ARTIGO ORIGINAL

Pollen viability of *Peltodon longipes* using distinct staining methods

Andrielle Wouters Kuhn[1]*; Marília Tedesco[2]; Jéssica Mena Barreto de Freitas[1]; H. Dail Laughinghouse IV[3]; Viviane Dal-Souto Frescura[1]; Thais Scotti do Canto-Dorow[4]; Antonio Carlos Ferreira da Silva[1]; Solange B. Tedesco[1]

*[1]Universidade Federal de Santa Maria. Santa Maria, RS. [2]Universidade Federal do Rio Grande do Sul. Porto Alegre, RS. [3]Institute for Bioscience & Biotechnology Research. Rockville, Estados Unidos. [4]Centro Universitário Franciscano. Santa Maria, RS. *Autor para correspondência: andri-kuhn@hotmail.com*

ABSTRACT: The pollen viability reveals the masculine breeding potential in plant species, and can be useful for taxonomic, ecological, genetic and palynological studies. In order to provide information on the medicinal species *Peltodon longipes* Kunth ex Benth., this study aimed at estimating the pollen viability in accessions of this species collected in the state of Rio Grande do Sul, by using three staining methods, as well as determining the most efficient stain. The inflorescences of 15 accessions were collected and fixed in ethanol/acetic acid (3:1 v/v) for 24 h and, subsequently, placed in 70% (v/v) ethanol under refrigeration until preparation of slides by the squashing technique. Three staining methods were used: acetic orcein 2%, acetic carmine 2%, and Alexander's stain. Pollen grains stained with acetic orcein 2% with intense coloring were considered viable, while those of weak coloring were considered non-viable. The grains treated with acetic carmine 2% were considered viable when they were stained red, whereas the transparent ones and those showing no staining were considered non-viable. The pollen grains stained with Alexander's stain were viable when the pollen was purple and non-viable when it was light blue-green. A completely randomized design was used and the data collected was submitted to analysis of variance (ANOVA) and compared by the Scott-Knott test at 5% probability of error. Of the 15 accessions of *P. longipes*, 13 showed high pollen viability, with values above 75% and the methods using acetic carmine 2% and Alexander reactive, were the most efficient technique for this species.
Keywords: *Peltodon longipes*, pollen, acetic orcein, acetic carmine, Alexander's stain.

RESUMO: Viabilidade polínica de *Peltodon longipes* usando distintos métodos colorímetros. A viabilidade polínica evidencia o potencial de reprodução masculina das espécies vegetais, podendo ser útil em estudos taxonômicos, ecológicos, genéticos e palinológicos. Visando fornecer informações a respeito da espécie medicinal *Peltodon longipes* Kunth ex Benth., objetivou-se estimar a viabilidade polínica de acessos da espécie do estado do Rio Grande do Sul, através de três métodos colorímetros, além de determinar o corante mais eficiente. As inflorescências de 15 acessos foram coletadas em cinco municípios sul-rio-grandenses, fixadas em etanol:ácido acético (3:1) por 24 h e, posteriormente, permaneceram em etanol 70% sob refrigeração até a preparação das lâminas pela técnica de esmagamento. Foram utilizados três métodos colorímetros: a orceína acética 2%, o carmim acético 2% e o reativo de Alexander. Para a orceína acética, foram considerados viáveis os grãos de pólen fortemente corados e, inviáveis, os de coloração fraca. Para o carmim acético, foram considerados viáveis os polens corados em vermelho, enquanto os transparentes ou não corados, foram ditos inviáveis. E, para o reativo de Alexander, os grãos de pólen de coloração púrpura foram considerados viáveis e, os grãos de pólen de coloração verde-claro-azulado, inviáveis. Foi utilizado um delineamento inteiramente casualizado e os dados foram submetidos à análise da variância (ANOVA) e comparados pelo teste Scott-Knott ao nível de 5% de probabilidade de erro. Dos 15 acessos de *P. longipes*, 13 apresentaram alta viabilidade polínica, com valores acima de 75% e os métodos usando carmim acético 2% e reativo de Alexander foram os mais eficientes para a técnica na espécie.
Palavras-chave: *Peltodon longipes*, pólen, orceína acética, carmim acético, Reativo de Alexander.

Recebido para publicação em 21/08/2016
Aceito para publicação em 12/04/2021
Data de publicação em 24/08/2021
ISSN 1983-084X

INTRODUCTION

For centuries, some plants have been recognised and used to treat various illnesses due to their medicinal properties Feijó et al. (2012). With the development of science, the therapeutic value of several medicinal plants has been evaluated and confirmed, increasing the use of these plants by health professionals Arnous et al. (2005). In Brazil, approximately half of plant species have beneficial properties Martins et al. (1994). Thus, the country has a responsibility to preserve and sustainably study these species, which suffer indiscriminate exploration that sometimes compromises entire populations Facanali (2008). Although these medicinal species are important, studies on their reproductive systems are extremely rare Moraes et al. (2002) e Almeida et al. (2004). Information on the species distribution and demographic, physiological, reproductive, and genetic diversity are important for both *in situ* and *ex situ* preservation. With this information, sustainable management programs and managed cultivating of these species are possible, reducing the impact of predatory exploration Facanali (2008). Knowledge on the reproductive systems of plant species is important to conserve the germplasm, management in cultivation and for breeding, as it defines strategies based on intra- and inter- population crosses Danner et al. (2011).

Estimating pollen viability is important for gene flow analyses in plants, as it highlights the male reproduction potential of the species and can be useful in taxonomic, ecological, genetic and palynological studies Frescura et al. (2012). Thus, it is possible to investigate genetic variability among populations or accessions of the same species, where pollen viability varies. The measurement of male fertility can be determined directly, using techniques to induce germination *in vitro* Pio et al. (2007) or *in vivo* Ferreira et al. (2007), or estimated indirectly, based on cytological parameters using diverse staining methods Munhoz et al. (2008), i.e. acetic orcein, acetic carmine, or Alexander reactive.

The Alexander reactive method differentiates between viable and unviable pollen by staining the cellulose of the pollen grain wall and protoplasm differently Alexander (1980). Acetic carmine stains the genetic material of the pollen cytoplasm red, which is why the viable grains are red, while the unviable are clear or non-stained Pagliarini & Pozzobon (2004). According to Paula (2009), acetic orcein stains both types of pollen grains red, with the difference in the intensity of the colour.

Peltodon longipes Kunth ex Benth. (Family Lamiaceae) is a plant species used in folk medicine to regulate menstruation, considered an emmenagogue, and is also used as a natural stimulant Mors et al. (2000). Furthermore, its roots are considered antiseptic and anti-inflammatory Fronza et al. (2011). With the aim to provide information on this species, we estimated the pollen viability of different *P. longipes* accessions from the state of Rio Grande do Sul (Brazil), using three staining methods, as well as determining the most effective method for this species.

MATERIAL AND METHODS

Plant material consisted of 15 *P. longipes* Kunth ex Benth. (Lamiaceae), accessions collected from five different municipalities: Santa Maria, Tupanciretã, São Pedro do Sul, São Vicente do Sul and Rosário do Sul, in the state of Rio Grande do Sul (RS), Brazil (Table 1). The municipalities are located at locations, 29° 41′ 2″ S, 53° 48′ 25″ W, 29° 4′ 51″ S, 53° 50′ 9″ W, 29° 37′ 15″ S, 54° 10′ 44″ W, 29° 41′ 31″ S, 54° 40′ 44″ W and 30° 15′ 28″ S, 54° 54′ 50″ W, respectively. These municipalities also differ in altitude: 151, 400, 173, 129, and 132 m, respectively. The experiment was undertaken in the Plant Cytogenetics and Genotoxicity Lab (LABCITOGEN), Centre of Natural and Exact Sciences, at the Federal University of Santa Maria (UFSM), Santa Maria, RS, Brazil.

The accessions of *P. longipes* were sampled during December 2013, according to Mori et al. (1989), with the aid Prof. Thais Scotti do Canto-Dorow for the identification of plants, and voucher material was deposited in the Herbarium SMDB of the Department of Biology, UFSM.

TABLE 1. Accessions of *Peltodon longipes* deposited in SMDB-UFSM as voucher material.

Acession	Herbarium SMDB Number	Municipality
1	15406	Santa Maria
2	15416	Santa Maria
3	15417	Santa Maria
4	15418	Santa Maria
5	15412	Tupanciretã
6	15407	São Pedro do Sul
7	15408	São Pedro do Sul
8	15413	São Pedro do Sul
9	15409	São Vicente do Sul
10	15410	São Vicente do Sul
11	15411	São Vicente do Sul
12	15414	São Vicente do Sul
13	15415	São Vicente do Sul
14	15419	Rosário do Sul
15	15420	Rosário do Sul

Inflorescences of the 15 accessions were immediately fixed after sampling in ethanol:acetic acid (3:1) for 24 h, and posteriorly, transferred to ethanol 70% and stored under refrigeration. The slides were prepared by the anther squashing technique and stained Guerra & Souza (2002). Three distinct staining methods were used, acetic orcein 2%, acetic carmine, and Alexander reactive, which allowed for determining pollen viability and the most efficient technique for *P. longipes*. For orcein, we considered viable those pollen grains strongly stained, and those unviable with lighter staining. For acetic carmine, viable pollen grains were those stained red, while unviable were clear or not stained, and for the Alexander reactive, viable pollen grains were purple, while unviable were those green to light-blue stained.

We used two replicates in a totally randomised experimental design. Two slides for each staining methods were prepared, counting 400 pollen grains per slide, totalizing 800 grains for each staining method, and 2400 per accession. The data were submitted to an analysis of variance (ANOVA) and compared by the Scott-Knott test at 5% probability of error, with the help of Assistat® statistical software, version 7.7 beta.

RESULTS AND DISCUSSION

We analysed the pollen viability in 15 accessions of *P. longipes.* The mean of viable pollen grains and the pollen viability for all three staining methods are found in Table 2. Pollen viability in the accessions of *P. longipes* varied from 57.5% to 100%, and a pairwise comparison of the means of pollen viability showed significant differences that are possibly consequences of the genetic variability between the distinct accessions. Neto et al. (2006) found similar results with differences in pollen viability of *Solanum paniculatum* L. populations, and credited these data as putative genetic variability.

Only accession 9, using acetic orcein 2%, had a lower viability (99.37%), statistically different from the rest. The same is observed for accessions 2 (75%), 11 (67.25%), 13 (75%), and 14 (47.5%) using acetic carmine 2% and only accession 14

TABLE 2. Means of viable pollen grains and pollen viability of the accesions of *Peltodon longipes* for the three staining methods used (SM = Santa Maria; TP = Tupanciretã; SPS = São Pedro do Sul; SVS = São Vicente do Sul; RDS = Rosário do Sul; OA = Acetic orcein 2%; CA = Acetic carmine 2%; RA = Alexander reactive).

Accession	Municipality	Mean of viable pollen grains			Mean of pollen viability (%)		
		OA	CA	RA	OA	CA	RA
1	SM	400 ^{aA}	347.5 ^{aB}	339.5 ^{aB}	100 ^{aA}	86.87 ^{aB}	84.87 ^{aB}
2	SM	400 ^{aA}	300 ^{bB}	342 ^{aB}	100 ^{aA}	75 ^{bB}	85.5 ^{aB}
3	SM	400 ^{aA}	374 ^{aB}	349 ^{aB}	100 ^{aA}	93.5 ^{aB}	87.25 ^{aB}
4	SM	400 ^{aA}	394.5 ^{aB}	394.5 ^{aB}	100 ^{aA}	98.62 ^{aB}	98.62 ^{aB}
5	TP	400 ^{aA}	393.5 ^{aB}	393 ^{aB}	100 ^{aA}	98.37 ^{aB}	98.25 ^{aB}
6	SPS	400 ^{aA}	389 ^{aB}	382.5 ^{aB}	100 ^{aA}	97.25 ^{aB}	95.62 ^{aB}
7	SPS	400 ^{aA}	361 ^{aB}	382 ^{aB}	100 ^{aA}	90.25 ^{aB}	95.5 ^{aB}
8	SPS	400 ^{aA}	348.5 ^{aB}	331 ^{aB}	100 ^{aA}	87.12 ^{aB}	82.75 ^{aB}
9	SVS	397.5 ^{bA}	346.5 ^{aB}	351.5 ^{aB}	99.37 ^{bA}	86.62 ^{aB}	87.87 ^{aB}
10	SVS	399.5 ^{aA}	396.5 ^{aB}	340 ^{aB}	99.87 ^{aA}	99.12 ^{aB}	85 ^{aB}
11	SVS	399.5 ^{aA}	269 ^{bB}	340 ^{aB}	99.87 ^{aA}	67.25 ^{bB}	85 ^{aB}
12	SVS	400 ^{aA}	363 ^{aB}	398 ^{aB}	100 ^{aA}	90.75 ^{aB}	99.5 ^{aB}
13	SVS	399 ^{aA}	300 ^{bB}	342 ^{aB}	99.75 ^{aA}	75 ^{bB}	85.5 ^{aB}
14	RDS	400 ^{aA}	230 ^{bB}	252 ^{bB}	100 ^{aA}	57.5 ^{bB}	63 ^{bB}
15	RDS	400 ^{aA}	393.5 ^{aB}	398 ^{aB}	100 ^{aA}	98.37 ^{aB}	99.5 ^{aB}

Means followed by the same lowercase letter in the column and uppercase in the row do not differ by the Scott-Knott test at 5% probabilty.

(63%) for Alexander reactive (Table 2). However, only accession 11 (São Vicente do Sul) with acetic carmine 2% and 14 (Rosário do Sul) with acetic carmine 2% and Alexander reactive had viabilities lower than 70%. For the other accessions, viability was higher than 70%, which Souza et al. (2002) denote as high pollen viability.

Other authors have also found high pollen viability for different species used in folk medicine. Frescura et al. (2012) observed high pollen viability for the majority of *Polygala paniculata* L. populations, analysed using the Alexander reactive. Studying accessions of *Crotalaria juncea* L., Coelho et al. (2012) found pollen viability higher than 80% for seven of the 10 accessions studied using the Alexander reactive method and higher than 96% viability for all 10 accessions using acetic orcein. Analysing different genotypes of *Eragrostis plana* Nees. Piccinini et al. (2012) observed high pollen viability in all genotypes using acetic orcein and Alexander reactive.

In relation to the different staining methods, we found that acetic carmine 2% and Alexander reactive were more efficient at distinguishing viable and unviable pollen grains compared to acetic orcein 2% that significantly differed for all *P. longipes* accessions (Table 2). In 11 of the 15 accessions of *P. longipes* stained using acetic orcein 2%, all the pollen grains were strongly red in colour, which indicate that all are viable. The same percentage of pollen viability was not found in accessions of *P. longipes* when analysed using the other two staining methods, where these methodologies provide better visualization of viable and unviable pollen. These results show that there is a possibility of overestimation of pollen viability using acetic orcein 2%, and subsequently, the efficiency of using acetic carmine 2% and Alexander reactive. There is a better distinction between viable and unviable pollen grains using the two latter methods (Figure 1).

By staining the pollen grain wall and protoplasm differently, Alexander reactive allows for an easier visualisation between aborted pollen and non-aborted pollen by the absence or presence of a nucleus Alexander (1980). Several studies have proved the efficiency of this staining technique in estimating pollen viability. Using carmine-propionic acid 2%, acetic orcein 2%, and Alexander reactive on the medicinal species *Baccharis trimera* (Less.) DC., Auler et al. (2006) concluded that the best staining method for estimating pollen grain viability was Alexander reactive. Coelho et al. (2012) also had a better results using Alexander reactive when analysing *Crotalaria juncea*.

Although other researchers have shown the efficiency of Alexander reactive, we found that acetic carmine was also able to sufficiently distinguish between viable and unviable accessions of *P. longipes* (Figure 1). Using acetic carmine, we were able to estimate the viability at the same rate as Alexander reactive, since these two did not significantly differ (Table 2).

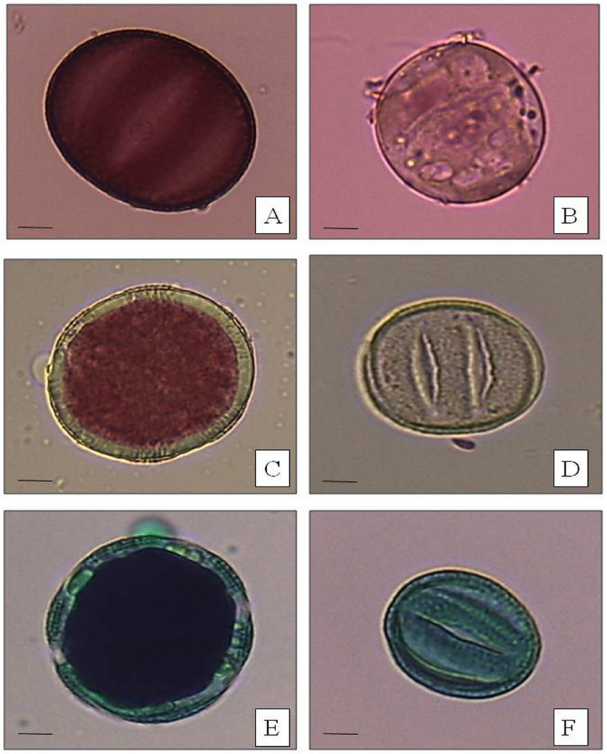

FIGURE 1. Pollen grains of *Peltodon longipes*, A) viable pollen grain using acetic orcein 2%; B) unviable pollen grain using acetic orcein 2%; C) viable pollen grain stained with acetic carmine 2%; D) unviable pollen grain with acetic carmine 2%; E) viable pollen grain using Alexander reactive; and F) unviable pollen grain using Alexander reactive. Scale is 10µm.

According to Almeida et al. (2006), acetic carmine was the best method for estimating pollen viability in *Ocimum officinalis* L., when compared to acetic orcein, lugol, and cotton-blue. Furthermore, a study on pollen viability in *Ricinus communis* L. cultivars found the use of acetic carmine 2% adequate, with good differentiation of viable and unviable grains Vargas (2006). Finally, acetic carmine was efficient in distinguishing pollen of *Capsicum* Martins (2010).

In 13 of the 15 accessions of *Peltodon longipes*, independent of the method used, we observed high pollen viability, with values above 75%. In addition, we found that acetic orcein 2% overestimated pollen viability. We found a better distinction between viable and unviable pollen grains using acetic carmine 2% or Alexander reactive, being the most efficient technique for this species.

ACKNOWLEDGEMENTS

We thank the Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS/CAPES) and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for financial support.

REFERENCES

ALEXANDER MPA (1980) Versatile stain for pollen fungi, yeast and bacterium. Stain Technol 5(1): 13-18. https://doi.org/10.3109/10520298009067890

ALMEIDA OS, SILVA AHB, SILVA AB, SILVA AB, AMARAL CLF (2004) Estudo da biologia floral e mecanismos reprodutivos do alfavacão (*Ocimum officinalis* L.) visando o melhoramento genético. Acta Sci Biol Sci 26(3): 343-348. https://doi.org/10.4025/actascibiolsci.v26i3.1598

ALMEIDA OS, RIBEIRO GS, ALVES JS, SILVA AB, REBOUÇAS TNH, AMARAL CLF (2006) Estimativa da viabilidade polínica em espécies do gênero *Ocimum* com diferentes corantes. In: 46º Congresso Brasileiro de Olericultura, Goiânia. Anais... Horticultura Brasileira. Brasília: ABH, 24: 242-242.

ARNOUS AH, SANTOS AS, BEINNER RPC (2005) Plantas medicinais de uso caseiro - conhecimento popular e interesse por cultivo comunitário. Revista Espaço para a Saúde 6(2): 1-6.

AULER NMF, BATTISTIN A, REIS MS (2006) Número de cromossomos, microsporogênese e viabilidade do pólen em populações de carqueja [*Baccharis trimera* (Less) DC.] do Rio Grande do Sul e Santa Catarina. Rev Bras Plantas Med 8: 55-63.

COELHO APD, MORAIS KP, LAUGHINGHOUSE IV HD, GIACOMINI SJ, TEDESCO SB (2012) Pollen grain viability in accessions of *Crotalaria juncea* l. (Fabaceae). Agrociencia 46: 481-487.

DANNER MA, CITADIN I, SASSO SAZ, SACHET MR, MALAGI G (2011) Modo de reprodução e viabilidade de pólen de três espécies de jabuticabeira. Rev Bras Frutic 33(2): 345-352. https://doi.org/10.1590/S0100-29452011005000077

FACANALI R (2008) Estudo da biologia reprodutiva, diversidade genética e química de populações de *Ocimum selloi* Benth. 145 f. Tese (Doutotrado em Agronomia - Horticultura) - Universidade Estadual Paulista "Júlio de Mesquita Filho" Faculdade de Ciências Agronômicas da UNESP - Câmpus de Botucatu, São Paulo.

FEREIRA CA, VON PINHO EVR, ALVIM PO, ANDRADE V, SILVA TTA, CARDOSO DL (2007) Conservação e determinação da viabilidade de grão de pólen de milho. Rev Bras Milho Sorgo 6: 159-173. https://doi.org/10.18512/1980-6477/rbms.v6n02p%25p

FEIJÓ AM, BUENO MEN, CEOLIN T, LONCK CL, SCHWARTS E, LANGE C, MEINCKE SMK, HECK RM, BARBIERI RL, HEIDEN G (2012) Plantas medicinais utilizadas por idosos com diagnóstico de Diabetes mellitus no tratamento dos sintomas da doença. Rev Bras Plantas Med 14(1): 50-56. https://doi.org/10.1590/S1516-05722012000100008

FRESCURA VD, LAUGHINGHOUSE IV HD, CANTO-DOROW TS, TEDESCO SB (2012) Pollen viability of *Polygala paniculata* L. (Polygalaceae) using different staining methods. Biocell 36(3): 143-145.

FRONZA M, MURILO R, SLUSARCZYK S, ADAMS M, HAMBURGER M, HEINZMANN B, LAUFER S, MERFORT I (2011) In vitro cytotoxic activity of abietane diterpenes from *Peltodon longipes* as well as *Salvia miltiorrhiza* and *Salvia sahendica*. Bioorg Med Chem 19: 4876–4881. https://doi.org/10.1016/j.bmc.2011.06.067

GUERRA M, SOUZA MJ (2002) Como observar cromossomos – Um guia de técnicas em citogenética vegetal, animal e humana. Ribeirão Preto: FUNPEC. 131p.

MARTINS ER, CASTRO DM, CASTELLANI DC, DIAS JE (1994) Plantas Medicinais, Universidade Federal de Viçosa, Viçosa, Minas Gerais. 220p.

MARTINS KC (2010) Palinologia de *Capsicum* spp.: caracterização, divergência e viabilidade polínica. Rio de Janeiro, RJ. Dissertação (mestrado em Genética e Melhoramento de Plantas) - Centro de Ciências e Tecnologias Agropecuárias, Universidade Estadual do Norte Fluminense Darcy Ribeiro, Campos dos Goytacazes.

MORAES PLR, DERBYSHIRE MTVC (2002) Estrutura genética de populações naturais de *Cryptocarya aschersoniana* Mez (Lauraceae) através de marcadores isoenzimáticos. Biota Neotropica, 2(2): 1-19. https://doi.org/10.1590/S1676-06032002000200011

MORI SA, SILVA LAM, LISBOA G, CORADIN L (1989) Manual de Manejo de Herbário Fanerogâmico. Bahia: Centro de Pesquisas do Cacau, ed. 104p.

MORS WB, RIZZINI CT, PEREIRA NA, DEFILIPPS RA (2000) Medicinal Plantas of Brazil. Reference Publications, Inc., Algonac, Michigan. 501p.

MUNHOZ M, LUZ CFP, MEISSNER FILHO PE, BARTH OM, REINERT F (2008) Viabilidade polínica de *Caryca papaya* L.: Uma comparação metodológica. Rev Bras Bot 31(2): 209-214. http://dx.doi.org/10.1590/S0100-84042008000200003

NETO ODS, KARSBURG IV, YOSHITOME MY (2006) Viabilidade e germinabilidade polínica de populações de jurubeba (*Solanum paniculatum* L.). Rev Ciên Agro-Amb 4(1): 67-74.

PAGLIARINI MS, POZZOBON MT (2005) Meiose em vegetais: Um enfoque para a caracterização de germoplasma. In: II Curso de citogenética aplicada a recursos genéticos vegetais, 1., Brasília. Brasília: Embrapa Recursos Genéticos e Biotecnologia, p. 24-41.

PAULA JM (2009) Caracterização e manejo de *Conyza* spp. resistente ao herbicida glifosato. 91 f. Dissertação (mestrado - Curso de Pós-Graduação em Fitossanidade), Universidade Federal de Pelotas, Pelotas.

PICCININI F, FRESCURA VDS, LAUGHINGHOUSE IV HD, PEREZ NB, TEDESCO SB (2012) Pollen viability of *Eragrostis plana* genotypes from different geographic populations in Rio Grande do Sul. Enc Biosfera 8(15): 13-16.

PIO LA, RAMOS JD, PASQUAL M, JUNQUEIRA KP, SANTOS FC, RUFINI JCM (2007) Viabilidade do pólen de laranjas doces em diferentes condições de armazenamento. Ciên Agrotec 31: 147-153. https://doi.org/10.1590/S1413-70542007000100022

SOUZA MM, PEREIRA TNS, MARTINS ER (2002)

Microsporogênese e microgametogênese associadas ao tamanho do botão floral e da antera e viabilidade polínica em maracujazeiro-amarelo (P*assiflora edulis* Sims f. *Flavicarpa degener*). Ciên Agrotéc 26(6): 1209-1217.

VARGAS DP (2006) Mamona (*Ricinus communis* L.): cultura de antera, viabilidade e conservação de pólen. 98 f. Dissertação (mestrado em Ciências), Universidade Federal de Pelotas, Pelotas.