Pollen morphology, meiotic index and pollen viability in individuals of *Vochysia divergens* Pohl. native to the Amazon and the Pantanal

Morfologia do pólen, índice meiótico e viabilidade polínica em indivíduos de *Vochysia divergens* Pohl. nativos da Amazônia e Pantanal

Morfología del polen, índice meiótico y viabilidad del polen en individuos de *Vochysia divergens* Pohl. nativa de la Amazonía y el Pantanal

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Abstract
Vochysia divergens Pohl. is a tree species used in folk medicine, landscaping and reforestation. Its occurrence is recorded in the Amazon, Pantanal and Cerrado biomes and, despite these uses and its wide distribution, studies regarding its reproductive aspects are nonexistent. Thus, this research aimed to study the morphology of the pollen of *V. divergens* and evaluate the pollen viability and its meiotic index, as well as compare these descriptors in two natural populations, those of the Amazon and the Pantanal. To characterize the morphology, pollen grains were subjected to acetolysis and subsequently photographed, measured and classified. The meiotic index (MI) was estimated by counting post-meiotic products. Pollen viability was evaluated via colorimetric testing and in vitro germination. Pollen grains do not differ in morphology between populations and are classified as: large, 3-colporate, isopolar, subprolate, with a small and exine polar area that has a pattern of ornamentation that is perforated in the polar region. Via the colorimetric testing, the MI and the pollen viability were greater than 95% and only the MI differed between populations. Pollen germination was low and it was found that the presence of boric acid and absence of agar positively influence germination. The pollens of *V. divergens* have starch and lipids as a reserve substance. It can therefore be concluded that the species has reproductive stability, which is important in the effectiveness of fertilization and generation of new individuals for the formation of new generations, thus continuing the processes of forest restoration and maintenance of genetic variability. The information obtained regarding the pollen morphology can be used to support taxonomic, paleoclimatic and paleovegetational studies.
Keywords: Cambará; Cytochemistry; Pollen germination; Palynology; Vochysiaceae.

Resumo
Vochysia divergens Pohl. é uma espécie arbórea utilizada na medicina popular, paisagismo e reflorestamento. Sua ocorrência é registrada nos biomas Amazônia, Pantanal e Cerrado e, apesar destes usos e sua ampla distribuição, estudos sobre seus aspectos reprodutivos são inexistentes. Assim, esta pesquisa objetivou estudar a morfologia do pólen e avaliar

a viabilidade polínica e índice meiótico de *V. divergens*, assim como, comparar esses descritores em duas populações naturais, da Amazônia e Pantanal. Para caracterizar a morfologia, os grãos de pólen foram submetidos a acetólise e posteriormente, fotografados, medidos e classificados. O índice meiótico (IM) foi estimado pela contagem de produtos pós-meióticos. A viabilidade polínica foi avaliada via teste colorimétrico e germinação *in vitro*. Os grãos de pólen não diferem quanto a morfologia entre as populações e são classificados como: grandes, 3-colporados, isopolares, subprolatos, de área polar pequena e exina com padrão de ornamentação perfurada na região polar. O IM e a viabilidade polínica via teste colorimétrico foram superiores a 95% e apenas o IM diferiu entre as populações. A germinação do pólen foi baixa e verificou-se que a presença do ácido bórico e ausência de ágar influenciam positivamente a germinação. Os polens de *V. divergens* possuem amido e lipídeos como substância de reserva. Conclui-se que a espécie possui estabilidade reprodutiva, a qual é importante na efetivação da fertilização e geração de novos indivíduos para formação de novas gerações, assim dando continuidade aos processos de restauração florestal e manutencão da variabilidade genética. As informações obtidas a respeito da morfologia polínica poderão ser utilizadas para subsidiar estudos taxonômicos, paleoclimáticos e paleovegetacionais.
Palavras-chave: Cambará; Citoquímica; Germinação do Pólen; Palinologia; Vochysiaceae.

Resumen

Vochysia divergens Pohl. es una especie de árbol utilizada en medicina popular, paisajismo y reforestación. Su ocurrencia está registrada en los biomas Amazonía, Pantanal y Cerrado y, a pesar de estos usos y su amplia distribución, los estudios sobre sus aspectos reproductivos son inexistentes. Por lo tanto, esta investigación tuvo como objetivo estudiar la morfología del polen y evaluar la viabilidad del polen y el índice meiótico de *V. divergens*, así como comparar estos descriptores en dos poblaciones naturales, de la Amazonía y el Pantanal. Para caracterizar la morfología, los granos de polen fueron sometidos a acetólisis y posteriormente fotografiados, medidos y clasificados. El índice meiótico (IM) se estimó contando los productos posmeióticos. La viabilidad del polen se evaluó mediante prueba colorimétrica y germinación in vitro. Los granos de polen no difieren en morfología entre poblaciones y se clasifican en: grandes, 3-colporados, isopolares, subprolatos, con área polar pequeña y exina con un patrón de ornamentación perforada en la región polar. IM y la viabilidad del polen mediante prueba colorimétrica fueron superiores al 95% y solo IM difirió entre poblaciones. La germinación del polen fue baja y se encontró que la presencia de ácido bórico y la ausencia de agar influyen positivamente en la germinación. Los pólenes de *V. divergens* tienen almidón y lípidos como sustancias de reserva. Se concluye que la especie presenta estabilidad reproductiva, lo cual es importante en la efectividad de la fecundación y generación de nuevos individuos para la formación de nuevas generaciones, continuando así los procesos de restauración forestal y mantenimiento de la variabilidad genética. La información obtenida sobre la morfología del polen se puede utilizar para apoyar estudios taxonómicos, paleoclimáticos y paleovegetacionales.
Palabras clave: Cambará; citoquímica; Germinación de polen; Palinología; Vochysiaceae.

1. Introduction

The family Vochysiaceae comprises eight genera and approximately 240 species, of which six genera and 163 species are found in Brazil (Shimizu & Gonçalves, 2017). *Vochysia* is the largest genus of this family with about 140 species. This genus is Neotropical and represented mainly by trees with lush yellow inflorescences that occur mainly in Brazil (Gonçalves et al., 2017). Among the 88 species of the genus *Vochysia* that occur in Brazil, we have *Vochysia divergens* Pohl, which is popularly known as "cambará" (Arieira & Cunha, 2012; Flora do Brasil, 2020), and is a species that is distributed in the Amazon, the Pantanal and the Cerrado biomes, though with greater abundance in the Pantanal. The uses of this species range from carpentry for the manufacture of troughs, boats and toys to applications in folk medicine, landscaping, reforestation, forest recovery and restoration (Hess et al., 1995; Lorenzi, 2002; Guarim Neto, 2006; Carvalho, 2015; Andrade Netto et al., 2015). In the Pantanal, it is considered the main timber species for rational use (Oliveira et al., 2018).

Species of the genus *Vochysia* have similar floral and pollen characteristics, and their adequate characterization facilitates the identification of species (Vianna, 1980; Oliveira & Gibbs, 1994; Vianna et al., 2002). Studies on the morphology of pollen in several species of the genus have already been carried out (Vianna, 1980; Vianna et al., 2002; Barth & Luz, 2014); however, studies on the morphology, viability and cytochemistry of pollen grains of *V. divergens* are not yet available.

The morphological characteristics of pollen grains can often have great variability (Plá Junior et al., 2009), are considered of great taxonomic value, and can be used to assist in the identification and classification of species in genera and families (Talledo et al., 2019). Palynology is also an important tool to help understand how climate change has shaped vegetation over time, and may indicate periods of higher or lower humidity and burning regimes (Cassino et al., 2020). In addition, pollen studies can help in understanding the propagation potential, which ensures the reproductive success of a species (Blackmore, 2007; Plá Junior et al., 2009; Luz et al., 2018).

The estimation of pollen viability evidences the male reproductive potential and interferes with the dispersion and gene flow of plant species (Frescura et al., 2012), since only viable pollen grains can perform fertilization. The viability of pollen is directly related to stable and normal meiotic divisions, since any change in the division process can lead to the formation of abnormal post-meiotic products and consequently generates unviable gametophytes (Pagliarini, 2000; Pagliarini & Pozzobon, 2005). In this sense, studies on the pollen viability of a species can provide basic information for application in conservation plans and ecosystem and population management studies (Costa et al., 2020).

The term viability is related to the ability to live, grow, germinate or develop (Dafni & Firmage, 2000), and has already been used to refer to pollen grains that germinate in stigma, to the number of viable seeds produced (Rathod et al., 2018), to the pollen grains that germinate *in vitro* (Marcellán & Camadro, 1996) and to pollens that stain in a specific way (Frescura et al., 2012). Therefore, there are several ways to test the viability of the pollen of a species, and, according to Rathod et al. (2018), it is important to carry out different methods to reach a more reliable result.

Colorimetric tests are widely used to evaluate pollen viability because they are relatively simple and present fast results, and many studies suggest that Alexander's reagent is the preferred colorimetric test, due to its ability to differentiate non-aborted pollen grains from aborted ones through different coloration (Alexander, 1980; Pagliarini & Pozzobon, 2005; Auler et al., 2006; Munhoz et al., 2008; Frescura et al., 2012; Hister & Tedesco, 2016). *In vitro* germination consists of subjecting the pollen grain to a condition similar to that found in the stigma (Sousa et al., 2010). However, the development of this method is influenced by different factors, such as the constituents of the culture medium, the temperature and the incubation time (Reis et al., 2011), which must be tested to establish the appropriate germination conditions for each species.

Other information about pollen grains that can help in understanding the ecology and dispersal of a species is the determination of their reserve content. As reserve substances, mature pollen grains usually have proteins, carbohydrates and lipids (Nepi & Franchi, 2000). These reserves provide energy so that the pollen grain can germinate, and can indicate the type of pollination of the species (Baker & Baker, 1979; Pacini et al., 2006).

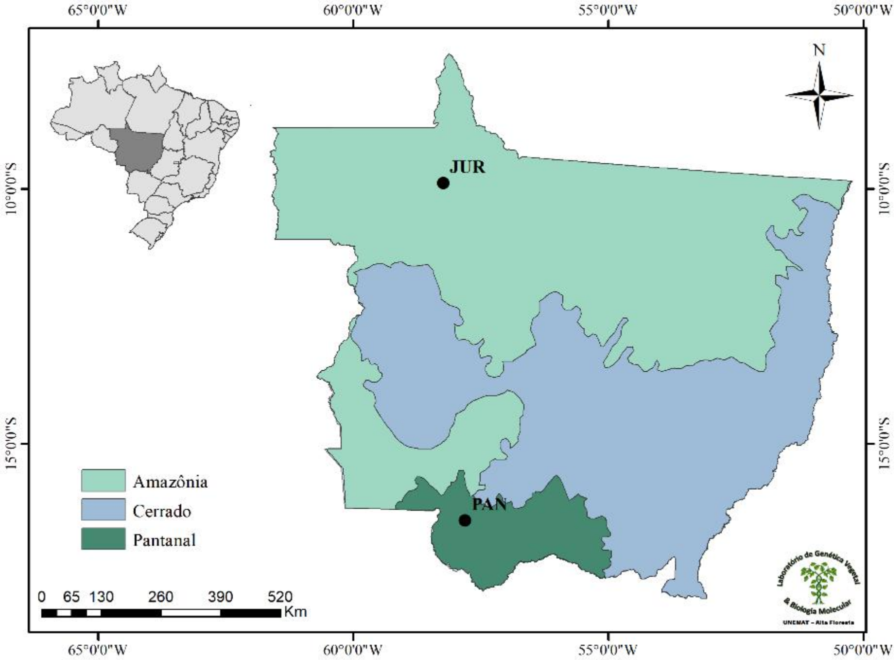
In view of the above, this study aimed to (i) examine the morphology of the pollen and estimate the meiotic index, viability and pollen cytochemistry of *V. divergens* in two natural populations of the Amazon and Pantanal biomes; (ii) verify the existence of differences between the analyzed characteristics between the two populations; (iii) generate knowledge to subsidize taxonomic, paleoclimatic, paleovegetational, ecological and dispersal studies of the species.

2. Methodology

2.1 Study area

The samplings for this study were carried out in two populations of *V. divergens* in the state of Mato Grosso, Brazil. The first population (JUR) is located in the Amazon biome, on the banks of the Juruena River between the municipalities of Cotriguaçu, and Nova Bandeirantes (58°13'56"S 9°53'05"W), and the second population (PAN) is located in the Pantanal biome on the banks of the Paraguay River, in the municipality of Cáceres (57°41'02"S, 15°58'13"W) (Figure 1).

Figure 1. Locations of the two populations of *Vochysia divergens* sampled in this study.



Source: Authors.

The region of the Amazon in which the collections were carried out has an *Am* climate type, with a well-defined dry and rainy season, average annual temperature over 26 °C and annual rainfall between 2,800 to 3,100 mm (Alvares et al., 2013). The vegetation is classified as open and dense ombrophilic forest, semi-deciduous and deciduous forest (Lira, 2011; Zappi et al., 2011). The sampling region in the Pantanal has an *Aw* climate type, with annual precipitation ranging from 1,300 to 1,600 mm and annual average temperature of 24 to 26 °C (Alvares et al., 2013).

2.2 Collection of flower buds

Flower buds at various stages of development were collected from five individuals in each population. At the time of collection, the flower buds were fixed in a solution of ethyl alcohol and glacial acetic acid (3:1 v/v). After 24 hours, they were transferred to 70% alcohol and kept under refrigeration at approximately 4 °C. For the *in vitro* germination, we used the pollen of flower buds in pre-anthesis of three specimens different from *V. divergens*, which were collected in the population of the Amazon region, one day before and stored under refrigeration (4 °C).

2.3 Morphological characterization of pollen

For the morphological analysis, the pollen grains were submitted to the acetolysis method, as described by Erdtman (1943), with modifications: a decrease in the amount of acetic acid from 1000 µl to 500 µl, an increase in the water bath temperature from 70 to 80 °C to 100 °C for two minutes, and an increase in the time in contact with the glycerin and distilled water mixture from ten minutes to one hour, without removal from the solution before the preparation of the slides.

The slides were prepared and the pollen grains were photographed on the same day that acetolysis was performed in order to avoid errors due to intumescence and changes in their size. The slides were observed in binocular optical microscope with magnitude of 400x (Biocam). The images were obtained by a camera (CMOS 1.3) coupled to the microscope and with the aid of an image capture system (Tsview 7).

The polar and equatorial diameters were measured in equatorial view (pollen grain perpendicular to the polar view), the equatorial diameter in polar view (pollen grain with the polar area facing the observer) of the pollen grains, the thickness of the exine layers (sexine and nexine) and the apocolpium. Measurements were made of 25 pollen grains in equatorial view and 25 pollen grains in polar view for each population (Amazon and Pantanal), with the aid of a quantitative analysis software program (Anati Quanti 2® UFV) (Aguiar et al., 2007).

Pollen grains were classified according to shape, through the relationship between the polar axis and the equatorial axis (P/E) in equatorial view, as proposed by Erdtman (1943). Pollen grain size was estimated according to Erdtman (1945), based on the length of the largest axis and classified according to the following classes: very small (< 10 µm), small (10-25 µm), medium (25-50 µm), large (50-100 µm), very large (100-200 µm) and giant (> 200 µm).

Pollen grains were also classified in relation to the polar area index (PAI) as proposed by Barth and Melhem (1988) and cited by Martins et al. (2010), which is given by the relationship between the ends of two adjacent apertures (or their margins) and the largest width of the pollen grain in polar view. The description of the pollen and the terminologies adopted were based on the glossary of Barth (1964) and Punt et al. (2007).

The measures were submitted to descriptive statistical analysis in the Genes program (Cruz, 2016), and the mean, standard deviation from the mean, coefficient of variation and confidence interval were obtained. After verifying the normality and homogeneity of the data, these were submitted to Student's t-test with 5% probability using the R program (RStudio Team, 2018).

2.4 Meiotic index and pollen viability via colorimetric test

The estimate of the meiotic index (MI) of *V. divergens* was performed with young flower buds of 10 individuals (5 of each population). To prepare the slides for the estimation of the MI, the anthers were removed and lightly macerated on the slide with a drop of 2% acetic carmine, according to the methodology described by Guerra and Souza (2002). The observation of the slides and counting of the post-meiotic products was carried out under a binocular optical microscope (Photonics Bio 2 LED, BEL) at a magnification of 400x. A total of 2,000 post-meiotic products were counted per individual. Tetrads with four cells of the same size were considered normal and any deviation (monad, dyad, triad and polyad) were considered abnormal, and the MI was obtained using the formula:

$$\text{MI}= \frac{\text{Number of normal tetrads}}{\text{Total number of post-meiotic products}} \text{ x } 100$$

Pollen viability was estimated in flower buds in pre-anthesis of the same individuals for which MI was estimated. The visualization of viable and unviable pollen grains was based on the method of Alexander (1969), which uses a solution containing two dyes: acid fuschin and malachite green. Via this staining, viable pollen grains present purple-colored protoplasms and green-colored walls and the unviable ones present a green coloration.

The slides were assembled following the maceration methodology proposed by Guerra and Souza (2002) and observation was carried out as previously described for the meiotic index. A total of 2,000 pollen grains per individual and 10,000 per population were counted. The percentage of pollen viability was obtained using the formula:

$$\text{Viability \%} = \frac{\text{Number of viable pollen grains}}{\text{Total number of pollen grains counted}} \text{ x } 100$$

The meiotic index and pollen viability data treatment was in a completely randomized design. The normality of the data was evaluated using the Shapiro-Wilk test and the homogeneity using the Levene test and, when necessary, data were transformed into arc-sen $\sqrt{\frac{x}{100}}$. Then, the data were submitted to the analysis of variance using the F test to verify whether there were differences between the individuals of each population. Significant means were submitted to the Tukey Test at 5% probability. The means of the populations were compared using the t-test and all statistical analyses were performed in the R software (RStudio Team, 2018).

2.5 *In vitro* germination of pollen grains

Due to the absence of information regarding the *in vitro* germination of pollen of *V. divergens* or species of the same genus, the methodology used for "cajazeira" (*Spondias mombin* L.) as described by Zortéa et al. (2019) was used as a basis, with modifications. A total of 12 culture media were tested with variations in sucrose and boric acid concentrations and the presence or absence of agar. Each culture medium was considered as a treatment: T1 - sucrose 5%, boric acid 50 mg mL^{-1} and agar at 0%; T2 - sucrose 5%, boric acid 50 mg mL^{-1} and agar at 0.25%; T3 - sucrose 10%, boric acid 50 mg mL^{-1} and agar at 0%; T4 - sucrose 10%, boric acid 50 mg mL^{-1} and agar at 0.25%; T5 - sucrose 20%, boric acid 50 mg mL^{-1} and agar at 0%; T6 - sucrose 20%, boric acid 50 mg mL^{-1} and agar at 0.25%; T7 - sucrose 5%, boric acid 0 mg mL^{-1} and agar at 0%; T8 - sucrose 5%, boric acid 0 mg mL^{-1} and agar at 0.25%; T9 - sucrose 10%, boric acid 0 mg mL^{-1} and agar at 0%; T10 - sucrose 10%, boric acid 0 mg mL^{-1} and agar at 0.25%; T11 - sucrose 20%, boric acid 0 mg mL^{-1} and agar at 0%; T12 - sucrose 20%, boric acid 0 mg mL^{-1} and agar at 0.25%.

Culture media were distributed on acrylic plates of 6 cm in diameter, and each plate received 10 mL of medium. The anthers were separated from the buds and lightly macerated to release the pollen grains, which were then distributed on the plates. The experiment was organized in a completely randomized design with 12 treatments and 3 repetitions, and was kept in a BOD chamber at a temperature of 25 °C (± 2 °C), in the dark, for 24 hours. After 24 hours, 500 pollen grains (germinated and non-germinated) were counted in each repetition. Pollen grains in which the length of the pollen tube reached or exceeded its diameter were considered germinated (Reis et al., 2011). The percentage of germinated pollens was obtained using the following formula:

$$\text{Germination }\% = \frac{\text{Number of germinated pollen grains}}{\text{Total number of pollen grains counted}} \times 100$$

The normality of the data was evaluated via the Shapiro-Wilk test and the homogeneity via the Levene test. Then, the data were submitted to analysis of variance, which used the F test to verify whether there were differences between the treatments. Significant means were submitted to the Scott-Knott test at 5% significance. Statistical analyses were performed in the R software (RStudio Team, 2018).

2.6 Pollen cytochemistry

The pollen grains of *V. divergens* were also subjected to staining with Lugol (Baker & Baker, 1979) and Sudan IV (Dafni, 1992) to verify the presence of starch and lipids, respectively. Lugol is specific for staining of pollen grains containing starch as a reserve material, and stains them a dark brown or black color, and this technique is based on the chemical reaction between iodine and starch (Pagliarini & Pozzobon, 2005). Sudan IV reacts with lipid molecules and the positive results are those that are colored red or light brown (Dafni, 1992).

In this analysis, a mix of flower buds from the five individuals collected from each population were used. A total of eight slides per population were prepared according to the methodology described by Guerra and Souza (2002), and 2,000 pollen grains per population were counted for each dye. The observation and counting of pollen grains were carried out under a binocular optical microscope (Photonics Bio 2 LED, BEL) at a magnification of 400x. The data obtained were transformed into percentage of positive starch and positive lipid pollens, and the number of stained pollens was divided by the total number of counted pollens and then multiplied by 100.

3. Results

3.1 Morphological characterization of pollen

The pollen grains of *V. divergens* are classified as 3-colporate, with long colpi and lalongate pores, isopolar, subprolate (subspheroidal) and have a small polar area due to the colpi being long. They are considered large, since the mean of the largest axis (polar axis) is between 50-100 µm (Table 1; Figure 2).

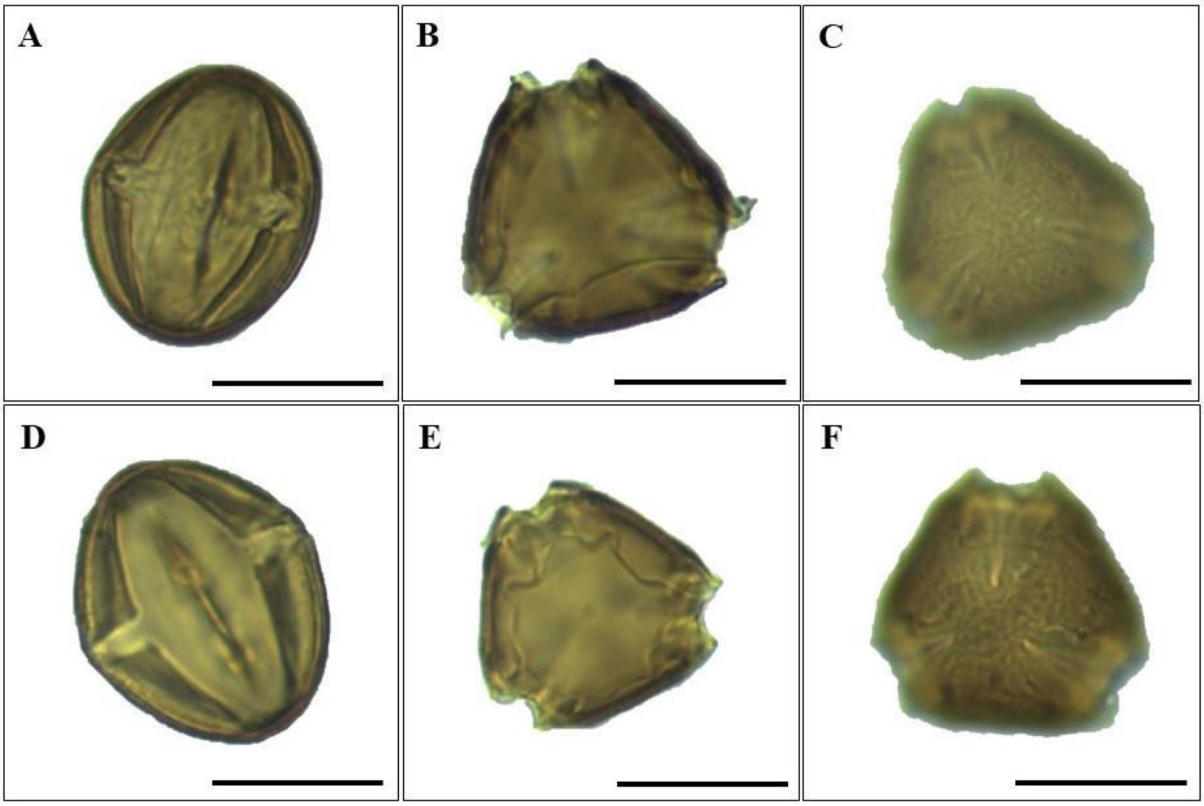
Table 1. Measurements of *Vochysia divergens* pollen collected in the Amazon and Pantanal biomes and submitted to the acetolysis method, with the t-test result for the means of the two populations.

Measure	Min. - Max. (µm)	x ± sx (µm)	CI 95% (µm)	C.V. (%)
		Amazon		
Equatorial diameter (EV)	65.21 - 79.61	71.49^{ns} ± 3.72	69.84 - 72.98	5.20
Polar axis (EV)	82.53 - 102.63	92.73* ± 5.28	90.38 - 94.84	5.69
Equatorial diameter (PV)	65.33 - 81.58	73.61^{ns} ± 4.16	71.77 - 75.28	5.65
Nexine	0.93 - 2.01	1.38^{ns} ± 0.27	1.26 - 1.49	19.27
Sexine	1.18 - 2.10	1.60* ± 0.27	1.47 - 1.71	17.17
Exine	2.29 - 3.91	2.98^{ns} ± 0.45	2.78 - 3.16	15.20
P/E	1.15 - 1.50	1.30^{ns} ± 0.09	1.26 - 1.34	6.84
P.A.I.	0.19 - 0.29	0.25^{ns} ± 0.03	0.23 - 0.26	11.68
		Pantanal		
Equatorial diameter (EV)	64.37 - 78.58	70.37^{ns} ± 3.64	68.75 - 71.82	5.17
Polar axis (EV)	75.58 - 97.58	88.60* ± 5.61	86.11 - 90.84	6.33
Equatorial diameter (PV)	66.39 - 80.99	73.51^{ns} ± 4.03	71.72 - 75.12	5.49
Nexine	1.05 - 1.90	1.38^{ns} ± 0.23	1.27 - 1.47	16.83
Sexine	1.49 - 2.17	1.75* ± 0.19	1.66 - 1.82	10.67
Exine	2.61 - 3.67	3.12^{ns} ±0.32	2.98 - 3.25	10.41
P/E	1.10 - 1.45	1.26^{ns} ± 0.10	1.22 - 1.30	8.23
P.A.I.	0.20 - 0.30	0.25^{ns} ± 0.26	0.24 - 0.26	10.30

x = mean; sx = tandard deviation from the mean; CI = confidence interval; C.V. (%) = coefficient of variation; EV = equatorial view; PV = polar view; P/E = ratio of polar axis to equatorial diameter; P.A.I. = polar area index. * significant at 5%; ^{ns} not significant by the t-test. Source: Authors.

The exine of the pollen grains of *V. divergens* has an average thickness of 3.05 µm, surface type DL (darkness and light), which indicates the presence of indentations and a pattern of ornamentation that is perforated in the polar region (Figure 2). The means of the polar axis in equatorial view and the thickness of the sexine were statistically different between the two populations analyzed (Table 1). There were no differences between the two populations analyzed regarding the number of apertures, shape, size and PAI of the pollen grains of *V. divergens*.

Figure 2. Photomicrographs of the pollen grains of *Vochysia divergens*. A-C = pollen of the Amazon population. D-F = pollen of the Pantanal population. A and D) equatorial view with detail of the colpi. B and E) polar view with detail of the colpi apertures. C and F) surface detail (ornamentation). Bar = 50μm.



Source: Authors.

3.2 Meiotic index

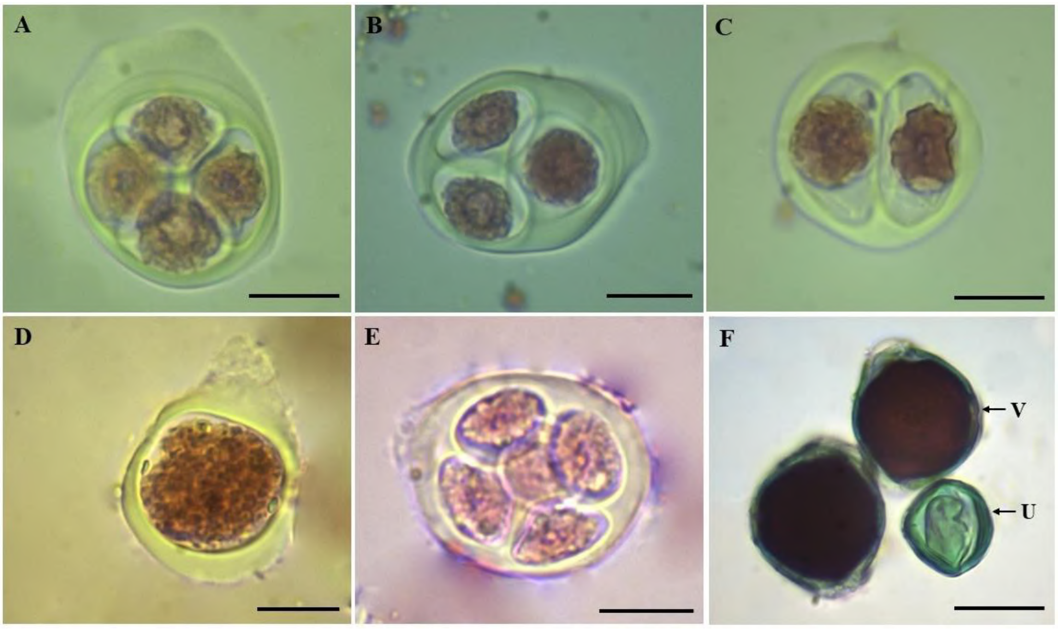
The meiotic index (MI) varied among individuals within each population, and were statistically different; however, all presented a high MI, which was always greater than 95% (Table 2). Via the analysis of the post-meiotic products of *V. divergens*, the presence of triads, dyads, monads and polyads was observed, with the triads being the most frequent (Table 2 and Figure 3).

Table 2. Post-meiotic products and meiotic index (MI) observed in *Vochysia divergens* individuals collected in the Amazon and Pantanal biomes.

Individuals	Tetrads	Monads	Dyads	Triads	Polyads	MI (%)
			Amazon			
1	1,909	8	13	69	0	95.45 b
2	1,926	9	7	57	1	96.30 b
3	1,946	13	13	27	1	97.30 ab
4	1,941	41	0	14	4	97.05 ab
5	1,974	1	0	24	1	98.70 a
Total	9,696	72	33	191	7	10,000
Mean	1,939.2	14.4	6.6	38.2	1.4	96.96
			Pantanal			
1	1,972	11	1	15	1	98.60 a
2	1,960	6	3	27	4	98.00 ab
3	1,938	2	11	30	19	96.90 b
4	1,979	2	1	16	2	98.95 a
5	1,975	3	0	16	6	98.75 a
Total	9,824	24	16	104	32	10,000
Mean	1,964.8	4.8	3.2	20.8	6.4	98.24

Means followed by the same lowercase letter in the column do not differ from each other by the Tukey test ($p < 0.05$). Source: Authors.

Figure 3. Post-meiotic products observed in *Vochysia divergens*. (A) Normal tetrad. (B) Triad. (C) Dyad. (D) Monad. (E) Polyad. F) Pollen grains stained with Alexander's reactive observed at a magnification of 400x. V = viable pollen grain; U = unviable pollen grain. Bar = 100 µm.



Source: Authors.

3.3 Pollen viability via colorimetric test

Alexander's reactive dye was efficient for distinguishing between viable and unviable pollen of *V. divergens* (Figure 3F) and enabled reliable analysis. The pollen viability was statistically different among *V. divergens* individuals from the Amazon population, but did not vary among individuals from the Pantanal population and, regardless of the statistical difference between individuals, both populations presented mean viability greater than 95% (Table 3). When the means of pollen viability between the Amazon and Pantanal populations were compared using the T-test, it was found that there is no statistical difference between the two populations ($p > 0.05$; $p = 0.3231$).

Table 3. Mean pollen viability (PV) for the five *Vochysia divergens* specimens evaluated within each population.

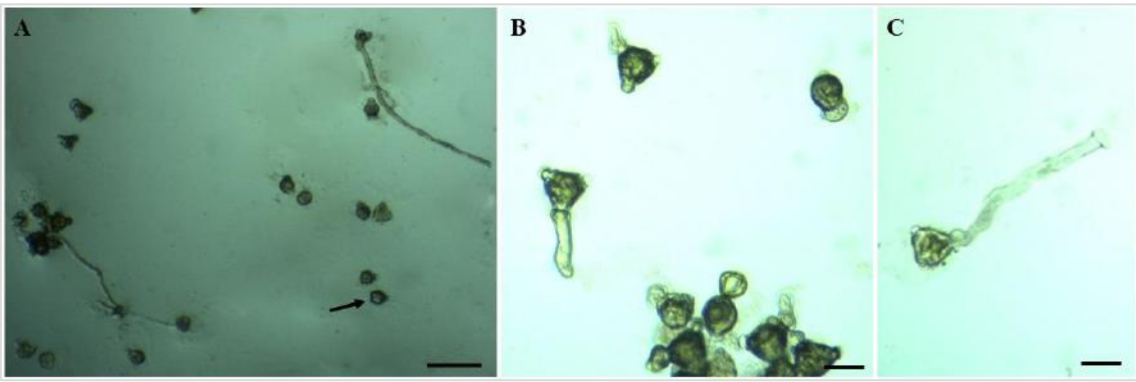
Individuals	PV (%) Amazon	PV (%) Pantanal
1	98.15 a	97.20
2	97.80 a	97.75
3	97.60 a	97.95
4	95.40 b	97.95
5	98.10 a	98.05
Mean	97.41	97.78

Means followed by the same lowercase letters in the column, do not differ from each other by the Tukey test at 5% probability. Source: Authors.

3.4 *In vitro* germination of pollen grains

The twelve culture media that were tested allowed us to germinate pollen grains of *V. divergens* (Figure 4); however, germination occurred at a low proportion (Table 4).

Figure 4. *Vochysia divergens* pollen germinated *in vitro*. A) Germinated and non-germinated pollen grains (arrow). Bar = 200 µm. B and C) Different stages of development of the pollen tube. Bar = 60 µm.



Source: Authors.

There was a significant statistical difference between the twelve culture media tested, though T1 enabled the highest percentage of germination (Table 4). The presence of boric acid positively influenced the germination of the pollen grains of *V. divergens*. Agar negatively interfered with the germination of pollen grains of *V. divergens* when in conjunction with boric acid. This interference can be verified by contrasting treatments T1 with T2, T3 with T4 and T5 with T6, which differed statistically. The increased sucrose concentration did not influence the germination of pollen grains (Table 4).

Table 4. Mean percentage of *in vitro* germination of pollen grains of *Vochysia divergens* in different culture media.

Treatment	Germination %
T1 (S 5%; BA 50 mg)	27.73 a
T2 (S 5%; BA 50 mg; agar 0.25%)	18.27 b
T3 (S 10%; BA 50 mg)	22.87 a
T4 (S 10%; BA 50 mg; agar 0.25%)	16.61 b
T5 (S 20%; BA 50 mg)	22.88 a
T6 (S 20%; BA 50 mg; agar 0.25%)	18.27 b
T7 (S 5%; BA 0 mg; agar 0.25%)	9.93 c
T8 (S 5%; BA 0 mg)	7.28 c
T9 (S 10%; BA 0 mg; agar 0.25%)	9.60 c
T10 (S 10%; BA 0 mg)	6.13 c
T11 (S 20%; BA 0 mg; Agar 0.25%)	8.2 c
T12 (S 20%; BA 0 mg)	7.13 c
Mean	14.58
C. V. (%)	25.38

S= Sucrose; BA= Boric acid; C.V. (%) = coefficient of variation. Means followed by the same lowercase letters in the column do not differ from each other by the Scott-Knott test ($p < 0.05$). Source: Authors.

3.5 Cytochemistry

The pollen grains of *V. divergens* present starch and lipids as reserve substances, according to the reactions evidenced by the Lugol and Sudan IV dyes, respectively. The Amazonian population presented 96.50% of the pollen grains as starch positive and 96.90% as lipid positive grains. In the Pantanal population, the values were 95.50% starch positive pollen grains and 94.35% lipid positive grains.

4. Discussion

Pollen grains collected in the Amazon and the Pantanal differed in relation to two morphological characteristics analyzed; however, these differences did not affect the classification. Differences in pollen grain sizes and shapes are common within the species (Salgado-Labouriau, 1973; Martins et al., 2010), and these differences may be derived from genetic variation among the individuals sampled (Martins et al., 2003). When dealing with individuals from populations located in different biomes, as in this study, this variation may be more significant, and may be related to adaptation to the environment.

The morphological characteristics of the pollen grains of *V. divergens* found in this study are similar to the morphology described for the genus *Vochysia* by Salgado-Labouriau (1973), Vianna (1980) and Vianna et al. (2002). According to these authors, the pollen grains of this genus are characterized by being 3-colporate, isopolar, subspheroidal, have a small to large size and perforated ornamentation, and a rough, hollow or reticulated exine. The characteristics that are generally constant in the species of this genus are the number and type of apertures, and the most contrasting are ornamentation, size and shape (which varies in four subclasses of the subspheroidal type – suboblate, oblate spheroidal, prolate-spheroidal and subprolate) (Vianna et al., 2002; Vianna, 1980). The differences observed in the pollen grains of the genus *Vochysia* reflect the variability of their morphology and may assist in the identification of some species (Vianna et al., 2002), and this work should contribute to enrich this knowledge.

With respect to other genera of the family Vochysiaceae, the pollens of *V. divergens* present important differences, such as size, aperture characteristics and ornamentation of the exine (RCPOL, 2020). Among the several existing examples, there are two species: *Qualea grandiflora* Mart and *Qualea parviflora* Mart. Pollen grains of *Qualea grandiflora* have perforated ornamentation and subprolate format, such as *V. divergens*, but have medium size, short colpi and lolongate pores (RCPOL, 2020). The pollen grains of *Qualea parviflora* have striated ornamentation, spheroidal shape, circular pore and medium size (RCPOL, 2020), all contrasting characteristics to *V. divergens*.

Pollen characteristics, such as exine ornamentation and apertures, are essential for the identification and separation of species (Blackmore, 2007; Martins et al., 2010). The correct identification of pollens is essential in order to subsidize taxonomic studies and this is also indispensable when using palynology to understand how climate change and humans have contributed to changing landscapes and vegetation over time, as in the study by Mottl et al. (2021), which evaluated 1,181 fossil pollen sequences to understand the vegetation changes that occurred around the world in the last 18,000 years. In this sense, it is hoped that the results of this research may contribute to future paleoclimatic and paleovegetational studies.

The meiotic index was high in all *V. divergens* samples. Despite statistical differences between individuals and populations, the MI was always greater than 95%. According to Love (1951), plants with an MI greater than 90% have high meiotic stability. Among the abnormalities found in post-meiotic products, triads were more common. These abnormalities derive from errors that occur during meiosis, such as irregularity in the orientation of the spindle fibers (Souza et al., 2014) and incorrect alignment of chromosomes in metaphases I and II (Shamina, 2005).

The pollen viability of *V. divergens*, estimated via the colorimetric test, was considered high and reflects the results of the meiotic index. In this study, the mean viability was greater than 95% and, according to Souza et al. (2002), estimates of pollen viability that reach values over 70% can be considered high and guarantee the reproductive success of the species.

The staining method used in this study made it possible to distinguish unviable pollen grains due to the absence of the nucleus (Alexander, 1980); however, it is important to remember that the pollen viability is influenced by several other factors that will determine whether the pollen will reach the stigma of the viable flower and if, upon arrival, it will have the ability to germinate, i.e., will be fertile. These factors include local environmental characteristics (humidity and temperature), level of dehydration and the reserve content of the pollen grains (Pacini & Franchi, 1993; Dafni & Firmage, 2000). This means that, although the colorimetric test indicates viability, other factors could prevent this pollen grain from germinating.

Pollen viability was statistically different among individuals in the Amazon population. As already mentioned, several factors can influence pollen viability, and it is thus expected that the pollen viability may be different between individuals of the same species or in samples of the same individual (Santos Neto et al., 2006). In addition to what has already been addressed, Shivana e Rangaswamy (1992) further suggest that the responses of pollen grains may be different depending on the period of the flowering season (early, medium and late), the different times of the day, the genotypic differences and the vigor and physiological state of the plants at the time of collection.

When the estimates of pollen viability between the two populations analyzed were compared, it was found that they do not differ statistically, despite the individuals coming from different biomes. Thus, it can be inferred that the differences in estimations for the viability of *V. divergens* are related to genotypic variations or the physiological state of individuals (Shivana & Rangaswamy, 1992) and not to the environment, and indicate the reproductive stability of the species, regardless of location. Data like these help explain the dispersal capacity of this species and its establishment in the tropical environment. The reproductive stability expressed by the result of the MI and the percentage of viable pollen is an important factor in species such as *V. divergens*, which can be used in reforestation aimed at forest restoration or recovery of degraded areas, since viable pollens lead to the production of equally viable seeds to be used in the production of seedlings or even for the establishment of new specimens in the recovering environment. The high pollen viability also guarantees the maintenance of genetic variability in cross-fertilization species, since each pollen grain carries with it genetic material resulting from recombinations, thus increasing the likelihood that these plants transmit highly diverse genotypes to the next generation.

All the culture media tested permitted the germination of the pollen grains of *V. divergens*; however, the percentage was very low compared to the pollen viability estimated via the colorimetric test. Decreased germination viability does not necessarily indicate that the pollen grain is dead or unviable, but that germination conditions may not be ideal (Mosquera et al., 2021). According to Dafni & Firmage (2000), even viable pollen grains may not germinate (*in vitro* or *in vivo*) if conditions are not adequate. In this sense, the low percentage of germination is probably related to the culture media and not to the unviability of the pollens, since the meiotic index was high. Therefore, it is necessary to test other means in order to establish the appropriate conditions for the *in vitro* germination of the pollen grains of *V. divergens*.

Boric acid positively influenced the germination of the *V. divergens* pollen and increasing its concentration could possibly improve germination. Franzon e Raseira (2006) state that boron stimulates the growth of the pollen tube and decreases the likelihood that it will rupture.

The increase in sucrose concentration did not influence the increase in the germination of the pollen grains, and it can be used in low concentrations. This component has the function of providing energy for the development of the pollen tube and promoting the osmotic balance between the pollen grain and the culture medium (Stanley & Linskens, 1974; Zambon et al., 2014). The need for low sucrose concentration in the culture medium may indicate that the pollen grain of *V. divergens* already has, in its reserve content, high concentrations of this compound.

The addition of agar in the culture medium negatively influenced the germination of the pollen grains of *V. divergens*. According to Ramos et al. (2008), the response of pollen grain germination to agar concentration varies among species. One explanation for the interference of agar in the germination of *V. divergens* pollen is its interaction with boron. The presence of boron, as seen, is important for the germination of pollen grains of this species but, according to Daher et al. (2008), agar molecules can sequester boron present in the culture medium, thus preventing pollens from using it for their development. As such, for *V. divergens*, it is recommended that lower concentrations be used or that the agar be completely removed from the culture medium to allow an increase in the percentage of germination.

Among the types of reserve substances commonly found in pollen grains, *V. divergens* presented starch and lipids. These substances are indispensable for providing the energy and materials necessary for the formation of the pollen tube during germination (Baker & Baker, 1979). In addition, these substances are important in the ecological, evolutionary and reproductive aspects of the species, and help in the maintenance and establishment of new generations through the maintenance of pollen viability and the attraction of specific pollinators.

Starch can be converted into sucrose before pollination so that this molecule can act in the protection of the membranes of pollen grains and prevent their desiccation and keep it viable for longer, which is a characteristic that is essential for species of cross-fertilization in which pollen must travel long distances for fertilization (Franchi et al., 1996). This condition may explain the high pollen viability according to the colorimetric test and the need for low sucrose concentrations in the culture medium for pollen germination of the species, as well as the wide distribution of *V. divergens* in tropical environments that generally present adverse conditions for the maintenance of the viability of pollen grains.

Lipids can also influence the viability of pollen and the fertilization process, because, according to Pacini & Hesse (2005), lipids promote better adhesion of grains to anthers and stigma, and have a protective function against dehydration and UV radiation. The presence of lipids in pollen grains is an indication of pollination by bees, since this substance can serve as an edible reward to pollinators seeking nectar. According to Baker & Baker (1979), bees may prefer lipid-containing pollens for feeding because lipids are more easily metabolized by them than starch. Starch, unlike lipids, is the substance that is preferentially found in anemophilous plants and pollinated by Lepidoptera or birds (Baker & Baker, 1979). There are no specific studies on the pollination of *V. divergens*; however, Lorenzi (2008) states that its flowers are good for beekeeping and also visited by hummingbirds. Some work that has been carried out with other species of the genus has indicated Anthophoridae and Eglossini bees and some moths as main pollinators, and hummingbirds as opportunists in some species and possible pollinators in others (Oliveira & Gibbs, 1994; Santos et al., 1997). Due to the similarity between the flowers of the species of the genus *Vochysia* (Oliveira & Gibbs, 1994), it is possible that they share the same types of pollinators. The sharing of pollinators among *V. divergens* and other species is an extra attribute for a species deployed in forest restorations, since it can generate an abundance of pollinators that will positively influence the entire ecosystem.

5. Conclusion

The populations showed no significant differences regarding pollen morphology, pollen viability, meiotic index and pollen cytochemistry. Only two measurements of the pollen grains differed between populations, but did not affect their final classification.

The pollen grains collected in the Amazon and the Pantanal biomes presented the same characteristics and are classified as large, 3-colporate, with long colpi and lalongate pores, isopolar, subprolate and small polar area. The pollen surface has indentations and the exine has a pattern of ornamentation that is perforated in the polar region. These results may support future taxonomic studies, as well as paleoclimatic and paleovegetational studies that seek to understand the influence of climate change and human activities on landscape change.

Although the MI was statistically different between the populations, it was high, as was the pollen viability according to the colorimetric test, thus indicating meiotic stability and regularity in the species. The pollen grains of *V. divergens* present starch and lipids as reserve material. These substances are related to maintaining the viability of the pollen grain and assist in the pollination process. The association of this set of factors in *V. divergens* contributes to its maintenance, genetic variability and the establishment of new generations in the habitat.

It is recommended that the culture media for *in vitro* germination tests of pollen grains of *V. divergens* be liquid or contain less than 0.25% agarose, as well as concentrations greater than 50 mg mL^{-1} of boric acid and at least 5% sucrose. More studies can be carried out to define better conditions for pollen germination, based on the results obtained in this study.

Researches on reproductive biology and pollination of the species may be carried out in order to complement the results obtained in this study.

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