39.3% (Table 7). There was also no difference between the solutions for seed germination, varying from 24.3 to 41.0% for the yatay palm.

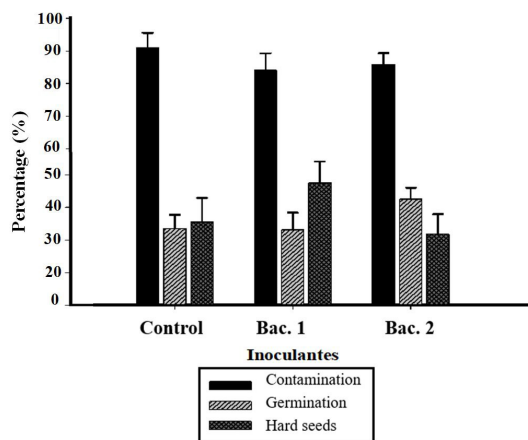
Contamination by fungi and bacteria was noted for *B. archeri* after one day of cultivation, mainly in the regions of lesions caused by the extraction and the scarification process. We observed that initially there was intense release of seed exudates, followed by fungal contamination. However, there was no difference in fungal and bacterial contamination between bacterial treatments, which varied between 60% and 63.3%.

A low percentage of germination was found, with a minimum of 16.6% and maximum of 22.5%. There was no difference in germination efficiency between the bacteria evaluated to control the contamination during the evaluated period (Figure 2).

**Table 7.** Percentage of germination and contamination in *Butia purpurascens* seeds treated with different inoculants.

	Germination (%)	Contamination (%)
Control	26.7 <sup>z</sup> ± 16.0	41.0 ± 8.2
<i>Bacillus amyloliquefaciens</i> BP1RB	39.3 ± 16.6	26.7 ± 6.1
<i>Bacillus amyloliquefaciens</i> BP70RB	25.1 ± 6.1	31.3 ± 8.2
<i>Bacillus subtilis</i> BP186RB	23.6 ± 5.3	29.3 ± 5.6
<i>Bacillus methylotrophicus</i> BP35EB	19.3 ± 3.5	24.3 ± 3.3

<sup>z</sup>Means followed by the same letter in the column are not significantly by Tukey test ( $P > 0.05$ ).



**Figure 2.** Percentage of germination, hard seeds and contamination by fungi in *Butia archeri* seeds treated with different inoculants. Control = Nutrient broth; Bac. 1 = Nutrient broth plus endophytic bacteria; Bac. 2 = Nutritious broth plus rhizosphere bacterium.

#### 4. Discussion

The results observed in this study for the percentage of germination of *B. purpurascens* seeds in different substrates was lower than that observed by Fior et al. (2011) with *Butia capitata* (Martius) Beccari seeds, which reached 90% germination when kept in Gerbox plastic boxes containing autoclaved sand for 150 days. The germination of wax palm (*Copernicia alba* Morong.) seeds was also favored: it reached 67% when sowed on paper at a temperature between 20 and 30 °C (Masetto et al., 2012). Rubio Neto et al. (2014) obtained 63.8% germination of *Acrocomia aculeata* (Jacq.) Lood. ex Mart. seeds when they were scarified and kept on rolls of Germitest® paper.

In this study, the seeds were not immersed in a gibberellic acid solution, which is an alternative to increase the germination percentages of seeds with physiological dormancy. Studies consider immersing the seeds in a gibberellic acid solution and removing the opercular tegument to be an alternative method that can promote increased germination in macaw palm seeds (Oliveira et al., 2013; Rodrigues Junior et al., 2013).

For the *B. purpurascens* seeds, the bacterial isolate *B. subtilis* BP186RB showed the best capacity for suppressing the growth of *Neodeightonia phoenicum* BP91DF and

*Penicillium purpurogenum* BP110DF, the two deteriorative fungi tested. However, for *B. archeri* seeds, no difference were observed between the microorganisms with *Bacillus* sp. BA68EB and *B. brevis* BA89RB compared to the control in inhibiting the growth of deteriorative fungi.

*Bacillus* sp. are capable of synthesizing a structurally diverse group of antimicrobial compounds of low molecular weight that suppress phytopathogens and cyclic peptide derivatives, especially lipopeptides (Kilimushi et al., 2017). Gond et al. (2015), through a MALDI-TOF-MS analysis, identified antifungal groups such as surfactin, iturin, and fengycin that exhibited strong antibacterial activity, consequently inhibiting the growth of filamentous fungi by antagonizing sterols, phospholipids, and oleic acid in fungal membranes, thereby protecting against pathogenic agents.

Song et al. (2014) performed a study for controlling ginseng root rot, caused by the fungus *Fusarium*, through the use of *B. amyloliquefaciens*. The study showed, through scanning electron microscopy, that the hyphae of the pathogen were twisted and withered with bacterial treatment, which can be a symptom of direct damage by antifungal substances.

In the microbialization of *B. purpurascens* and *B. archeri* seeds performed with microbiological solutions produced based on endophytic and rhizospheric strains of *Bacillus* sp., no differences were observed in the percentages of germination and contamination by fungi.

For *B. archeri*, there was contamination by fungi and bacteria after one day of cultivation, mainly in the regions with lesions caused by the extraction and scarification process.

This is the first study for *B. purpurascens* and *B. archeri* that shows major problems with contamination during germination, exhibiting low percentages of normal seedlings. However, further studies should be carried out to further validate the use of biological control in these species.

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