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Seed traits of species from South Brazilian grasslands with contrasting distribution

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ABSTRACT

South Brazilian grasslands are among the most species-rich grasslands worldwide yet they have been suffering ongoing degradation due to land-use. Little is known about the reproductive ecology of the native species of these grasslands. Thus, our goal was to characterize seed traits and germination requirements of three native species of the tribe Tigridieae (Iridaceae, Iridoideae) due to its richness in the Pampa biome and the contrasting morphology, cytogenetics, and geographic distributions of its representatives. We tested if closely related species possess similar seed traits and whether species with wider distributions have broader germination requirements. Seed production and mass were estimated, and morphological analyses, germination experiments and viability tests were performed. Principal component analysis (PCA) was used to describe correlations between seed traits and species' distributions. Germination was assessed using time-to-event analysis and the Cox model. All seed traits differed among the analyzed species/cytotypes. Final germination percentage (FGP) averaged 39.1 % and with overall viability of 89.9 %. Germination tests showed that seeds benefit from negative photoblasty. Species/cytotypes with wider distributions and heavier and larger seeds generally had better germination performances than narrower distributed species/cytotypes with lighter and smaller seeds.

Keywords: dormancy, functional traits, germination, grasslands, Iridaceae, photoinhibition, regeneration niche, reproductive ecology, seed morphology, time-to-event analysis

Introduction

Located in Rio Grande do Sul (RS), the southernmost state of Brazil, the Pampa biome is part of the Río de la Plata ecoregion (shared with Uruguay and Argentina), the largest continuous grassland in the Americas and whose species-richness within Brazil was estimated at 3,530 species, of which 278 are endemic (Andrade *et al.* 2018). Despite being among the most species-rich grasslands in the world (Overbeck *et al.* 2007; Andrade *et al.* 2015), less than 31 % of the original native vegetation cover of the Brazilian Pampa remains, which has the lowest proportion covered by conservation units with only 0.3 % of its area under full protection, compared to other biomes in Brazil (Brentano *et al.* 2015; ICMBio 2016). Unfortunately, basic data on reproductive ecology (*e.g.*, aspects of seed biology) for native grassland species of the South Brazilian Pampa are scarce and so research efforts must continue to advance

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restoration (Shivanna & Tandon 2014; Thomas *et al.* 2019). For effective restoration projects to be designed with the aim of conserving the biodiversity and ecosystem services of the Brazilian Pampa, native species that occupy its grassland remnants need to be studied (Overbeck *et al.* 2013; Andrade *et al.* 2015; Havens *et al.* 2015; Andrade *et al.* 2019).

Research on seed traits provides important knowledge for biodiversity conservation and can contribute to decision-making regarding *in situ* management of species, populations or communities, and *ex situ* seed conservation and ecological restoration (Barak *et al.* 2018; Saatkamp *et al.* 2018). Knowledge about seed production, size and mass (Paulů *et al.* 2017; Alstad *et al.* 2018; Chen *et al.* 2018), and recognition of characteristics related to germinability, may be key for determining species suitability for restoration (Perring *et al.* 2015; Carta *et al.* 2016; Ladouceur *et al.* 2018; Scotton 2018). In general, seed traits are likely to be more similar in more closely related species due evolutionary conservatism (Burns & Strauss 2011; Gabrielová *et al.* 2013; Zhang *et al.* 2014). Studies have been investigating whether common species with broader distributions possesses patterns of seed traits and germination characteristics that favor their occurrence compared to more narrowly distributed close relatives (Lavergne *et al.* 2004; Mattana *et al.* 2010; Youssef *et al.* 2011; Imbert *et al.* 2012). For instance, Paulů *et al.* (2017) found significant differences in seed mass, while other studies showed that rare species could possess either lighter or heavier seeds than their common congeners (Brown *et al.* 2003; Hewitt *et al.* 2015). There is no consensus about possible patterns of species distributions determined by seed features since patterns are largely species-specific (Paulů *et al.* 2017). Therefore, seed traits, such as morphology and ecophysiological requirements for germination, need to be characterized at intra- and interspecific levels to make restoration outcomes more predictable and to employ effective niche regeneration strategies (Barak *et al.* 2018; Saatkamp *et al.* 2018).

Iridaceae is the botanical family with the greatest number of exclusive Pampa biome representatives (Zappi *et al.* 2015), comprising perennial herbaceous plants, mostly geophytes (Rodriguez & Sytsma 2006). Although several studies have been developed in recent years on seed morphology and ecophysiology in the Old World, such as for *Crocus* (Carta *et al.* 2014; 2015; Erol *et al.* 2015; Skourti & Thanos 2015; Karaismailoğlu *et al.* 2018) and *Romulea* (Swart *et al.* 2011; Karaismailoğlu 2015; Carta *et al.*2016), little is known about seed features of South American species of Iridaceae (Goldblatt *et al.* 1989; Schiappacasse *et al.* 2005). The seed dormancy classification system of Baskin & Baskin (2004) suggests seeds of Iridaceae have some level of physiological dormancy. Nevertheless, detailed information on dormancy-breaking and germination requirements for wild species is essential to develop efficient and cost-effective propagation techniques (Fu *et al.* 2013). Therefore, basic imbibition and viability tests are still needed to confirm the presence, type, and level of seed dormancy of native species (Baskin & Baskin 2014; Marin *et al.* 2017). Furthermore, Merritt & Dixon (2011) reported that typical establishment rates for biodiversity restoration are less than 10 % due to substandard storage before seed use, lack of pre-treatments of seeds for dormancy release and precision in delivering seeds to sites at the appropriate time. Thus, dry storage, move-along, photoinhibition and cold stratification experiments have been used to more accurately detect specific seed germination requirements and determine to what extent storage and variation in temperature and light conditions affect species germination performance (Baskin & Baskin 2003a; Fu *et al.* 2013; Skourti & Thanos 2015).

Approximately 200 species of Iridaceae, belonging to the tribes Sisyrinchieae, Trimezieae, and Tigridieae, are found in Brazil (Eggers *et al.* 2015). Tigridieae is the second most species-rich tribe in South Brazil, with its representatives being characterized by the presence of bulbs and plicate leaves (Souza-Chies *et al.* 2012). We selected the single species of the monotypic genus *Kelissa* and two from *Herbertia* to characterize seed traits, due to their evolutionary proximity and their contrasting morphological and cytogenetic characteristics and geographic distributions, to explore their future use in restoration (Nevill *et al.* 2016; Ladouceur *et al.* 2018).

Kelissa brasiliensis occurs in open grasslands and is considered endemic to these environments in RS (Eggers 2015). Despite its high degree of vulnerability, knowledge about the species is very limited (Chauveau *et al.* 2012; Moraes *et al.* 2015). Only diploid individuals of the species have been found in RS thus far (Moraes *et al.* 2015), and pollination experiments have evidenced it as self-incompatible (Oleques *et al.* 2020).

There are species delimitation problems for *Herbertia* since its representatives exhibit little vegetative distinction, high floral morphological variability (inter- and intraspecific), differences in reproductive behavior (self-compatible and self-incompatible) and genetic variation among populations (Souza-Chies *et al.* 2012; Stiehl-Alves *et al.* 2016; 2017). The two most common species are *Herbertia lahue* and *H. pulchella* (Souza-Chies *et al.* 2012). The former has a broader distribution than the latter and can be found in almost all regions of RS, often in sympatry with *H. pulchella*, which is restricted to the Río de la Plata grassland ecoregion (Souza-Chies *et al.* 2012; Stiehl-Alves *et al.* 2016; 2017; Andrade *et al.* 2018) and is more recurrently found in the Brazilian Pampa, especially in regions at higher altitudes. Intraspecific polyploid series are particularly common in the genus: *H. pulchella* has cytotypes 2x, 4x, and 6x, with 4x individuals being found in RS; and *H. lahue* has 2x, 4x, 6x and 8x populations, with 6x and 8x individuals being found in RS (Moraes *et al.* 2015). According to Stiehl-Alves *et al.*

(2016), subtle morphological variation among *H. lahue* cytotypes is detectable. Morphological differences have yet to be recognized among *H. pulchella* cytotypes. Given the complex evolutionary scenario for representatives of Tigridieae, and knowing that the reproductive system plays an important role in shaping variation among genetic lineages (Chauveau *et al.* 2012; Moraes *et al.* 2015; Stiehl-Alves *et al.* 2016; 2017), detecting seed traits that may be related to reproductive success would be of great value for a better understanding of habitat specialization in regeneration niche (Ten-Brink *et al.* 2013; Carta *et al.* 2016).

In this study, we characterized seed traits and germination requirements of three native species of Tigridieae seeking to identify possible inter- and intraspecific trade-offs that may influence their reproductive performance. Our main goal was to test whether closely related species possess similar seed traits and whether species with broader distributions have broader germination requirements. Thus, we selected *H. lahue*, *H. pulchella*, and *K. brasiliensis* to be characterized by (1) estimating seed production and viability, (2) determining seed dormancy, (3) evaluating seed morphological variation and (4) assessing germination response under distinct temperature, photoperiod and storage time conditions. We predict that common species/cytotypes with broader distributions will possess larger and heavier seeds than their more narrowly distributed relatives. At the same time, we expect the species/cytotypes with broader distributions to germinate better under broader temperature and light conditions than their narrowly distributed relatives, which will germinate only under more specific conditions.

Materials and methods

Seed collection

Seed collection sites were selected prioritizing co-occurrence records of populations of at least two of the studied species — *K. brasiliensis*, *H. lahue*, and *H. pulchella* (speciesLink 2002 onwards). Field sampling was performed between 14 October 2017 and 14 December 2017, when 20–30 individual flowers and mature fruits (capsules) from each population were wrapped in fine mesh plastic bags, tagged with colorful plastic glue and collected. Seeds were collected from a total of 20 populations. Geographic coordinates and elevation for all collection sites were recorded with GPS (Tab. 1). Due to the recognition of populations of a new *H. lahue* cytotype in recent years, 2x (with similar morphology to *H. lahue,* hereafter *H.* aff. *lahue*), 6x and 8x seeds were collected separately and treated as distinct groups. Voucher specimens were deposited in the ICN Herbarium of Universidade Federal do Rio Grande do Sul (UFRGS) (Tab. 1). Dried fruits were stored in an airy place with standard temperature and humidity conditions (~25 °C, 20–30 % RH) to avoid changes in seed quality (BRASIL 2009).

Flow cytometry

Flow cytometry (FCM) assays were carried out following the protocol proposed by Doležel *et al.* (2007) to confirm ploidy level of the sampled populations of *H.* aff. *lahue* (2x) and *H. lahue* (6x and 8x), initially identified and collected based on their floral morphology as described in Stiehl-

Table 1. Collection sites of all sampled species populations.

Species	Ploidy level	Municipality	Latitude	Longitude	Elevation (m)	Voucher [1]
Herbertia aff. *lahue*	2x	Porto Alegre	-30.06768	-51.11988	95	C01
	2x	Porto Alegre	-30.06747	-51.11942	90	C02
	2x	Porto Alegre	-30.07224	-51.11841	80	CM03
	2x	Guaíba	-30.180114	-51.392645	27	CMGA01
Herbertia lahue	6x	Porto Alegre	-30.07136	-51.11923	63	CM01
	6x	Porto Alegre	-30.07229	-51.11847	80	CM02
	6x	Porto Alegre	-30.030971	-51.170317	45	C03
	6x	Porto Alegre	-30.07168	-51.11992	62	CM05
	8x	Caçapava do Sul	-30.89532	-53.43036	139	CSAM07
	8x	Caçapava do Sul	-30.89366	-53.42928	149	CSAM09
	8x	Porto Alegre	-30.03083	-51.170065	50	C04
	8x	Porto Alegre	-30.07211	-51.11941	68	CM04
	8x	Porto Alegre	-30.07155	-51.11963	76	CM06
Herbertia pulchella		Caçapava do Sul	-30.69351	-53.39317	142	CSAM02
		Caçapava do Sul	-30.89499	-53.42707	167	CSAM06
		Porto Alegre	-30.122003	-51.23361	90	CBS02
		Porto Alegre	-30.06066	-51.12307	234	CdaSMOB01
Kelissa brasiliensis		Caçapava do Sul	-30.6929	-53.39234	146	CSAM03
		Caçapava do Sul	-30.89515	-53.42904	146	CSAM08
		Caçapava do Sul	-30.86648	-53.28816	230	CSAM11

[1] Voucher acronyms refer to members of collector teams. C: Cristante, A. M.; M: Marchioretto, R. M.; G: Garcia V. O.; A: Alves, M. E. O.; SA: Stiehl-Alves, E. M; BL: Báez-Lizarazo, M. R.; S: Silva, D. F.; daS: da Silva, L. N.; O: Oliveira, R.; B: Buchoski, M. G.

Alves *et al.* (2016). Fresh young leaves of the target species were obtained from newly developed seedlings resulting from germinated seeds (see Fig. S1 for detailed internal standards information).

Seed traits

Seed set

After fruits underwent natural dehiscence due to ripening in the laboratory, 30 individual capsules were randomly and proportionally sampled to estimate the number of seeds per fruit from all populations found for each species/cytotype. Remaining seeds were separated in lots and replaced in dry storage for the experiments described below.

Seed mass

Seed mass was determined by weighing six replicates of 100 fresh seeds from the aforementioned capsules for each species/cytotype on a precision balance (0.0001 g) under laboratory conditions (~ 25 °C, 20–30 % RH).

Seed morphology

Thirty seeds per species/cytotype were analyzed using a stereomicroscope with a digital camera (Leica M165FC) and integrated analysis software (LAS V4.5) to measure their length (µm), width (µm), thickness (µm) and umbilicus area (µm²), an area which represents the inner part of the seed pit borders, located near the hilum that straddles the raphe (Goldblatt *et al.* 1989; 1990). Seed shape (unitless) was calculated according to data standards of LEDA Traitbase (Kleyer *et al.* 2008) as the variance of the three dimensions (length, width, and thickness), each divided by seed length so that length is unity.

Imbibition test

An imbibition test was performed to determine if seeds have physical dormancy. Four replicates of 25 seeds per species/cytotype were placed in plastic Petri dishes (90 mm diameter) on two hydrated filter paper sheets (6 ml of distilled water). Plates with hydrated seeds were then closed, had their ends wrapped with Parafilm (hereafter, incubation) and kept in the laboratory at room temperature (~25 °C, 20–30 % RH) for 24 hours. Seeds were then removed from the plates, wiped with paper towels, and reweighed. Percentage increase of seed mass due to water uptake was calculated using the formula proposed by Fu *et al.* (2013). This preliminary test evaluated whether the seed coat allowed water to enter. A seed mass increase of more than 20% was considered sufficient to characterize seeds as being permeable to water. If not, seeds were considered to have physical dormancy and must undergo a scarification process to allow the embryo access to water.

Germination experiments

Four different treatments were performed to evaluate seed germination response under laboratory conditions (Tab. S1 in supplementary material). To indicate precisely when each germination experiment started, the numbers 0 to 6 were presented aside acronyms for the germination experiments to represent storage time experienced by seed lots in months before incubation (Tab. S1 in supplementary material).

Dry storage

To evaluate whether a prolonged dry storage period affects the germination performance of mature seeds, 600 fresh seeds per species/cytotype were stored in six individual paper bags with 100 seeds each. Stored seeds were kept in a chamber with temperature and humidity control (18–30 °C, 20–30 % RH) until being removed after 0 (control), 1, 2, 3, 4, and 6 months of storage. After the storage period, the lots of 100 seeds were distributed in four plastic Petri dishes with 25 seeds each and incubated in a germination chamber with light (12 h photoperiod, hereafter light) under variable temperature conditions at 25 °C/15 °C (hereafter day/night) simulating a summer season (hereafter SS) when seeds would naturally be dispersed. Plates were checked weekly for 140 days or until all seeds germinated. During each observation, seeds with emerged radicles (considered germinated) were removed from the plates and reserved for flow cytometry analyses (see above). Plates were watered when needed as a maintenance procedure.

A disinfection procedure was implemented before the incubations of the seed lots to prevent pathogen development during the experiments. The disinfection procedure consisted of placing seed lots in 2 ml Eppendorf tubes with 2 % sodium hypochlorite solution for 15 min and 70 % ethanol for 1 min, with washing three times with distilled water between and after each solution. Seed lots were hydrated first with a 0.2 % nystatin solution to ensure plate sterilization (Iossi *et al.* 2016). The standard germination measurements of initial germination day (IGD) and final germination day (FGD) were made, and mean germination time (MGT) and final germination percentage (FGP) were calculated, for each germination treatment per species/cytotype tested (Ranal *et al.* 2009).

Move-along experiment

To determine the temperature or temperature sequence necessary to break physiological dormancy in water-permeable seeds (non-physical dormancy), a move-along experiment (Baskin & Baskin 2003a) was performed to simulate the natural sequence of temperature variation experienced by grassland species in the Pampa biome. Three lots of 100 seeds (four replicates of 25 seeds) per species/cytotype were established one month after the collection date. Initially, two of these lots (with four replicates of 25 seeds) were incubated under a variable temperature

of 25 °C/15 °C, simulating one SS, and the third under variable temperature conditions of 20 °C/10 °C, simulating an autumn season (AS), both under light. Seed plates kept in SS were incubated for 12 weeks. After this period, one SS lot was exposed to alternating temperatures (SS → AS, hereafter AT), being transferred to AS conditions for eight weeks (totaling 20 weeks) to simulate the natural transition of the seasons. The remaining SS lot was maintained under its initial condition (SS control). Seed plates incubated initially in AS were kept under the same conditions during the entire 20 weeks duration of the experiment (AS control). Checking, observation, and maintenance of the plates were performed as described above.

Photoinhibition

A photoinhibition experiment was conducted to better understand seed germination response and light requirement. Four months after the collection date, four lots of 100 seeds per species/cytotype were divided and placed into two germination chambers operating at different variable temperatures, one simulating a SS and another an AS. In each chamber, one of the seed lots was incubated in light and the other in continuous darkness (SD and AD), which was achieved by wrapping plates with aluminum foil. Plate checking, observation and maintenance were performed as previously described except for the photoinhibition lots, for which observations were done in a dark room under a dim green safelight to prevent light exposure.

Cold stratification

A cold stratification test was performed to stimulate germination by alleviating non-deep physiological dormancy in seeds. Two weeks before completing four months of dry storage, four lots of 100 seeds per species/cytotype were placed in a refrigerator at a constant temperature of 5 ºC. After two weeks of cold stratification, seed lot incubation in germination chambers at variable AS temperature conditions under light and darkness (CS and CSD) and plate checking, observation, and maintenance followed the methodology of the photoinhibition experiment.

Viability tests

Seeking to assess the viability of non-germinated seeds from all germination experiments after a 140 day incubation period, a cut test (CT) followed by a tetrazolium test (TZ) were performed according to Newton *et al.* (2014) and Marin *et al.* (2017), respectively. The CT consists of visually assessing the coat, endosperm, and embryo health of seeds after lengthwise dissection with a sharp blade and recording each seed as fresh (firm coat with a creamy-white colored inner part), moldy, empty, insect-infested or abnormal. Empty and insect-infested seeds were excluded from further analysis. Abnormal and moldy were considered as ungerminated. The TZ was applied to determine whether ungerminated seeds were still alive (viable) or dead. Cut seeds were immersed in 1 % buffered 2,3,5-triphenyl tetrazolium chloride solution and maintained in the dark at room temperature (~25 °C, 20–30 % RH) for at least six hours. Living tissue stained red due to the action of dehydrogenase enzymes confirmed seeds as viable (but dormant) from a physiological perspective.

Data analyses

Flow cytometry statistics, dot plots, and histograms were generated in BD FACSDiva Version 6.1.3. Ploidy screening and total holoploid nuclear DNA content (2C) were assessed through relative calculation assuming a linear relationship between fluorescent signals from target-specimen stained nuclei and its internal standard using the formula proposed by Galbraith *et al.* (1998). Conventionally, relative nuclear DNA amounts are presented in pg and Mbp (1 pg = 978 Mbp) as proposed by Doležel *et al.* (2007). Only measurements with coefficients of variation (CV) less than 5 % were taken into account.

Numerical data obtained from seed traits and germination experiments were described as mean ± 95 % confidence interval (CI). Normality of the distribution of variables was graphically diagnosed with histogram/quantile-quantile plots and further assessed with the Shapiro-Wilk test. In the case of non-normality, the degree of skewness was quantified to evaluate if non-normality was significant (Crawley 2012). Variables were assessed for homogeneity of variance with the Fligner-Killeen test. Linear models were fitted for each variable and the assumption of homoscedasticity was graphically diagnosed by extracting and plotting residuals against fitted models. Variables were log-transformed in case of the need for parametric testing. For seed set (count data) and water uptake (percentage data) comparisons, estimated marginal means were extracted from Poisson and Beta regression models, respectively (Mangiofico 2016). One-way ANOVA followed by Tukey post-hoc test (with Bonferroni correction) were performed to compare differences between seed trait means per species/cytotype. Pearson correlation was used to detect correlations among seed mass, shape, and water uptake. Principal component analysis (PCA) was applied using *FactoMineR* and *factoextra* packages (Lê *et al.* 2008; Kassambara & Mundt 2020) to describe correlations between seed traits and species distribution after hierarchical clustering implementing Ward's minimum variance criterion using the *hclust()* function from the R *stats* package. The significance of PCA clustering was tested with PerMANOVA using the *adonis()* function from the *vegan* package (Oksanen *et al.* 2019).

Time-to-event analysis was performed to evaluate seed germination response for each germination experiment (McNair *et al.* 2012). Survival curves were generated with the non-parametric Kaplan-Meier estimator of survival functions using the *survfit()* function and differences between germination treatments were assessed through survival

curve comparisons with the Mantel-Haenszel homogeneity test using the *survdiff()* function, both functions from the *survival* package (Therneau & Lumley 2018). Survival curves Pairwise comparisons of species/cytotype survival curves were assessed by Log-Rank tests (with the Benjamini & Hochberg method for *p* value adjustment) using the *pairwise_survdiff()* function of the *survminer* package (Kassambara *et al.* 2017). Cox proportional hazards regression models were created and tested using, respectively, *coxph()* and *cox.zph()* from the *survival* package (Therneau & Lumley 2018) to identify the most effective germination treatment per species/cytotype and assess the potential of seed traits as predictive covariates. Akaike information criterion (AIC) was implemented in a stepwise model-building procedure using the *step()* function from the R *stats* package to select the best fitting Cox models. All above mentioned analyses were performed with R software version 3.6.3 (R Development Core Team 2020).

Results

Flow cytometry

Ploidy levels of five *Herbertia* aff. *lahue* (2x) and *H. lahue* (6x and 8x) samples distinguished only by floral morphology in the field were confirmed through estimated 2C results (Tab. S2 in supplementary material) and histograms of DNA content (Fig. S1 in supplementary material) obtained from flow cytometry.

Seed traits

A total of 3,650 seeds representing *H. lahue*, *H. pulchella*, and *K. brasiliensis* were used for seed trait measurements (Tab. S3 in supplementary material). The distributions of all seed trait variables met normality and homogeneity of variances except for seed shape (Shapiro-Wilk, $p < 0.001$; Fligner-Killeen, $p < 0.001$), which required log transformation to meet the assumptions of parametric testing. All seed trait mean values differed significantly among groups (one-way ANOVA, $p < 0.01$). Differences between species/cytotypes for seed traits after Tukey post-hoc analysis are summarized in Figure 1.

Seed set

Mean seed set for *K. brasiliensis,* hexaploid *H. lahue* and *H. pulchella* were very similar in comparison to diploid *H.* aff. *lahue* and octoploid of *H. lahue*, which have a significantly higher number of seeds per capsule (Tab. S3 in supplementary material).

Seed mass

Seed mass differed among all species except for *K. brasiliensis* and diploid *H.* aff. *lahue* (one-way ANOVA with Tukey post-hoc method, $p = 0.7240$). Among the three *H. lahue* cytotypes analyzed, the largest difference in average seed weight was between diploid *H.* aff. *lahue* and hexaploid *H. lahue*, with hexaploids being almost twice as heavy (Tab. S3 in supplementary material).

Seed morphology

Differences in morphological seed traits were found among the studied species ($p < 0.05$, Fig. 1). Both umbilicus area and seed shape had significant interspecific variation for some taxa (Fig. 1). Comparing *H. lahue* cytotypes revealed diploid *H.* aff. *lahue* to have smaller dimensions for all seed traits than both hexaploid and octoploid *H. lahue* (one-way ANOVA with Tukey post-hoc method, $p < 0.001$) while no differences in shape were detected (one-way ANOVA with Tukey post-hoc method, $p > 0.05$). There was no correlation between seed mass and shape ($r = 0.1021$, $p = 0.5913$). In addition, the seed shapes and colors observed for the studied species varied from angular to barrel-shaped and from dark brown to light brown, respectively (Fig. S2 in supplementary material).

Imbibition test

None of the species exhibited seed physical dormancy since the average mass of all fresh seed lots tested increased by at least 48 % (Tab. S3 in supplementary material) due to water uptake. *Herbertia* aff. *lahue* had the highest water uptake percentage (0.6576 ± 0.0553). Seed water uptake capacity was moderately negatively correlated with seed mass ($r = -0.5870$, $p = 0.0065$) and seed shape ($r = -0.6669$, $p = 0.0013$).

Principal component analysis (PCA)

Principal component analysis of 150 individual seeds described by eight seed trait variables explained about 85.7 % of the variance in the dataset with the first five dimensions (Fig. 2A). Seed mass, shape, set and thickness contributed the most to dimensions one and two (Fig. 2B), which were the most important dimensions in explaining the overall variability of the dataset. Seed weight and thickness were positively correlated with dimension one, while seed shape and seed set were negative correlated with it (Fig. 2C), and thus represent the main traits responsible for successfully grouping our dataset into two clusters. Ward's hierarchical clustering algorithm grouped species by distribution factor (narrow or broad) as the best explanation of seed trait dissimilarity in the PCA (Fig. 2D). The narrow cluster includes *Herbertia* aff. *lahue*, *H. pulchella*, and *Kelissa brasiliensis* while the broader cluster includes *Herbertia lahue* hexaploid and octoploid. Clusters were significantly different (PerMANOVA, $df = 1$, $R^2 = 0.26$, $p < 0.001$).

Germination experiments

A total of 6,000 seeds were tested for germination and the standard germination measurements calculated for each

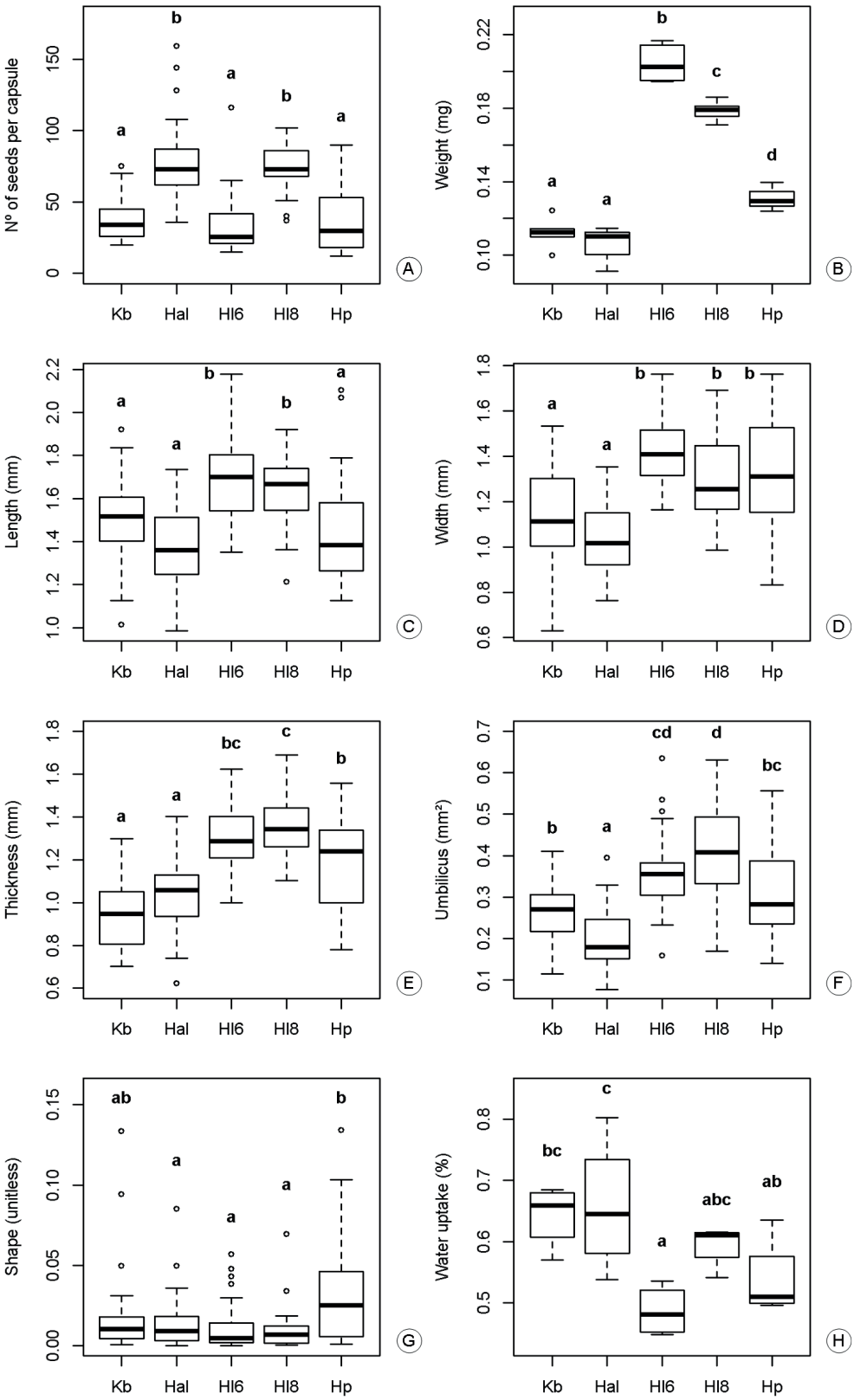

Figure 1. Boxplot of mean values for seed traits of the studied species. **A:** seed set; **B:** seed mass; **C:** seed length; **D:** seed width; **E:** seed thickness; **F:** seed umbilicus area; **G:** seed shape; **H:** seed water uptake capacity. Kb: *Kelissa brasiliensis*; Hal: diploid *Herbertia* aff. *lahue*; Hl6: hexaploid *H. lahue*; Hl8: octoploid *H. lahue*; Hp: *H. pulchella*. Different letters indicate significant differences (one-way ANOVA with Tukey post-hoc method, $p < 0.05$).

lot are compiled in Table 2. Among all 60 lots assessed under distinct treatment conditions, seeds started to germinate only after four weeks of incubation. The FGP was on average at 39.1 %, while the mean FGP for each species varied greatly depending on the germination treatment applied (Tab. 2).

Dry storage

The first lot of freshly harvested seeds tested in the dry storage germination experiment (SS0, representing storage time = 0) did not germinate during the first four weeks of incubation with seeds dying due to mold development shortly thereafter, revealing the need to implement a disinfection procedure for all further seed lots before incubation. Lot SS0 was discarded from the analysis. Comparisons among survival curves for storage time intervals revealed differences in temporal germination patterns (Fig. 3A, Mantel-Haenszel, $p < 0.0001$), with the six months storage treatment differing (List S1 in supplementary material, Log-Rank, $p < 0.0001$) and outperforming all other lots,

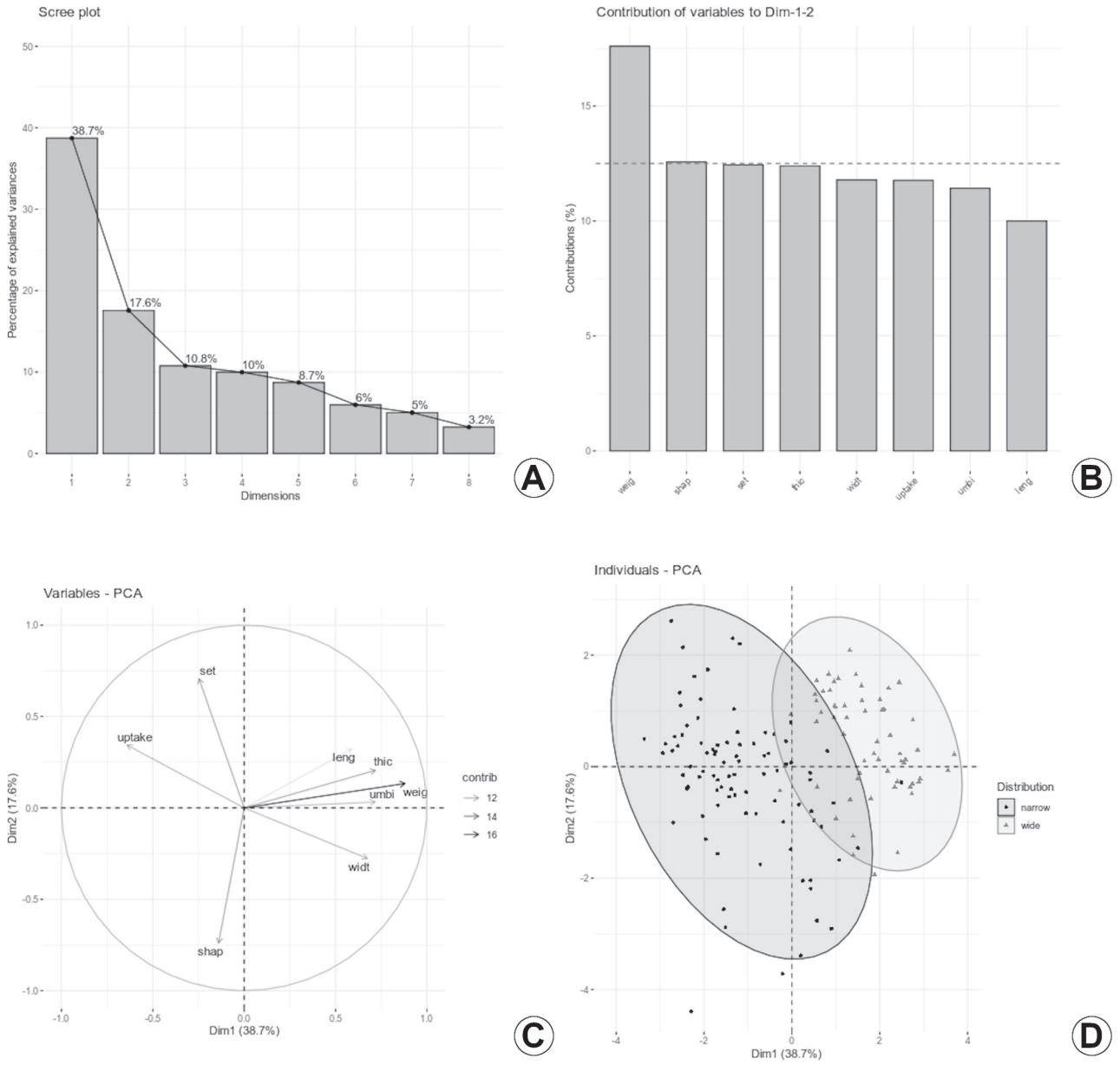

Figure 2. Results of the principal component analysis (PCA) of seed trait data. **A:** scree plot; **B:** contribution histogram. Contribution percentages for representative variables to the first two dimensions where the dashed line indicates the expected average contribution; weig: seed mass; shap: seed shape; set: seed set; thick: seed thickness; widt: seed width; uptake: seed water uptake capacity; umbi: seed umbilicus area; leng: seed length; **C:** variables correlation plot. Transparency of arrows are according to projection contribution values of variables; Dim1: dimension 1, Dim2: dimension 2; **D:** graph of individuals. Concentration ellipses were added by species distribution categories after hierarchical clustering (PerMANOVA, $df = 1$, $R^2 = 0.26$, $p < 0.001$); narrow: *Herbertia* aff. *lahue*, *H. pulchella* and *Kelissa brasiliensis*, cluster of species with narrow distributions; wide: *H. lahue* hexaploid and octoploid, cluster of species with broad distributions.

Table 2. Germination treatments measurements and viability tests results (mean ± CI margin of error). IGD: Initial germination date; FGD: Final germination date; MTG: Mean time germination; FGP: Final germination percent; CT: Cut test; TZ: Tetrazolium test.

Species	Ploidy level	Germination treatment	IGD (days)	FGD (days)	MGT (days)	FGP (%)	CT (%)	TZ (%)
Herbertia aff. *lahue*	2x	SS1	-	-	-	-	94 ± 0.1	100 ± 0
		AS1	-	-	-	-	86 ± 0.2	100 ± 0
		AT	98 ± 4.8	131.3 ± 7.8	114 ± 5.7	35 ± 9.4	88 ± 0.2	100 ± 0
		SS2	-	-	-	-	82 ± 0.1	100 ± 0
		SS3	127.8 ± 6.2	133 ± 0	130.4 ± 3.1	6 ± 4.7	92 ± 0.1	100 ± 0
		SS4	108.5 ± 2.9	108.5 ± 2.9	108.5 ± 2.9	2 ± 2.8	60 ± 0.3	100 ± 0
		SD	75.3 ± 17.6	126 ± 4.8	96.1 ± 9.1	33 ± 9.3	87 ± 0.5	100 ± 0
		AS4	43.8 ± 3.9	120.8 ± 9.7	78.1 ± 8.9	22 ± 8.2	96 ± 0.2	100 ± 0
		AD	33.3 ± 3.9	75.3 ± 7	52.1 ± 0.9	47 ± 9.8	94 ± 0.1	100 ± 0
		CS	43.8 ± 6.2	98 ± 20.2	64.2 ± 5.4	35 ± 9.4	92 ± 0.2	100 ± 0
		CSD	35 ± 4.8	61.3 ± 3.9	48.3 ± 2.1	46 ± 9.8	89 ± 0.2	100 ± 0
		SS6	81.7 ± 29.2	137.7 ± 2.4	112.6 ± 12.4	11 ± 6.2	90 ± 0.3	85.7 ± 0.3
Herbertia lahue	6x	SS1	80.5 ± 2.4	126 ± 10.1	102.2 ± 2.8	33 ± 9.3	85 ± 0.1	90.9 ± 0.4
		AS1	77 ± 7.5	126 ± 9.5	100.9 ± 10	68 ± 9.2	82 ± 0.4	59.2 ± 0.7
		AT	68.3 ± 9.1	120.8 ± 3.9	91.5 ± 6.3	89 ± 6.2	89 ± 0.1	-
		SS2	80.5 ± 4.1	129.5 ± 5.3	104.9 ± 6.4	61 ± 9.6	86 ± 0.1	95.8 ± 0.2
		SS3	66.5 ± 2.4	138.3 ± 2.1	107.3 ± 6.9	46 ± 9.8	81 ± 0.3	100 ± 0
		SS4	66.5 ± 5.3	129.5 ± 4.1	96.1 ± 4.9	67 ± 9.3	92 ± 0.1	100 ± 0
		SD	47.3 ± 3.9	66.5 ± 5.3	54.9 ± 4.2	90 ± 5.9	93 ± 0.1	100 ± 0
		AS4	45.5 ± 2.4	89.3 ± 6.2	64.6 ± 4.1	65 ± 9.4	91 ± 0.2	100 ± 0
		AD	36.8 ± 2.1	68.3 ± 9.1	47 ± 1.8	51 ± 9.9	99 ± 0	100 ± 0
		CS	36.8 ± 2.1	99.8 ± 14	54.6 ± 4.1	89 ± 6.2	99 ± 0.1	100 ± 0
		CSD	40.3 ± 9.7	57.8 ± 6.2	48.9 ± 7.1	28 ± 8.8	92 ± 0.1	100 ± 0
		SS6	68.3 ± 9.7	117.3 ± 9.1	90.4 ± 4.4	91 ± 5.6	94 ± 0.1	100 ± 0
	8x	SS1	101.5 ± 14.6	122.5 ± 2.9	112 ± 5.8	3 ± 3.4	84 ± 0.2	93.4 ± 0.2
		AS1	112 ± 7.5	129.5 ± 5.3	120.5 ± 2.2	16 ± 7.2	79 ± 0.2	100 ± 0
		AT	87.5 ± 12.8	138.3 ± 2.1	116.1 ± 2.7	56 ± 9.8	74 ± 0.2	60 ± 1
		SS2	101.5 ± 8.7	126 ± 11.6	116.8 ± 4	9 ± 5.6	97 ± 0.1	84.3 ± 0.1
		SS3	133 ± 0	133 ± 0	133 ± 0	2 ± 2.8	92 ± 0.1	100 ± 0
		SS4	113.8 ± 6.2	115.5 ± 5.3	114.6 ± 5.7	6 ± 4.7	55 ± 0.4	100 ± 0
		SD	54.3 ± 3.9	115.5 ± 15.6	84.2 ± 9	80 ± 7.9	92 ± 0.1	100 ± 0
		AS4	49 ± 3.4	115.5 ± 12.8	81.2 ± 7.8	39 ± 9.6	85 ± 0.2	100 ± 0
		AD	35 ± 4.8	73.5 ± 5.3	49.6 ± 3.3	59 ± 9.7	91 ± 0.1	100 ± 0
		CS	57.8 ± 5.2	129.5 ± 7.1	92.9 ± 11.5	37 ± 9.5	76 ± 0.3	100 ± 0
		CSD	40.3 ± 2.1	70 ± 12.1	51.7 ± 4.8	41 ± 9.7	94 ± 0.1	100 ± 0
		SS6	91 ± 12.1	133 ± 5.8	119 ± 2.5	32 ± 9.2	97 ± 0.2	92.2 ± 0.2
Herbertia pulchella		SS1	89.3 ± 7.8	119 ± 10.1	97.8 ± 9.6	14 ± 6.8	81 ± 0.4	71.1 ± 0.5
		AS1	49 ± 6.7	127.8 ± 5.2	86 ± 10.4	42 ± 9.7	85 ± 0.3	78.6 ± 0.9
		AT	73.5 ± 12.8	131.3 ± 5.2	105.5 ± 6.6	61 ± 9.6	82 ± 0.2	56.3 ± 1
		SS2	61.3 ± 2.1	99.8 ± 20.8	78.2 ± 8.9	18 ± 7.6	76 ± 0.2	66.5 ± 0.5
		SS3	75.3 ± 11.3	96.3 ± 10.8	87.2 ± 10	13 ± 6.6	86 ± 0.2	100 ± 0
		SS4	56 ± 3.4	122.5 ± 13.7	86.1 ± 8.6	34 ± 9.3	67 ± 0.3	100 ± 0
		SD	57.8 ± 6.2	113.8 ± 12.3	87.4 ± 6.1	50 ± 9.9	92 ± 0.3	100 ± 0
		AS4	26.3 ± 2.1	105 ± 13	50.7 ± 1.3	77 ± 8.3	99 ± 0	100 ± 0
		AD	22.8 ± 2.1	63 ± 5.8	40.8 ± 1.6	91 ± 5.6	96 ± 0.1	100 ± 0
		CS	29.8 ± 2.1	87.5 ± 12.8	51.4 ± 1.6	67 ± 9.3	75 ± 0.5	100 ± 0
		CSD	26.3 ± 3.9	68.3 ± 2.1	42.2 ± 1.9	84 ± 7.2	95 ± 0.1	100 ± 0
		SS6	52.5 ± 8.6	134.8 ± 3.9	100.7 ± 4.2	47 ± 9.8	93 ± 0.1	76.6 ± 0.5
Kelissa brasiliensis		SS1	115.5 ± 8.7	136.5 ± 2.9	126 ± 5.8	4 ± 3.9	90 ± 0.2	100 ± 0
		AS1	85.8 ± 2.1	101.5 ± 12.4	92.5 ± 6.8	7 ± 5	90 ± 0.1	100 ± 0
		AT	85.8 ± 9.1	133 ± 4.8	108.8 ± 1.8	37 ± 9.5	93 ± 0.1	100 ± 0
		SS2	101.5 ± 15.2	113.8 ± 15.5	106.8 ± 13.6	9 ± 5.6	84 ± 0.2	83.2 ± 0.3
		SS3	91 ± 4.8	126 ± 7.5	109.5 ± 4.8	17 ± 7.4	93 ± 0.1	100 ± 0
		SS4	98 ± 21.3	108.5 ± 21.7	103.3 ± 20.6	5 ± 4.3	78 ± 0.2	100 ± 0
		SD	61.3 ± 3.9	101.5 ± 12.4	79.2 ± 3.4	22 ± 8.2	92 ± 0.3	100 ± 0
		AS4	42 ± 5.8	120.8 ± 14.8	78.6 ± 8	20 ± 7.9	87 ± 0.1	100 ± 0
		AD	38.5 ± 4.1	78.8 ± 18.5	55.1 ± 8.2	40 ± 9.7	88 ± 0.3	100 ± 0
		CS	42 ± 5.8	96.3 ± 14.8	65.1 ± 4.2	24 ± 8.4	88 ± 0.4	100 ± 0
		CSD	38.5 ± 4.1	61.3 ± 7	50.3 ± 4.6	35 ± 9.4	99 ± 0	100 ± 0
		SS6	78.8 ± 7	140 ± 0	113.5 ± 3.6	51 ± 9.9	93 ± 0.1	77.4 ± 0.4

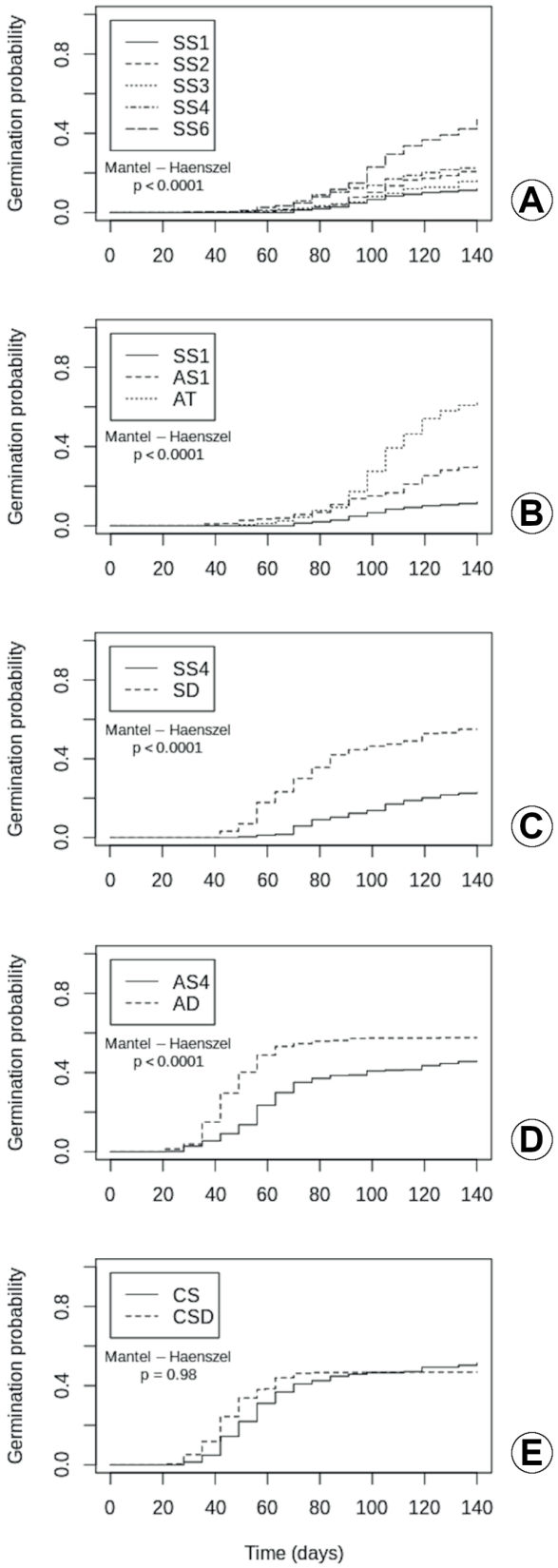

Figure 3. Survival curves generated for each germination treatment tested with Kaplan-Meier estimator of survival functions. **A:** dry storage; **B:** move-along; **C-D:** photoinhibition, respectively, under summer and autumn condition; **E:** cold stratification.

despite the poor FGP achieved by diploid *H.* aff. *lahue* and octoploid *H. lahue* (Tab. 2).

Move-along

Results from the move-along experiment demonstrated that seed germination response differed significantly among all treatment temperatures (Fig. 3B, Mantel-Haenszel, $p < 0.0001$). Due to lack of germination, seeds subjected to treatments SS1 and AS1 did not differ for *K. brasiliensis* (List S2 in supplementary material, Log-Rank, $p = 0.3137$) and for diploid *H.* aff. *lahue* (List S2 in supplementary material, Log-Rank, $p = 0.3137$). The AT treatment differed (List S2 in supplementary material, Log-Rank, $p < 0.0001$) and outperformed SS1 and AS1, with the highest FGP for all species/cytotypes (Tab. 2). The *H. pulchella* seed lot under AS1 had the lowest IGD (49 ± 6.7) followed by hexaploid *H. lahue* under AT (68.3 ± 9.1), which also achieved the highest FGP (89 ± 6.2) among all the lots tested in the move-along experiment.

Photoinhibition

Photoinhibition tests recorded the lowest IGDs, FGDs and MGTs of all the germination treatments (Tab. 2), demonstrating the faster germination response of species/cytotypes in the absence of light. Treatments differed under SS temperature conditions, (Fig. 3C, Mantel-Haenszel, $p < 0.0001$) with seed lots under darkness outperforming those under light for all species/cytotypes except *H. pulchella* (List S3 in supplementary material, Log-Rank, $p = 0.0559$), which showed no difference between treatments. Hexaploid and octoploid *H. lahue* had their highest FGPs of all tested germination treatments under SD conditions (6x = 90 ± 5.9, 8x = 80 ± 7.9). Treatments also differed under AS temperature conditions, (Fig. 3D, Mantel-Haenszel, $p < 0.0001$) with seed lots under darkness outperforming those under light for all species except hexaploid *H. lahue* (List S4 in supplementary material, Log-Rank, $p = 0.6828$), which obtained even a better germination response when exposed to photoperiod (AS4) instead of continuous darkness (AD) (Tab. 2), revealing a possible high-temperature-dependent mechanism for the initiation of germination rather than a light sensitive one. Interestingly, *H. pulchella* and *H.* aff. *lahue* achieved their highest FGPs of all tested germination treatments under AD conditions (91 ± 5.9 and 47 ± 9.8, respectively), exhibiting a potential low-temperature affinity and photosensitivity in the control of germination timing.

Cold stratification

No differences were found between cold stratification pre-germination treatments tested under AS (Fig. 3E, Mantel-Haenszel, $p = 0.98$). When CS and CSD treatments were compared pairwise between species/cytotypes, only hexaploid *H. lahue* and *H. pulchella* responded differently (List S5 in supplementary material, respectively Log-Rank test results,

$p < 0.0001$ and $p < 0.01$) with opposite preferences regarding light exposure. While hexaploid *H. lahue* achieved a higher FGP under CS treatment conditions (89 ± 6.2), *H. pulchella* performed better under CSD (84 ± 7.2).

Viability tests

After 140 days of incubation of 3,447 ungerminated seeds, 3,017 (87.5 %) were visually recognized as viable, with viability of 2,846 seeds (94.3 %) being confirmed by TZ. Combining the total number of seeds capable of germinating during experimental incubation time (2,553) with seeds censored (2,846 viable but dormant) detected through viability tests, an overall seed viability of 89.9 % was assumed for the tested species/cytotypes. Polyploids from *H. lahue* had the highest and lowest seed viability percentages, with hexaploid (90.2 %) and octoploid (84.7 %) cytotypes, respectively.

Cox proportional hazards regression model

Final Cox regression models after model-building stepwise selection with the dataset of all germination treatments and seed traits, retained species/cytotype-specific models shown in Table 3 as the best-ranking germination prediction models (Likelihood-ratio tests, $p < 0.001$). Predictors with significant ($p < 0.01$) and large effect sizes (exp(β) = hazard ratio, hereafter HR) were germination treatments (Tab. 3) that also had the highest MGT and FGP values (Tab. 2). Although the seed traits of length, thickness, water uptake capacity, and umbilicus area were selected by models, none had significant predictive effects regarding germination time ($p > 0.05$). The signs of the coefficient β values show that all germination treatments contributed positively towards species germination success except SS4, which proved to be the only treatment that negatively affected *Herbertia* aff. *lahue* and octoploid *H. lahue* (Tab. 3). Comparing the HR per species/cytotype for the covariates, Cox models indicate that the most effective germination treatments were: CSD for *Herbertia* aff. *lahue* (HR increase by 4.1 times), SD for both hexaploid and octoploid *H. lahue* (HR increase by 4.3 and 5.6 times, respectively), AD for *H. pulchella* (HR increase by 9.9 times) and SS6 for *Kelissa brasiliensis* (HR increase by 4.1 times). Taken together, these results summarize species/cytotype-specific temperature and light conditions and storage time preferences for germination.

Table 3. Final multivariate species/cytotype-specific Cox regression models (Likelihood-ratio tests, $p < 0.001$) for germination treatment and seed trait data after model-building using Akaike information criterion (AIC) stepwise selection. SE: Standard error; AD: Autumn season under darkness; CSD: Cold stratification before autumn season under darkness; SS4: Summer season after 4 month dry storage; leng: Seed length; thic: Seed thickness; uptake: water uptake capacity; AT: Alternating temperature from summer to autumn season; SD: Summer season under darkness; SS6: Summer season after 6 month dry storage; AS4: Autumn season after 4 month dry storage; CS: Cold stratification before autumn season; umbi: Seed umbilicus area.

Species	Ploidy level	Covariate	Coefficient β	exp(β)	SE of β	z	p [1]
Herbertia aff. *lahue*	2x	AD	1.382	3.983	0.169	8.191	<0.001 ***
		CSD	1.427	4.166	0.171	8.358	<0.001 ***
		SS4	-2.18	0.113	0.712	-3.061	0.002 **
		leng	0.567	1.764	0.331	1.712	0.087 .
		thic	-0.575	0.563	0.387	-1.485	0.137 ns
		uptake	1.13	3.097	0.601	1.881	0.059 .
Herbertia lahue	6x	AT	0.788	2.2	0.117	6.751	<0.001 ***
		SD	1.473	4.364	0.118	12.492	<0.001 ***
		SS6	0.649	1.914	0.115	5.622	<0.001 ***
		uptake	1.286	3.619	0.896	1.436	0.151 ns
	8x	SD	1.727	5.621	0.137	12.628	<0.001 ***
		AT	1.241	3.459	0.154	8.066	<0.001 ***
		AD	1.516	4.554	0.15	10.076	<0.001 ***
		SS4	-1.468	0.23	0.415	-3.536	<0.001 ***
Herbertia pulchella		AD	2.297	9.946	0.126	18.271	<0.001 ***
		CSD	1.916	6.795	0.126	15.154	<0.001 ***
		AS4	1.585	4.881	0.131	12.098	<0.001 ***
		CS	1.283	3.606	0.137	9.376	<0.001 ***
		umbi	-0.662	0.516	0.376	-1.76	0.078 .
Kelissa brasiliensis		AT	1.144	3.141	0.191	5.996	<0.001 ***
		AD	1.397	4.044	0.186	7.498	<0.001 ***
		CSD	1.225	3.406	0.195	6.289	<0.001 ***
		SS6	1.41	4.096	0.17	8.275	<0.001 ***
		umbi	1.459	4.304	0.807	1.808	0.071 .

[1] Significance codes: 0 to 0.001 '***', 0.001 to 0.01 '**', 0.01 to 0.05 '*', 0.05 to 0.1 '.', 0.1 to 1 'ns'.

Discussion

This study can be considered a pioneering attempt at characterizing seed traits of native grassland species of Iridaceae of the Pampa biome in Rio Grande do Sul. Until now, similar studies have only been carried out with native grasses of the Brazilian Cerrado biome (Correia-Lima *et al.* 2014; Kolb *et al.* 2016; Escobar *et al.* 2018). Our results show several differences among the studied species of the genera *Kelissa* and *Herbertia* regarding seed traits (*e.g.*, seed production, mass, morphology, and viability) and ecophysiological requirements (*e.g.*, optimal germination conditions).

Plants with high seed production but low viability have been reported for congeneric perennial grasses (Peters 2002). This pattern has been documented for some native Cerrado grasslands where high investment in seed production acts as a compensatory mechanism for low germination potential (Aires 2013). Our seed set and viability results are in agreement with those of Ashman *et al.* (2004), suggesting that trade-offs between seed production and vital proportion (*e.g.*, germination success) may be related to a possible reproductive strategy adaptation in which species dedicate greater resources to seed quantity than quality. Conversely, *K. brasiliensis,* hexaploid *H. lahue*, and *H. pulchella* presented lower seed production, with the latter two recording better final germination percentages. *Kelissa brasiliensis* and *H. pulchella* also present narrower distributions than octoploid *H. lahue*. Even knowing that seed production alone cannot explain species endemism, rarity or restricted natural distribution (Münzbergová 2005; Powell *et al.* 2011; Gabrielová *et al.* 2013; Janišová *et al*. 2018), rarer species often produce fewer seeds than their common relatives (Murray *et al.* 2002; Lavergne *et al.* 2004). Moreover, seed mass, which is expected to be inversely proportional to the number of seeds produced (Moles & Westoby 2004), was another almost fulfilled trade-off premise, except for the octoploid *H. lahue* (*i.e.*, with a large production of heavy seeds).

Seed mass was one of the most variable seed traits among the species of Tigridieae evaluated here. This was a surprising outcome, as seed mass is considered a rather evolutionarily stable seed trait with variation being more comprehensively expected for higher taxonomic levels than between species of closely related genera (Fenner 2000). Similar to other ecophysiological studies (Carta *et al.* 2016; Paulů *et al.* 2017), species of the present study with heavier seeds, such as *H. lahue* (hexaploid and octoploid cytotypes) and *H. pulchella*, were also the ones with the best germination performances. Those were also the species with larger seeds, although no correlation between mass and seed shape could be identified.

It was not possible to identify a pattern of seed morphology variation that enables a clear distinction among the studied species of Tigridieae. However, the PCA successfully grouped species by distribution, recognizing seed mass, shape, set, and thickness as traits that contributed the most to distinguishing the clusters. Umbilicus area, which also varied greatly, has potential as a feature with considerable taxonomic significance since some taxa could be differentiated. As already reported by Goldblatt *et al.* (1989; 1990) for other Iridoideae (tribe Sisyrinchieae and its allies), black-colored umbilicoid seeds with a globose shape appear to be an apomorphic condition in *Sisyrinchium* and *Echthronema*, representing their basic seed types. Despite the fact that morphological seed traits are notoriously conserved phylogenetically and have potential usage as characteristics for taxa delimitation at different taxonomic levels (Jacobs *et al.* 2010; Karaismailoğlu 2015; Vandelook *et al*. 2018), seed morphology has been underexploited in Iridaceae taxonomy (Erol & Küçüker 2003). To our knowledge, no further systematic investigations have been done regarding umbilicus trait features in Iridaceae. Remarkably, comparing seed morphological traits across *H. lahue* cytotypes distinguished diploid *Herbertia* aff. *lahue* in the PCA probably due to its smaller overall seed morphology. In spite of the conserved similar shape among *H. lahue* ploidies, hexaploid *H. lahue* is the one with the larger seed size, instead of the octoploid. This result is somewhat counterintuitive since positive relationships between genome size and seed mass are often verified (Bretagnolle *et al*. 1995; Beaulieu *et al.* 2007; Carta *et al.* 2014).

The PCA clustering result is also noteworthy for the breeding system of *H. lahue* since previous studies have found that *H. lahue* polyploids are self-compatible and non-dependent on pollination vectors (Stiehl-Alves *et al.* 2016). The relationship between ploidy level, breeding system, and geographic distribution is being studied in *H. lahue* and preliminary analyses suggest a correlation among these characters (Stiehl-Alves *et al.* unpublished data). Indeed, previous studies have highlighted that selfing taxa may give rise to successful polyploid lineages more often than outcrossing taxa (Barringer 2007). Considering that polyploidy is important in the evolution of species of Tigridieae, our PCA results open the way for further studies that seek to analyze the relationship between breeding system, ploidy level, and ecology of grassland species.

The laboratory experiments performed here demonstrated that longer dry storage favors seed germination performance. This has already been noticed for other grassland species from open habitats (Liu *et al.* 2011), including Brazilian savannas (Ramos 2015). Although our species were analyzed only for a maximum dry storage period of six months, there was a clear promotion of germination. Our results highlight that *K. brasiliensis* achieved its best germination result after six months dry storage (treatment SS6), which is useful information for conservation strategies (Galmés *et al.* 2006; Copete *et al.* 2011; Saatkamp *et al.* 2018).

The move-along experiment showed that seeds were favored when submitted to a temperature transition sequence. Seeds initially incubated under summer season (SS) conditions and further exposed to autumn season (AS) conditions reached higher germination percentages. This indicates that the physiological dormancy of these seed can be overcome through this treatment (Baskin & Baskin 2003a). Nevertheless, Cox models demonstrated that the most effective treatments, considering seeds necessary incubation time to obtain a satisfactory average germination result in a shorter time interval, were the ones without temperature transition. Intriguingly, *H. pulchella* presents low-temperature sensitivity, which could be interpreted as a mechanism to rapidly colonize available space promptly after recognizing suitable temperature conditions to avoid competition (Carta *et al.* 2016).

Our study pointed out that the seeds from the studied species of Tigridieae obtained faster and more efficient germination responses in photoinhibition experiments. Due to the scarcity of data on seed germination for species of *Herbertia* (Schiappacasse *et al.* 2005; Forgiarini *et al.* 2017; Kew RBG 2017) and *K. brasiliensis* (Barroso 2006), all that was known was that their seeds have a late germination response and some preference for low temperatures. To our knowledge, this is the first record of photoinhibition affinity for representatives of Tigridieae. Recent studies have shown that negative photoblastic seeds are common among other geophytic species of Iridaceae and closely related families (Carta *et al.* 2014; Copete *et al.* 2014; Newton *et al.* 2015; Carta *et al.* 2017; Vandelook *et al.* 2018). In addition, all species tested under photoinhibition started to germinate much earlier in the absence of light and demonstrated different preferences for incubation temperatures.

Under photoinhibition, hexaploid and octoploid *H. lahue*, with broader distributions and heavier and larger seeds, had better germination performances at elevated temperatures while *H.* aff. *lahue*, *H. pulchella*, and *K. brasiliensis*, which have narrower distributions and lighter and smaller seeds, had preferences for lower temperatures. Such adaptations demonstrate habitat specialization through germination cueing (Ten-Brink *et al.* 2013), where hexaploid and octoploid *H. lahue* seeds may be more tolerant to drought, soil burial depth and high-temperature exposure, with greater plasticity to fluctuations in environmental conditions during colonization and establishment in diverse open grassland habitats (Westoby *et al.* 1992; Carta *et al.* 2014; Zhang *et al.* 2014; Skourti & Thanos 2015; Limón & Peco 2016; Vandelook *et al.* 2018).

The cold stratification experiments demonstrated that such exposure to low temperature is not a prerequisite to alleviate seed dormancy. However, our results indicate that the studied species probably possess only a non-deep physiological dormancy instead of a combined morphophysiological dormancy (Baskin & Baskin 2003b). Predictably, seeds present some dormancy mechanisms to avoid germination during summer, which would expose seedlings to unfavorable conditions that threaten their survival (Copete *et al.* 2011; Herranz *et al.* 2013; Carta *et al.* 2014). This has been identified as a typical spring geophyte germination behavior, where seeds germinate in autumn after dormancy break in summer and seedlings emerge in early spring (Vandelook *et al.* 2012; Newton *et al.* 2015). To precisely classify seed dormancy, further tests taking into account embryo growth in *in situ* germination experiments should be conducted in the future (Baskin & Baskin 2004; Carta *et al.* 2014). Notwithstanding, in combination with the results from the dry storage assays performed here, the cold stratification experiments also suggest species of Tigridieae may possess an after-ripening requirement (Skourti & Thanos 2015), meaning that their seeds need to undergo a gradual release of primary physiological dormancy, which starts during seed maturation and continues as a post-maturation event in dry and warm conditions. To confirm seed dormancy classification and after-ripening requirement, further germination tests that consider aspects of seed embryo development must be performed with species of Tigridieae.

Complementarily, flow cytometry results confirmed that the morphological floral variation between cytotypes prescribed by Stiehl-Alves *et al.* (2016) for *H. lahue* can be effectively used to distinguish their populations in the field. Remarkably, diploid *H.* aff. *lahue* populations were found, which have gone unrecorded since Winge (1959). Floral characters, coupled with differences identified among seed traits and ecophysiological behavior for *H. lahue* ploidies, suggest that a further survey that expands diploid *H.* aff. *lahue* sampling must be done.

Concluding, we identified several inter- and intraspecific differences regarding seed traits and germination requirements for closely related species of Tigridieae. Taken together, trade-offs between seed traits and germination requirements confirmed our hypothesis, since species/cytotypes with broader distributions presented larger and heavier seeds and better general germinative performance under broader temperature and light conditions than their relatives with narrower distributions, which seem to have more specific germination requirements. We hope our findings will encourage further investigations to better understand the reproductive ecology of South Brazilian grassland species, which is required knowledge for the elaboration of effective management plans and restoration strategies to promote conservation of the Pampa biome.

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