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**Caracterização ecofisiológica de três espécies da tribo
Tigridieae (Iridaceae, Iridoideae)**

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*Dedico este trabalho à minha família e minha esposa
por todo o companheirismo e apoio.*

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RESUMO

A ecologia reprodutiva das plantas está intimamente relacionada com suas adaptações locais a diferentes condições de habitat. Investimentos na produção de sementes viáveis e em mecanismos adaptativos para promover a germinação são essenciais para garantir o sucesso reprodutivo e apresentam requerimentos ecofisiológicos específicos, cruciais para a compreensão do histórico evolutivo associado às adaptações das espécies aos seus ambientes. Apesar da importância da investigação de tais aspectos, pouco se conhece sobre características ecofisiológicas para as espécies da tribo Tigridaeae (Iridaceae, Iridoideae) e como elas possivelmente interferem na manutenção e sobrevivência de seus representantes. Assim, realizamos um estudo sobre aspectos relacionados à biologia reprodutiva de três espécies pertencentes à tribo Tigridaeae com registro de ocorrência para o Rio Grande do Sul: *Herbertia lahue*, que apresenta ampla distribuição geográfica e grande diversidade genética, fenotípica e citotípica; *H. pulchella* e *Kelissa brasiliensis*, com distribuições restritas ao domínio fitogeográfico do Bioma Pampa e na Depressão Central, sendo esta última exclusiva dos campos limpos desta região. Para avaliar suas performances reprodutivas, foram realizadas estimativas de produção e peso de sementes, análises morfológicas, testes de viabilidade e experimentos de germinação sob diferentes condições de armazenamento, temperatura e fotoperíodo. Identificamos várias diferenças significativas entre as espécies quanto às características das sementes analisadas. Nossos resultados sugerem que as sementes de Tigridaeae testadas possuem dormência fisiológica, podendo ser superada através do armazenamento a seco estendido ou estratificação alternada expondo-as primeiramente a condições quentes de temperatura. Além disso, os testes de germinação mostraram que as sementes se beneficiam da fotoinibição como uma estratégia para evitar condições ambientais instáveis na superfície do solo. Em conjunto, “trade-offs” entre produção de sementes, dimensões, biomassa e exigências de germinação, foram interpretados como adaptações explicativas influenciando as espécies na ocupação de seus habitats e, portanto, em sua distribuição natural atual. Por fim, o presente estudo proporcionou um incremento quanto ao conhecimento da ecologia reprodutiva e sucesso de estabelecimento de espécies campestres nativas do bioma Pampa e poderá auxiliar na elaboração de futuros planos de manejo e estratégias de restauração eficazes que promovam a conservação de sua flora.

Palavras-chave: Performance reprodutiva, Germinação, Dormência, Fotoinibição, South Brazilian grasslands, Iridaceae

ABSTRACT

The reproductive ecology of plants is closely related to their local adaptations to different habitat conditions. Investments in viable seed production and in adaptive mechanisms to promote germination are essential to ensure reproductive success, which present specific ecophysiological requirements, crucial to understand the evolutionary history associated with species adaptations to their environments. Little is known about ecophysiological traits for Tigridaeae (Iridaceae, Iridoideae) species and how they possibly interfere in the maintenance and survival of their representatives. We conducted a study on aspects related to the reproductive biology of three species which belong to the Tigridaeae tribe with occurrence record for Rio Grande do Sul: *Herbertia lahue*, which presents a wide geographic distribution and great genetic, phenotypic and cytotypic diversity; *H. pulchella* and *Kelissa brasiliensis*, with distributions restricted to the phytogeographical domain of the Pampa biome and Depressão Central, where the latter is exclusive of open grasslands. To evaluate reproductive performance of these species, seed production and mass estimates, morphology analyzes, viability tests and germination experiments were performed under different storage, temperature and photoperiod conditions. We identified several significant differences among species in relation to the analyzed seed traits. Our results suggest that the seeds of Tigridaeae tested have physiological dormancy which can be overcome through extended dry storage or alternate stratification after primarily exposition to higher temperature conditions. In addition, germination tests showed that seeds benefit from photoinhibition as a strategy to avoid unstable environmental conditions on the soil surface. Altogether, trade-offs between seed production, size, biomass and germination requirements were interpreted as explanatory adaptations that influence species habitat occupation, therefore, their current natural distribution. Finally, a better understanding of the reproductive ecology and success in establishment of native species of the Pampa biome can help in the elaboration of future management plans and effective restoration strategies that can promote the conservation of its flora.

Keywords: Reproductive performance, Germination, Seed dormancy, Photoinhibition, South Brazilian grasslands, Iridaceae

CAPÍTULO I

Introdução Geral

1. INTRODUÇÃO

1.1 ECOLOGIA REPRODUTIVA, EVOLUÇÃO E CONSERVAÇÃO DE ESPÉCIES DE PLANTAS

A ecologia reprodutiva em plantas abrange todos os aspectos dos eventos reprodutivos e suas interações com componentes bióticos e abióticos do meio ambiente. As plantas diferem em suas estratégias reprodutivas visando maximizar seu sucesso reprodutivo e diversidade genética (SHIVANNA; TANDON, 2014). A propagação através de sementes e propágulos vegetativos (reprodução assexuada/vegetativa) são duas das principais estratégias reprodutivas utilizadas de forma combinada por plantas com flores para maximizar sua performance (JACQUEMYN et al., 2011; SHIVANNA; TANDON, 2014). Apesar da propagação vegetativa facilitar a colonização e dominância local de populações em nichos aos quais estão bem adaptados, ela limita sua variabilidade genética devido à falta de troca gênica. Essa estratégia assexuada de reprodução pode limitar a habilidade competitiva das plantas de se adaptarem a novos nichos e assim, restringir suas populações a habitats especializados, que a longo prazo podem inviabilizar sua sobrevivência devido a modificações em seus ambientes naturais (SHIVANNA; TANDON, 2014; VALLEJO-MARÍN; DORKEN; BARRETT, 2010). Já a reprodução sexuada é o único método que permite a recombinação gênica (a partir da polinização e fertilização), resultando em variações herdáveis que subsidiam a evolução das espécies, apesar de serem conhecidos consequentes efeitos de processos como a endogamia, que por muitas gerações pode levar à perda de variabilidade genética, ao efeito de depressão por endogamia, a uma progênie com *fitness* e vigor reduzido e a consequente perda do potencial evolucionário de lidar com alterações de hábitat (RICHARDS, 1997; SHIVANNA; TANDON, 2014). Conhecimento sobre estratégias reprodutivas básicas torna-se crucial para o adequado planejamento de atividades de conservação de espécies (ROSSI et al., 2015).

A reprodução sexuada em plantas com flores envolve amplamente uma série de eventos sequenciais: o desenvolvimento das flores, a produção de gametas funcionais, a transferência dos grãos de pólen da antera ao estigma receptivo (polinização), a germinação dos grãos de pólen para o crescimento dos tubos polínicos que possibilitam a fertilização, o

desenvolvimento de sementes e frutos e, por fim, a dispersão e germinação das sementes que levam ao estabelecimento de descendentes viáveis (ASHMAN et al., 2004; SHIVANNA; TANDON, 2014). Qualquer interrupção provocada nestes eventos resultam na finalização precoce do processo de reprodução.

A persistência de populações de plantas está intimamente ligada ao seu tempo de geração, de modo que estudos sobre sua história de vida geralmente apresentam uma abordagem integrada unindo demografia, biologia reprodutiva e genética para avaliar a persistência de populações de plantas em ecossistemas (OHARA et al., 2006). Apesar dessas contribuições fornecerem informações importantes sobre a distribuição, o habitat e a sistemática das espécies, muitos aspectos ecológicos, incluindo o comportamento reprodutivo, ainda são desconhecidos (ASTUTI et al., 2018; MURRAY et al., 2002). Conhecimento dos detalhes sobre a ecologia reprodutiva das espécies torna-se vital para sua gestão, conservação e utilização sustentável (SHIVANNA; TANDON, 2014). O sucesso na restauração de populações de espécies é maior quando bem estudadas, particularmente no que tange a ecologia reprodutiva (WASER, 2006). Na ausência destes dados, esforços de conservação podem permanecer ineficazes. Aspectos da ecologia reprodutiva das plantas devem ser estudados interdisciplinarmente integrando técnicas aplicadas em diversas áreas da botânica como a ecologia, genética, fisiologia, biologia molecular e biologia da conservação a fim de preencher cada lacuna de conhecimento (ASTUTI et al., 2018; SHIVANNA; TANDON, 2014).

Todas as regiões do planeta fornecem exemplos de restrições ambientais e históricas nos processos de evolução das plantas à medida que as populações se adaptam, sobrevivem e diversificam (ARROYO; THOMPSON, 2018; VAN ANDEL; ARONSON, 2012). Extensos dados estão disponíveis sobre a ecologia reprodutiva de espécies que ocorrem em regiões temperadas (KIEHL et al., 2010; LADOUCEUR et al., 2018; SHIVANNA; TANDON, 2014; VAN ANDEL; ARONSON, 2012). Algumas espécies representam objetos de estudo de especial importância para a ciência da conservação por serem típicas de habitats ameaçados, portanto, fornecendo dados indicativos de como mudanças de habitat podem ter efeitos em escala de comunidade (ARROYO; THOMPSON, 2018). Da mesma forma, faz-se necessário iniciar estudos em larga escala nos trópicos com o objetivo principal de coletar dados básicos sobre a ecologia reprodutiva de espécies e comunidades de plantas, inestimáveis para o gerenciamento eficaz e a conservação de recursos vegetais (KRICHEN; BEN MARIEM;

CHAIEB, 2014; NEVILL et al., 2016; SHIVANNA; TANDON, 2014). Existe muito interesse em compreender como os processos relacionados à ecologia reprodutiva das plantas promovem divergência e contribuem para a persistência de populações e suas linhagens em regiões sob o efeito de mudanças climáticas graduais (ARROYO; THOMPSON, 2018; DAYRELL et al., 2017; WILLIS et al., 2014). Dados sobre ecologia reprodutiva das espécies também são necessários para monitorar o sucesso de qualquer esforço de conservação, possuindo implicações diretas para a ciência da conservação (ARROYO; THOMPSON, 2018; NEVILL et al., 2016; SHIVANNA; TANDON, 2014) em busca de seu aprimoramento.

1.2 CONSERVAÇÃO E RESTAURAÇÃO DO BIOMA PAMPA

Os ecossistemas campestres estão entre os que possuem maior riqueza específica no mundo (WILSON et al., 2012) e providenciam uma grande variedade de serviços ecológicos, como o sequestro de carbono, controle de erosão, infiltração de água no solo e a produção forrageira (PILLAR; TORNQUIST; BAYER, 2012). Quatro dos seis biomas brasileiros terrestres reconhecidos, possuem uma maior predominância de ecossistemas não-florestais (IBGE, 2004; OVERBECK et al., 2007). Entre eles está o Bioma Pampa, que apesar de pouco conhecido internacionalmente, está entre os ambientes campestres mais ricos em espécies do mundo (ANDRADE et al., 2015; OVERBECK et al., 2007). Situado no estado mais ao Sul do Brasil, o Rio Grande do Sul (RS), o Bioma Pampa integra a maior ecorregião campestre contínua das Américas (compartilhada com o Uruguai e a Argentina), denominada de região campestre de Río de la Plata, cuja riqueza específica de sua flora em território brasileiro foi estimada em 3.530 spp., das quais 278 spp. são endêmicas (ANDRADE et al., 2018). Estes campos têm sido a principal fonte de forrageio para a pecuária no RS, sendo uma de suas principais atividades econômicas (DE PATTA PILLAR, 2009). Apesar de sua riqueza e de sua extensão original ter representado cerca de 63% da área do RS, atualmente o Bioma Pampa permanece com menos de 31% de sua cobertura vegetal nativa e possui a menor proporção de território abrangido por Unidades de Conservação (UC) a nível nacional (BRENTANO; FOLLMANN; FOLETO, 2015), com apenas 0,3% de sua área sob proteção integral e 2,4% em áreas de uso extensivo, totalizando 2,7% de sua extensão total (ICMBIO, 2016). A conversão de suas áreas campestres em campos aráveis para produção agrícola e

plantio de espécies exóticas de árvores para extração da madeira têm sido apontadas como as principais causas da perda e fragmentação dos campos nativos (ANDRADE et al., 2015; OVERBECK et al., 2007). Por outro lado, o manejo desses ambientes campestres através de distúrbios como o pastejo e o uso do fogo são reconhecidos como vitais para manter sua diversidade, serviços ecossistêmicos e conservação, devido à dinâmica sucessional de expansão natural das florestas, quando em clima favorável, sobre o campo (OVERBECK et al., 2013; PILLAR; TORNQUIST; BAYER, 2012). Concentrar esforços na restauração da vegetação não florestal do Bioma Pampa pode ajudar a reduzir e, eventualmente, impedir a contínua perda da biodiversidade campestre e suas funções ambientais (OVERBECK et al., 2013). Para que estes objetivos sejam alcançados com sucesso, reunir um acervo de técnicas de manejo que reconheçam as funções e necessidades essenciais da biodiversidade campestre, promovam sua efetiva restauração, incentivem sua conservação através do manejo adequado e possibilitem o desenvolvimento de atividades econômicas sustentáveis, são medidas imprescindíveis para a longevidade do Bioma Pampa (ARONSON; VAN ANDEL, 2012; BULLOCK et al., 2011; DE FREITAS; DE OLIVEIRA; DE OLIVEIRA, 2019; OVERBECK et al., 2013; PILLAR; TORNQUIST; BAYER, 2012).

Existem muitos exemplos de restauração bem sucedida em ecossistemas campestres desenvolvidos em outros países, sob uma ampla gama de condições climáticas, diferentes regimes de manejo e processos de degradação (ARONSON; VAN ANDEL, 2012; CONRAD; TISCHEW, 2011; KIEHL et al., 2010; MULLER et al., 1998; RUPRECHT, 2006; STROMBERG et al., 2007; TÖRÖK et al., 2011). Porém, estudos que evidenciem experiências em restauração com campos sulinos no RS ainda são muito escassos (THOMAS et al., 2018), pois muitas das abordagens desenvolvidas para outros tipos de vegetação campestre necessitam de adaptações, já que as características abióticas e bióticas assim como a dinâmica sucessional entre os campos e florestas no RS são bastante singulares (OVERBECK et al., 2013, 2018). Além disso, outros fatores que vem restringindo o avanço desses estudos são: o escasso conhecimento sobre a ecologia reprodutiva e requerimentos específicos das espécies nativas dos campos sulinos (THOMAS et al., 2018), e a falta de sementes nativas regionais comercialmente disponíveis (OVERBECK et al., 2013). Para que a produção de sementes nativas seja viável e áreas de produção de sementes sejam criadas no RS, mostra-se necessário compreender melhor os processos ecossistêmicos relacionados com a ecologia reprodutiva e ecofisiologia das espécies (MATTHEW; PEREIRA, 2017; NEVILL

et al., 2016; SHIVANNA; TANDON, 2014). Realizar estudos sobre produção, viabilidade, morfologia e germinação polínica, para compreensão do sucesso reprodutivo das plantas (MAITI; GONZALEZ-RODRIGUEZ; OJHA, 2016), assim como estudos sobre estes mesmos aspectos para sementes, que contemplem a caracterização de dormência e dos requerimentos ecofisiológicos específicos para que a germinação ocorra (AIRES; SATO; MIRANDA, 2013; LE STRADIC et al., 2015; SAATKAMP et al., 2018) em espécies nativas do Bioma Pampa. A obtenção de conhecimento suficiente sobre as sementes campestres nativas para que a produção, o armazenamento, a dispersão e a manutenção de sua viabilidade no banco de sementes do solo até que as condições ambientais necessárias para a germinação e estabelecimento de plântulas estejam adequadas viabiliza um recrutamento bem sucedido (SHIVANNA; TANDON, 2014) e, conseqüentemente a restauração de campos degradados.

Neste âmbito, a família Iridaceae mostra-se promissora, pois é a família botânica com maior número de representantes exclusivos do Bioma Pampa (ZAPPI et al., 2015). De acordo com o recente levantamento realizado por Ladouceur *et al.* (2018), que identificou a proporção de espécies nativas por família botânica com sementes comercialmente disponíveis no continente europeu, a família Iridaceae está entre as dez famílias taxonômicas com menor disponibilidade. Até o presente momento, não foi realizado nenhum levantamento equivalente que contemple espécies nativas brasileiras comercialmente disponíveis, e o único registro similar que existe atualmente contempla apenas quatro gêneros de espécies exóticas de Iridaceae (*Crocus* L., *Freesia* Klatt, *Gladiolus* L. e *Iris* L.) (BRASIL, 2009).

1.3 A FAMÍLIA IRIDACEAE E A TRIBO TIGRIDIEAE

A família Iridaceae é um grupo de plantas herbáceas perenes que inclui espécies de grande valor econômico (RODRIGUEZ; SYTSMA, 2006). Dentre elas, destacam-se *Crocus sativus* L., o açafrão, por sua importância econômica na indústria alimentícia como aromatizante culinário (MATHEW, 1977) e espécies dos gêneros *Iris*, *Gladiolus* e *Freesia* pelo potencial ornamental de suas flores (GOLDBLATT; MANNING; RUDALL, 1998).

Estima-se que esta família apresenta aproximadamente 2.030 espécies de plantas, divididas entre 65 e 75 gêneros (GOLDBLATT et al., 2008; STEVENS, 2001 onwards).

Pertencente à ordem Asparagales, é reconhecida como uma das maiores famílias de monocotiledôneas, que difere das demais pelas flores de seus representantes possuírem três estames no androceu (GOLDBLATT; MANNING, 2008), e perianto petaloide com três tépalas internas e três externas geralmente similares em textura, formato e coloração (SOUZA-CHIES et al., 2012). A família apresenta distribuição global, concentrando-se no Hemisfério Sul, com seus dois maiores centros de diversidade, respectivamente, no Sul da África e região neotropical (GOLDBLATT, 1982; GOLDBLATT; MANNING, 2008).

A partir das análises filogenéticas de cinco regiões do DNA plastidial realizada por Goldblatt *et al.* (2008), a família Iridaceae foi subdividida em sete subfamílias: Isophysidoideae, Patersonioideae, Geosiridoideae, Aristeoideae, Nivenioideae, Crocoideae e Iridoideae, sendo esta última a única com representantes na América do Sul. A subfamília Iridoideae, definida morfológicamente por suas flores com tépalas fendidas, estiletos ramificados e ausência de nectários septais, compreende ao menos 900 espécies de plantas, o que representa cerca de 40% da riqueza específica em Iridaceae (GOLDBLATT et al., 2008). Por sua vez, Iridoideae se subdivide em cinco tribos: Diplarreneae, Irideae, Sisyrinchieae, Trimezieae e Tigridieae.

No Brasil, são encontradas aproximadamente 200 espécies de Iridaceae pertencentes às tribos Sisyrinchieae, Trimezia e Tigridieae (FLORA DO BRASIL, 2020 em construção), que compartilham características florais marcantes, como a presença de glândulas produtoras de óleos em suas tépalas ou coluna estaminal (CHAUVEAU et al., 2012). Na região sul do país, Sisyrinchieae e Tigridieae são as tribos com maior riqueza específica, cujos representantes em Sisyrinchieae são caracterizados pela presença de rizomas ou sistemas radiculares fibrosos e folhas planas ou teretes, enquanto, em Tigridieae, pela presença de bulbos e folhas plicadas (SOUZA-CHIES et al., 2012).

1.3.1 Tribo Tigridieae

A tribo Tigridieae compreende vários gêneros de plantas com características vegetativas muito similares, sendo possível diferenciá-los apenas durante o período de floração (RODRIGUEZ; SYTSMA, 2006). Verifica-se também uma ampla variabilidade

morfológica nas flores de uma mesma espécie, o que dificulta a circunscrição taxonômica das mesmas (SOUZA-CHIES et al., 2012).

Em abordagem filogenética recente, baseada em sete marcadores moleculares obtidos de 97 espécies que contemplam a maior parte dos gêneros das tribos da subfamília Iridoideae do Novo Mundo, Chauveau *et al.* (2012) reconheceram a divisão da tribo Tigridieae em dois clados bem suportados, denominados A e B. Dos dez gêneros pertencentes atualmente ao Clado A da tribo Tigridieae, seis possuem representantes com registro de ocorrência nas paisagens naturais do Rio Grande Sul: *Cypella* Herb. (17 spp.), *Calydorea* Herb. (12 spp.), *Herbertia* Sweet (8 spp.), *Onira* Ravenna (1 spp.), *Catila* Ravenna (1 spp.) e *Kelissa* Ravenna (1 spp.) (FLORA DO BRASIL, 2020 em construção). Sugere-se que delimitações alternativas sejam propostas para alguns dos gêneros dentro destes clados por terem se revelado não monofiléticos, como *Cypella* e *Calydorea*, ou por estarem incluídos dentro destas linhagens, como as espécies do clado que compreende *Kelissa*, *Herbertia* e *Onira* (CHAUVEAU et al., 2012). Buscando compreender melhor os processos evolutivos relacionados com a diversificação das espécies destes gêneros, estudos com análises de dados citogenéticos, tamanho genômico e genética de populações vêm sendo realizados (MORAES et al., 2015; STIEHL-ALVES et al., 2016, 2017). Neste contexto, foram selecionadas espécies dos gêneros *Kelissa* e *Herbertia* para este estudo que busca a caracterização ecofisiológica devido à proximidade evolutiva das espécies escolhidas para estes gêneros e suas distintas características de distribuição geográfica, morfológicas e citogenéticas.

1.3.1.1 Gênero *Kelissa* Ravenna

Com base em análises de morfologia floral, Ravenna (1981) modificou a classificação de *Herbertia brasiliensis* Barker, criou o gênero monotípico *Kelissa* e descreveu sua única espécie *Kelissa brasiliensis* (Barker) Ravenna. Esta planta pode ser reconhecida por suas flores com tépalas livres (sendo as externas maiores que as internas) de cor violeta pálido, cuja extensão da lâmina possui máculas púrpuras com tricomas esparsos na base, áreas laterais amarelas, área glandular longitudinal densa e esbranquiçada (elaióforos), coluna estaminal cilíndrica e inteira, anteras lineares eretas e gineceu com estilete filiforme fino

(FLORA DO BRASIL, 2020 em construção; GOLDBLATT; MANNING, 2008; GOLDBLATT; MANNING; RUDALL, 1998). Sua principal região de ocorrência são os campos limpos da Serra do Sudeste, sendo considerada endêmica destes ambientes no RS (FLORA DO BRASIL, 2020 em construção).

Apesar de seu potencial ornamental (Barroso, 2006) e elevado grau de ameaça (vulnerável) (LIVE, 2014), *Kelissa brasiliensis* é uma espécie pouco estudada, sendo nosso conhecimento acerca desta espécie bastante limitado (CHAUVEAU et al., 2012; MORAES et al., 2015).

Barroso (2006) avaliou a qualidade fisiológica de sementes e estratégias reprodutivas de *Kelissa brasiliensis*, determinando que 10 °C seria a temperatura ótima para germinação de suas sementes (que iniciam o processo entre o vigésimo e sexagésimo dia de incubação em substrato de papel na presença de luz), sendo que temperaturas superiores a 20 °C estimulam a dormência. O padrão de distribuição de suas populações foi interpretado como gregário devido à capacidade de dispersão limitada de suas sementes (BARROSO, 2006). Chauveau *et al.* (2012) em sua abordagem filogenética para a subfamília Iridoideae, demonstraram que *Kelissa brasiliensis* forma um clado bem suportado junto com *Cypella hauthalli* (Kuntze) R.C. Foster e *Onira unguiculata* (Baker) Ravenna, representando um clado próximo aos demais representantes dos gêneros *Cypella* e *Herbertia*. No RS, foram encontrados apenas indivíduos diploides até o momento (MORAES et al., 2015).

1.3.1.2 Gênero *Herbertia* Sweet

O gênero *Herbertia* tem se mostrado um interessante objeto de estudo devido à problemática relacionada com a delimitação de suas espécies, que exibem pouca distinção vegetativa, alta variabilidade morfológica floral (inter e intraespecífica), diferenças de comportamento reprodutivo (autocompatível e auto-incompatível), diferentes citótipos para algumas de suas espécies e variações genéticas significativas entre populações (SOUZA-CHIES et al., 2012; STIEHL-ALVES et al., 2016, 2017). As flores de *Herbertia* apresentam tépalas desiguais arranjadas em dois verticilos (externo maior e interno menor), androceu com coluna estaminal formada pela união de três filamentos que culminam em

antras divergentes, e gineceu com três ramos bifurcados nas extremidades, onde se encontram apicalmente as regiões estigmáticas (GOLDBLATT; MANNING, 2008; STIEHL-ALVES et al., 2016). As duas espécies mais comuns são *Herbertia lahue* (Molina) Goldblatt e *H. pulchella* Sweet, que podem ser prontamente distinguidas pela presença de uma listra branca longitudinal nas tépalas do verticilo externo de *H. pulchella* (SOUZA-CHIES et al., 2012).

Quanto à distribuição destas espécies, *H. lahue* possui maior amplitude de ocorrência (sul do Brasil, Uruguai, Norte da Argentina e sul do Chile), sendo encontrada praticamente em todas as regiões do RS, muitas vezes em simpatria com *H. pulchella*, que se restringe ao Pampa brasileiro (SOUZA-CHIES et al., 2012; STIEHL-ALVES et al., 2016, 2017). Séries poliploides são particularmente comuns em ambas as espécies - *H. pulchella* ($2x$, $4x$ e $6x$, sendo indivíduos $4x$ encontrados no RS) e *H. lahue* ($2x$, $4x$, $6x$ e $8x$, sendo indivíduos $6x$ e $8x$ encontrados no RS). No caso dos citótipos hexaploides e octoploides, estes apresentam valores de tamanho genômico monoploide menores que os diploides, possivelmente originados a partir de um fenômeno de redução após poliploidia (MORAES et al., 2015). De acordo com Stiehl-Alves et al. (2016), variações morfológicas sutis entre os citótipos de *H. lahue* são detectáveis, onde indivíduos hexaploides possuem tépalas internas lanceoladas e as tépalas externas, frequentemente com constrição na base, e ápice uncinado. Já as octoploides possuem as tépalas dos dois verticilos obovadas com constrição basal ou ápice uncinado ausentes (STIEHL-ALVES et al., 2016). Diferenças de morfologia entre os citótipos de *H. pulchella* até então não foram reconhecidas.

Experimentos de viabilidade polínica indicam que *H. lahue* e *H. pulchella* produzem pólen viável (> 90%) (MORAES et al., 2015), porém os citótipos de *H. lahue* são autocompatíveis e os de *H. pulchella* são auto-incompatíveis (STIEHL-ALVES et al., 2016, 2017). Através da identificação de polimorfismos entre regiões de microssatélites com o uso de marcadores moleculares *Inter Simple Sequence Repeats* (ISSR) e *Analysis of Molecular Variance* (AMOVA), detectou-se que a maior fonte de variação genética em *H. lahue* reside entre suas populações e quando analisadas isoladamente as populações hexaploides exibem menor variação genética do que as octoploides (STIEHL-ALVES et al., 2017). Este comportamento autogâmico, somado à distância geográfica e à capacidade de dispersão de sementes limitada, possivelmente contribuem para o isolamento reprodutivo entre populações e consequente restrição de fluxo gênico (STIEHL-ALVES et al., 2016). Em abordagem

semelhante empregando marcadores ISSR para estimar a variação genética em populações de três espécies de *Herbertia*, Stiehl-Alves *et al.* (2017) detectaram três grupos genéticos estatisticamente divergentes (AMOVA, $P = 0.001$), sendo um deles, próprio de *H. pulchella*. Apesar das análises moleculares distinguirem grupos genéticos tanto para *H. lahue* como *H. pulchella*, não têm sido possível definir diferenças morfológicas claras entre estes grupos genéticos para cada espécie através de análises morfológicas quantitativa para caracteres florais (STIEHL-ALVES *et al.*, 2016, 2017). No caso de *Herbertia lahue*, para a qual são conhecidos diferentes citótipos, destaca-se um grande polimorfismo floral intraespecífico (STIEHL-ALVES *et al.*, 2016, 2017). Devido a este polimorfismo floral intraespecífico, a identificação de novas características (ex. morfológicas, anatômicas, fisiológicas, etc) que sirvam de diagnóstico para delimitar estas linhagens genéticas distintas são necessárias.

1.4 CARACTERES ECOFISIOLÓGICOS E A INFLUÊNCIA DO FITNESS NA EVOLUÇÃO ADAPTATIVA DE LINHAGENS DE PLANTAS

Caracteres ecofisiológicos em plantas são considerados como todos os aspectos de sua fisiologia relacionados com a captação e utilização de recursos (nutrição, metabolismo, alocação de biomassa, etc) que costumam influenciar seu *fitness*, adaptando as espécies às circunstâncias locais dos ambientes em que vivem (ACKERLY *et al.*, 2000). Por ser muito ambíguo e com uma série de definições, o conceito de *fitness* já foi interpretado de diversas maneiras (BROWN; MARQUET; TAPER, 1993; HECHT; MACINTYRE; CLEGG, 1998; WIKBERG, 1995). Resumidamente, o termo *fitness* pode ser entendido como uma medida de curto prazo do sucesso reprodutivo das espécies, levando em consideração aspectos relativos à genética populacional (DE JONG, 1994; HANSEN, 2018). De acordo com Younginger *et al.* (2017), o termo *fitness relativo* vem sendo utilizado atualmente para referir-se ao sucesso reprodutivo de um genótipo particular comparado a todos os genótipos de uma população. O sucesso reprodutivo de uma espécie em seu habitat pode ser avaliado através de seu desempenho levando em consideração sua habilidade de se desenvolver, reproduzir e sobreviver (JACQUEMYN *et al.*, 2011; VIOLLE *et al.*, 2007). Diversos caracteres ecofisiológicos são utilizados como preditores de desempenho (VIOLLE *et al.*, 2007; YOUNGINGER *et al.*, 2017). Portanto, conjuntos de caracteres que permitam avaliar

investimentos realizados pelas espécies em prol de seu desempenho reprodutivo (produção, massa, morfologia, germinabilidade de sementes) têm sido reconhecidas como importantes medidas de *fitness* relativo (ACKERLY et al., 2000; SHIVANNA; TANDON, 2014; YOUNGINGER et al., 2017). Atualmente muitos deles vêm sendo resgatados de estudos sobre pólen e sementes (DELPH; JOHANNSSON; STEPHENSON, 1997; JOHNSTON; BARTKOWSKA, 2017; PÉLABON et al., 2015; WILLIAMS; MAZER, 2016) para serem utilizados em abordagens filogenéticas, devido ao potencial que possuem para esclarecer melhor o histórico evolutivo de linhagens de espécies (VANDELOOK; NEWTON; CARTA, 2018). Um pressuposto central da ecologia baseada em caracteres é que a morfologia determina o desempenho fisiológico, influenciando taxas vitais (sobrevivência e fecundidade) na manutenção de indivíduos em seus ambientes e conseqüentemente na evolução da história de vida das espécies (ACKERLY; MONSON, 2003; ADLER et al., 2014; VIOLLE et al., 2007). Novas abordagens vêm buscando evidências quanto à evolução adaptativa de caracteres ecofisiológicos em populações naturais associadas à diferenciação de nichos ecológicos para corroborar divergências entre espécies (ACKERLY et al., 2000; CARTA et al., 2014). Estudos recentes com representantes da família Iridaceae demonstram divergências evolutivas entre espécies filogeneticamente próximas com relação a caracteres ecofisiológicos (germinação, dormência e crescimento embrionário de sementes) em resposta a diferenças de fotoperíodo, temperatura e distribuição geográfica (CARTA et al., 2014; CARTA; HANSON; MÜLLER, 2016; FORGIARINI et al., 2017).

Diante do cenário evolutivo complexo dos representantes da tribo Tigridieae (CHAUVEAU et al., 2012; MORAES et al., 2015; STIEHL-ALVES et al., 2016, 2017) e sabendo que o sistema reprodutivo desempenha um papel importante na configuração das variações entre suas linhagens genéticas (STIEHL-ALVES et al., 2017), a detecção de características diagnósticas ligadas ao sucesso reprodutivo podem ser de grande valia para uma melhor compreensão do histórico evolutivo de suas espécies e como possivelmente influenciaram sua adaptabilidade na conquista de ambientes naturais. Considerando os avanços com foco na análise do valor adaptativo de caracteres ecofisiológicos somados a variações morfológicas e genéticas de suas populações, uma abordagem integrativa que estime os investimentos reprodutivos das espécies *Herbertia lahue*, *H. pulchella* e *K. brasiliensis* pode auxiliar a contextualizar impactos evolutivos em linhagens de espécies intimamente relacionadas da tribo Tigridieae.

2. OBJETIVO GERAL

Esta dissertação tem por objetivo caracterizar três espécies da tribo Tigridaeae, sendo duas pertencentes ao gênero *Herbertia* (*H. lahue* e *H. pulchella*) e uma ao gênero *Kelissa* (*K. brasiliensis*), todas ocorrentes no Rio Grande do Sul, quanto a aspectos ecofisiológicos e morfológicos importantes para seu desempenho reprodutivo.

2.1 OBJETIVOS ESPECÍFICOS

- a) Estimar o investimento em produção de sementes das espécies *Herbertia lahue*, *H. pulchella* e *Kelissa brasiliensis*.
- b) Avaliar variações morfológicas das sementes das espécies *Herbertia lahue*, *H. pulchella* e *Kelissa brasiliensis*.
- c) Verificar a existência de dormência nas sementes das espécies *Herbertia lahue*, *H. pulchella* e *Kelissa brasiliensis*.
- d) Avaliar a germinação das sementes das espécies *Herbertia lahue*, *H. pulchella* e *Kelissa brasiliensis* após intervalos distintos de armazenamento e sob diferentes condições de temperatura e fotoperíodo.
- e) Determinar o percentual de sementes viáveis provenientes das espécies *Herbertia lahue*, *H. pulchella* e *Kelissa brasiliensis*.

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CAPÍTULO II

Original research to be submitted to Flora, drafted according to norms of the journal.

How do ecophysiological differences contribute to grassland species habitat adaptation? A case study with three species of the tribe Tigridieae (Iridaceae) from South Brazilian grasslands

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Abstract

The reproductive ecology of plants is related to their local adaptations to different habitat conditions. Our study goal was the ecophysiological characterization of native South Brazilian grassland species to clarify how seed traits possibly interfere in reproductive performance and habitat adaptation. We selected three close related species from Tigridieae (Iridaceae, Iridoideae): *Herbertia lahue*, which presents a wide geographic distribution and great genetic, phenotypic and cytotypic diversity; *H. pulchella* and *Kelissa brasiliensis*, with distributions restricted to the phytogeographical domain of the Pampa biome, were the latter is exclusive of open grasslands. Species reproductive performance was evaluated through: seed production and mass estimates, morphology analyzes, viability tests and germination experiments. Our results suggest that the sampled seeds have physiological dormancy which can be overcome through extended dry storage or alternate stratification primarily exposing them to higher temperature. Germination tests shown that seeds benefit from photoinhibition, interpreted as an adaptive mechanism to avoid seedling emergence during unstable environmental conditions. Altogether, trade-offs between seed production, size, biomass and germination requirements were interpreted as explanatory adaptations influencing species *fitness* in the occupation of their habitats, therefore, their current natural distribution. Understanding the reproductive ecology and ecophysiological aspects of native grassland species can subsidize knowledge for further conservation projects.

Keywords

Reproductive performance, Germination, Seed dormancy, Photoinhibition, South Brazilian grasslands, Iridaceae

1. Introduction

Ecophysiological traits in plants are considered as all aspects of their physiology related to the uptake and utilization of resources (e.g. nutrition, metabolism, biomass allocation, etc.) that usually influence their *fitness*, adapting species to the local circumstances of the environments where they live (Ackerly et al., 2000). The term *fitness* can be understood as a short-term measure of reproductive success linked to population-genetics dynamics (De Jong, 1994; Hansen, 2018). The reproductive success of a species in its habitat can be assessed through its performance taking into account its ability to develop, reproduce and survive (Jacquemyn et al., 2011; Violle et al., 2007).

Several ecophysiological traits can be used as performance predictors (Violle et al., 2007). Therefore, trait sets that allow the evaluation of species investments in favor of their reproductive performance (e.g. production, fertility and germinability of pollen and viable seeds) have been recognized as important *relative fitness* measurements (Ackerly et al., 2000; Shivanna and Tandon, 2014; Younginger et al., 2017). According to Younginger *et al.* (2017), the term *relative fitness* has been recently used to refer to the reproductive success of a particular genotype compared to all genotypes in a population. Currently, many of those traits have been revisited from pollen and seed studies to be used in phylogenetic approaches, due to their potential to clarify the evolutionary history of species lineages (Delph et al., 1997; Johnston and Bartkowska, 2017; Pélabon et al., 2015; Vandeloos et al., 2018; Williams and Mazer, 2016). A central assumption of trait-based ecology is that morphology determines physiological performance, influencing vital rates (e.g. survival and fecundity) in determining individual *fitness* and evolution of the species life history (Ackerly and Monson, 2003; Adler et al., 2014; Violle et al., 2007).

New approaches have been seeking evidence regarding the adaptive evolution of ecophysiological traits in natural populations associated to the differentiation of ecological niches to corroborate divergences among species (Ackerly et al., 2000; Carta et al., 2014). Recent studies with representatives of Iridaceae from the Mediterranean Basin demonstrated evolutionary divergences among these phylogenetically close grassland species by examining their ecophysiological traits (e.g. seed germination, dormancy and embryo growth) in response to different photoperiod and temperature conditions, which reflected some species local adaptations to their habitats (Carta et al., 2016, 2014). Unfortunately, basic data on

reproductive ecology for plants of various regions are still unknown (Shivanna and Tandon, 2014), including native grassland species from the South America.

Located in the southernmost state of Brazil, Rio Grande do Sul (RS), the Pampa biome integrates the Río de la Plata ecoregion (shared with Uruguay and Argentina) the largest continuous grassland in the Americas, whose species-richness in the Brazilian territory was estimated at 3.530 spp., of which 278 spp. are endemic (Andrade et al., 2018). Despite being among the most species-rich grassland environments in the world (Andrade et al., 2015; Overbeck et al., 2007), the Brazilian Pampa remains with less than 31% of its original native vegetation cover and has the lowest proportion of territory covered by conservation units in Brazil with only 0.3% of its area under full protection (Brentano et al., 2015; ICMBio, 2016). Iridaceae is the botanical family with the most exclusive representatives of the Pampa biome (Zappi et al., 2015), comprising perennial herbaceous plants, mostly geophytes (Rodríguez and Sytsma, 2006). The family presents a global distribution, concentrating on the Southern Hemisphere, with its two largest centers of diversity, respectively, in South Africa and the neotropical region (Goldblatt, 1982; Goldblatt and Manning, 2008). In Brazil, approximately 200 species of Iridaceae belonging to the tribes Sisyrinchieae, Trimezieae and Tigridieae are found (Flora do Brasil, 2020 under construction). In the south of Brazil, Tigridieae is the second species-rich tribe, whose representatives are characterized by the presence of bulbs and plicate leaves (Souza-Chies et al., 2012).

Tigridieae comprises several genera encompassing species with very similar vegetative characteristics, being possible to differentiate them only during the flowering period (Rodríguez and Sytsma, 2006). There is a wide morphological variability in the flowers within the same species, which hinders their taxonomic circumscription (Souza-Chies et al., 2012). In order to understand the evolutionary processes related to their diversification, cytogenetic, and population genetics studies have been carried out (Moraes et al., 2015; Stiehl-Alves et al., 2017, 2016). However, the knowledge about seed viability and other ecophysiological aspects is scarce. We selected one species from the monotypic genus *Kelissa* Ravenna and two from *Herbertia* Sweet for ecophysiological characterization study due to their evolutionary proximity and distinct, morphologic, cytogenetic and geographic distribution characteristics.

Kelissa brasiliensis (Barker) Ravenna can be recognized by its flowers with free tepals (the external ones being larger than the inner ones) of pale violet color, whose extension of the

blade has purple macules with sparse trichomes in the base, yellow lateral areas, dense and whitish longitudinal glandular area (elaiophores), cylindrical and whole staminal column, erect linear anthers and gynoecium with fine filiform stylet (Flora do Brasil, 2020 under construction; Goldblatt et al., 1998; Goldblatt and Manning, 2008). Its main region of occurrence are the open grasslands of Serra do Sudeste region, being considered endemic of these environments in RS (Flora do Brasil, 2020 under construction). Despite its ornamental potential (Barroso, 2006) and a high vulnerable degree of threat (LIVE, 2014), our knowledge about *K. brasiliensis* is very limited (Chauveau et al., 2012; Moraes et al., 2015). So far, only diploid individuals were found in RS (Moraes et al., 2015).

Herbertia has shown species delimitation problems, 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 variations among populations (Souza-Chies et al., 2012; Stiehl-Alves et al., 2017, 2016). The two most common species are *Herbertia lahue* (Molina) Goldblatt and *H. pulchella* Sweet, which can be readily distinguished by the presence of a longitudinal white stripe on the external whorl tepals of *H. pulchella* (Souza-Chies et al., 2012). In respect to their distribution, *H. lahue* has a wider range of occurrence (southern Brazil, Uruguay, northern Argentina and southern Chile) and can be found in practically all regions of RS, often in sympatry with *H. pulchella*, which is restricted to the Brazilian Pampa (Souza-Chies et al., 2012; Stiehl-Alves et al., 2017, 2016) and more recurrently found in regions with higher altitude, such as hilltops. Polyploid series are particularly common in both *H. pulchella* species ($2x$, $4x$ and $6x$, being $4x$ individuals found in RS) and *H. lahue* ($2x$, $4x$, $6x$ and $8x$, being $6x$ and $8x$ individuals found in RS) (Moraes et al., 2015). According to Stiehl-Alves et al. (2016), subtle morphological variations between *H. lahue* cytotypes are detectable (Stiehl-Alves et al., 2016). Until now, differences in morphology between *H. pulchella* cytotypes have not been recognized. Cytotypes from *H. lahue* are self-compatible, while *H. pulchella* is self-incompatible (Moraes et al., 2015; Stiehl-Alves et al., 2017, 2016). This autogamous behavior, coupled with geographic distance and limited seed dispersal capacity, possibly contribute to interpopulation breeding isolation and consequent restriction of gene flow (Stiehl-Alves et al., 2016). Although molecular analyzes could distinguish genetic groups for both *H. lahue* and *H. pulchella*, it has not been possible to define for *H. lahue* clear morphological differences between these genetic groups through quantitative morphological

analyses for floral characters (Stiehl-Alves et al., 2017, 2016). In view of the complex evolutionary scenario of the representatives of Tigridae and knowing that the reproductive system plays an important role in shaping the variations between genetic lineages (Chauveau et al., 2012; Moraes et al., 2015; Stiehl-Alves et al., 2017, 2016), the detection of diagnostic traits which can be related to reproductive success may be of great value for a better understanding of their evolutionary history and how they possibly influenced their adaptability in the conquest of natural environments.

The main goal from our study was to characterize species from Tigridae regarding seed traits using ecophysiological and morphological aspects related to their reproductive performance intending to clarify their habitat adaptation. Here, we selected *H. lahue*, *H. pulchella* and *K. brasiliensis* to be characterized by: (1) estimating seed production and viability, (2) verifying the existence of seed dormancy, (3) evaluating seed morphological variations and (4) assessing germination response under distinct temperature, photoperiod and storage time conditions.

2. Materials and methods

2.1. Seed collection

Collection sites were selected prioritizing where co-occurrence records of populations from at least two species belonging to *K. brasiliensis*, *H. lahue* and *H. pulchella* (speciesLink, 2002 onwards) were identified. Field expeditions were 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 20 populations found in the municipalities of Porto Alegre, Guaíba and Caçapava do Sul. Due to the recognition of new *H. lahue* cytotypes populations 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. Geographic coordinates and elevation of all sampled sites were recorded with GPS. 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 conditions of temperature and humidity (~25 °C, 20-30 % RH) to avoid changes in seed quality (BRASIL, 2009).

2.2. Flow cytometry

To confirm the ploidy level from 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-Alves *et al.* (2016), assays for ploidy estimation through flow cytometry (FCM) were carried following the protocol proposed by Doležal *et al.* (2007). Fresh young leaf material of target species was obtained from newly developed seedlings resulting from germinated seeds. Cultivated *Solanum lycopersicum* L. ‘Stupické polní rané’ (1.96 pg) was used as internal standard for *H. aff. lahue* (2x) and *Zea mays* L. ‘CE-777’ (5.43 pg) for *H. lahue* (6x and 8x). Cell nuclei were released by chopping target and standard leaves in a glass Petri dish with a sharp blade immersed in 2 ml LB01 buffer solution (Doležal *et al.*, 2007). After filtration of the homogenate through a 50 µm nylon mesh in a glass cytometer tube, nuclei were stained with 25 µL propidium iodide DNA fluorochrome and for some samples we added 5 µL RNase, following the reagent and nuclear suspension preparation using the one-step protocol procedure explained in detail by Doležal *et al.* (2007). Nuclei were analyzed for each sample using a BD FACSAria III cytometer.

2.3. Seed traits

2.3.1. Seed set

After all fruits undergone their natural dehiscence process due to ripen in laboratory, seeds from 30 individualized capsules selected randomly and proportionally from all populations found for each species / cytotype were counted. Remaining seeds were separated in lots for the following activities and replaced in dry storage.

2.3.2. Seed mass

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

2.3.3. Seed morphology

Thirty seeds per species / cytotype were analyzed using an stereomicroscope with digital camera (Leica M165FC) and integrated analysis software (LAS V4.5) to measure their length (μm), width (μm), thickness (μm) and umbilicus area (μm^2), a large pit near the hilum that straddles the raphe (Goldblatt et al., 1990). Seed shape (unitless) was calculated according to data standards from LEDA Traitbase (Kleyer et al., 2008) as the variance of the three dimensions (length, width and thickness), dividing each by the seed length so that length is unity.

2.3.4. 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 sheets of hydrated filter paper (6 ml of distilled water). Plates with hydrated seeds were closed and their ends wrapped with Parafilm (hereafter, incubation), remaining in laboratory at room temperature (~25 °C, 20-30 % RH) for 24 hours. After one day incubation, seeds were removed from the plates, wiped with paper towel and reweighed. The increased percentage 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. An increase of more than 20% in the mass of the seeds was considered sufficient to

characterize them as being permeable to water. If not, these seeds have a physical dormancy and must undergo a scarification process to allow the embryo to have access to water.

2.4. Germination experiments

Four different treatments were performed to evaluate seed germination response under laboratory conditions (Tab. 2). Numbers (0 to 6) presented aside germination experiments acronyms representing the storage time experienced by seed lots in months before incubation.

2.4.1. Dry storage

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

When necessary, a disinfection procedure was implemented before following seed lots incubations to prevent pathogens development during experiments. Disinfection procedure consisted on previously putting seed lots in 2 mL Eppendorf tubes with 2% sodium hypochlorite solution for 15 min and 70% ethanol for 1 min, being washed between and after the application of each solution three times with distilled water. To ensure plates sterilization, seed lots were hydrated for the first time with 0.2% nystatin solution (Iossi et al., 2016). Standard germination measurements such as initial germination day (IGD), final germination day (FGD), mean germination time (MGT) and final germination percentage (FGP) were calculated for each germination treatment per species / cytotype tested (Ranal et al., 2009).

2.4.2. Move-along

To determine the temperature, or temperature sequence, necessary for physiological dormancy break in seeds that are water-permeable (non physical dormant), we performed a move-along experiment (Baskin and Baskin, 2003a). Three lots with 100 seeds per species / cytotype were set up after one month from 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 of the SS lots was exposed to an alternating temperature (SS → AS, hereafter AT), respecting the natural duration of the seasons being simulated in natural environment for the species under experimentation, being transferred to the conditions of the AS for 8 weeks (totalizing 20 weeks). The other SS lot was maintained on its initial condition (SS control). Seed plates incubated initially in AS were kept under the same conditions during the whole 20 weeks experiment duration (AS control). Plates checking, observation and maintenance were made as described above.

2.4.3. Photoinhibition

To detect whether photoinhibition promotes germination and is rather more advantageous as an adaptive mechanism compared with light requirement, a photoinhibition experiment was conducted. After four months from the collection date, four lots of 100 seeds per species / cytotype were divided and placed in two germination chambers operating at different variable temperatures, one simulating a SS and other an AS. On each chamber, one of the seed lots was incubated in light and the other in continuous darkness (SD and AD), achieved by wrapping plates with aluminium foil. Plates checking, observation and maintenance were made as previously described except for the photoinhibition lots, in which observations were done in a dark room under dim green safelight to prevent light exposition.

2.4.4. Cold stratification

A cold stratification test was held 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 constant 5 °C temperature. After two weeks of cold stratification, seed lots incubation in germination

chambers at variable AS temperature conditions under light and darkness (CS and CSD). plates checking, observation and maintenance followed the prescribed methodology from the photoinhibition experiment.

2.4.5. Viability tests

To assess viability of non-germinated seeds from all germination experiments after the 140 days incubation period, a cut test (CT) followed by a tetrazolium test (TZ) were performed. The CT consists on visually assessing seeds coat, endosperm and embryo health after lengthwise dissection with a sharp blade and record each seed as fresh (firm coat with creamy-white inner part in colour), mouldy, empty, insect-infested or abnormal. Empty and insect-infested seeds were excluded from further analysis. Abnormal and mouldy were considered as ungerminated. The TZ was applied to determine whether ungerminated seeds were still alive (viable) or dead. Cutted seeds were immersed in 1% buffered 2,3,5-triphenyl tetrazolium chloride solution and maintained in dark under laboratory at room temperature (~25 °C, 20-30 % RH) for at least six hours. Seeds living tissue stain red, due to the action of dehydrogenase enzymes, enabling to confirm which seeds were viable (but dormant) from a physiological perspective.

2.5. Data analysis

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 the stained nuclei of the target specimen 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) smaller than 5% were accepted.

Numerical data obtained from seed traits and germination experiments were described as mean \pm standard error of the mean (SE). Normal distribution of variables were graphically diagnosed with histogram / quantile-quantile plots and further assessed with a Shapiro-Wilk test. In case of non-normality distribution detection, the degree of skewness from curves were quantified to evaluate if non-normality was significant (Crawley, 2012). Variables homogeneity of variance were assessed with a Fligner-Killeen test. Linear models were fitted

for each variable and homoscedastic assumption was graphically diagnosed by extracting and plotting its residuals against fitted models. Variables were log transformed in case of need for parametric testing. For seed set (count data) and water uptake (percentage data) comparisons, estimated marginal means were extracted respectively from a Poisson and Beta regression models (Mangiofico, 2016). We performed a one-way ANOVA followed by Tukey post-hoc test (with Bonferroni correction) to compare the differences between seed traits means per species / cytotype. Pearson correlation was used to detect correlation between seed mass, shape and water uptake.

Time-to-event analysis was performed to evaluate seed germination response from 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 curves comparisons with Mantel-Haenszel homogeneity tests using the *survdif()* function, both functions from the *survival* package (Therneau and Lumley, 2018). Survival curves pairwise comparisons per species / cytotype were assessed by Log-Rank tests (with Benjamini & Hochberg method for p values adjustment) using the *pairwise_survdif()* function from the *survminer* package (Kassambara et al., 2017). All analyses above mentioned were performed with R software version 3.5.3 (R Development Core Team, 2019).

3. Results

3.1. Flow cytometry

Ploidy levels from five samples of *H. aff. lahue* (2x) and *H. lahue* (6x and 8x) distinguished only by floral morphology in the field were confirmed through estimated 2C results (Appendix A, Tab. S1) and histograms of DNA content (Appendix B, Fig. S1) obtained from flow cytometry. Samples which received RNase retrieved better nuclei measurements than those without the enzyme solution.

3.2 Seed traits

A total of 3.650 seeds were used for seed traits measurements (Appendix A, Tab. S2). All seed traits variables revealed normal distribution and homogeneity of variance except for seed shape (Shapiro-Wilk, $p < 0.001$; Fligner-Killeen, $p < 0.001$) that required log transformation to fulfill parametric testing assumptions. All seed traits mean values presented statistically significant differences among groups (one-way ANOVA, $p < 0.01$). Differences between species / cytotypes for seed traits after Tukey post-hoc analysis are summarized in Fig. 1.

3.2.2. Seed set

The mean seed set for *K. brasiliensis*, hexaploid *H. lahue* and *H. pulchella* species were very similar in comparison with the diploid *H. aff. lahue* and octoploid cytotype from *H. lahue*, that have shown significantly higher investments in number of seeds per capsule (Appendix A, Tab. S2).

3.2.3. Seed mass

Seed mass presented differences among all species, except for *K. brasiliensis* and diploid *H. aff. lahue* (one-way ANOVA with Tukey post-hoc method, $p = 0.7240$). From the three cytotypes analysed of *H. lahue*, a larger distinction between the average weight of diploid *H. aff. lahue* and hexaploid *H. lahue* seeds was found, where hexaploids were almost twice as heavier (Appendix A, Tab. S2).

3.2.4. Seed morphology

From the morphological seed traits assessed (Fig. 1), no clear applicable differences for species determination were recognised. When only *H. lahue* cytotypes were compared, the

diploid *H. aff. lahue* presented 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 ($r = 0.1021$, $p = 0.5913$).

3.2.5. Imbibition test

Imbibition tests shown that none of the species present seed morphological dormancy since the average mass of all fresh seed lots tested increased at least 48 % (Appendix A, Tab. S2) due to water uptake. Diploid *H. aff. lahue* presented the higher percentage of water uptake (0.6576 ± 0.0553). Seed water uptake capacity presented moderate negative correlations with seed mass ($r = -0.5870$, $p = 0.0065$) and seed shape ($r = -0.6669$, $p = 0.0013$).

3.3. Germination experiments

A total of 6.000 seeds were tested and standard germination measurements calculated for each lot were compiled in Tab. 3. From all 60 lots assessed under distinct treatment conditions, seeds started to germinate only after four weeks of incubation, indicating the probable presence of a physiological dormancy mechanism at some level. FGP was on average at 39.11%, were mean FGP for each species varied greatly depending on the germination treatment applied (Tab. 3).

3.3.1. Dry storage

The first lot of freshly harvested seeds tested in the dry storage germination experiment (SS0, representing storage time = 0) have not germinated during the first four weeks of incubation and seeds died due to mold development shortly after, revealing the need of a disinfection procedure implementation for all further seed lots before incubation. SS0 lot was discarded from analysis. Comparisons among survival curves for storage time intervals revealed differences in temporal pattern of germination (Fig. 2A, Mantel-Haenszel, $p < 0.0001$), which six month storage treatment differed (Appendix C, S1, Log-Rank, $p < 0.0001$) and outperformed all other lots, despite the poor FGP achieved by diploid *H. aff. lahue* and octoploid *H. lahue* (Tab. 3).

3.3.2. Move-along

Results from move-along experiment demonstrated that seed germination response among all treatment temperatures were different (Fig. 2B, Mantel-Haenszel, $p < 0.0001$). Due to the lack of germination, seeds subjected to treatments SS1 and AS1 had no difference for *K. brasiliensis* (Appendix C, S2, Log-Rank, $p = 0.3137$) and diploid *H. aff. lahue* (Appendix C, S2, Log-Rank, $p = 0.3137$) when compared. AT treatment differed (Appendix C, S2, Log-Rank, $p < 0.0001$) and outperformed SS1 and AS1 retrieving the highest FGP for all species / cytotypes (Tab. 3). The seed lot from *H. pulchella* under AS1 had the lower IGD (49 ± 2.09) followed by hexaploid *H. lahue* under AT (68.25 ± 2.83) which also achieved the highest FGP (0.89 ± 0.01) from all lots tested in the move-along experiment.

3.3.3. Photoinhibition

Photoinhibition tests recorded the lowest IGD's, FGD's and MGT's of all germination treatments (Tab. 3), demonstrating a faster germination response of the species / cytotypes tested in the absence of light. Under SS temperature conditions, treatments differed (Fig. 2C, Mantel-Haenszel, $p < 0.0001$) and seed lots under darkness outperformed those over light for all species / cytotypes except for *H. pulchella* (Appendix C, S3, Log-Rank, $p = 0.0559$), which showed no difference between treatments. Hexaploid and octoploid *H. lahue* achieved their highest FGP's from all tested germination treatments under SD conditions ($6x = 0.9 \pm 0.0094$, $8x = 0.8 \pm 0.0119$). Under AS temperature conditions, treatments also differed (Fig. 2D, Mantel-Haenszel, $p < 0.0001$) and seed lots under darkness outperformed those over light for all species except for the hexaploid *H. lahue* (Appendix C, S4, Log-Rank, $p = 0.6828$), which even obtained a better germination response when exposed to photoperiod (AS4) than under continuous darkness (AD) (Tab. 3), revealing a possible high temperature dependent mechanism to ignite germination rather than a light sensible one. Interestingly, *H. pulchella* and diploid *H. aff. lahue* achieved their highest FGP's from all tested germination treatments under AD conditions (respectively, 0.91 ± 0.007 and 0.47 ± 0.0183), exhibiting a potential low temperature affinity and photosensitivity to control germination timing.

3.3.4. Cold stratification

No differences between cold stratification pre-germination treatments tested under AS were found (Fig. 2E, 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 (Appendix C, S5, respectively Log-Rank test results, $p < 0.0001$ and $p < 0.01$) and they showed opposite preferences regarding light exposure. While hexaploid *H. lahue* achieved a higher FGP under CS treatment conditions (0.89 ± 0.0162), *H. pulchella* performed better under CSD (0.84 ± 0.0158).

3.3.5. Viability tests

From the 3.447 ungerminated seeds which reached the 140 days of incubation and were subjected to CT's, 3.017 (87.52%) were visually recognized as viable. After the following TZ's 2.846 seeds (94.33%) had their viability confirmed. Combining the total number of seeds capable of germination during experimental incubation time (2.553) with those censored (2.846 viable but dormant) detected through viability tests, an overall seed viability of 89.98% was assumed for the species / cytotypes tested. The different ploidies from *H. lahue* holded the highest and lowest seed viability percentages, respectively with the hexaploid (90.25%) and octoploid (84.67%) cytotypes.

4. Discussion

This study can be considered a pioneer attempt in the ecophysiological characterization of native grassland Iridaceae species from the Pampa biome in Rio Grande do Sul. Until now, similar studies have only been carried out with native grasses from the Brazilian Cerrado biome (Carmona et al., 1999; Correia Lima et al., 2014; Escobar et al., 2018; Kolb et al., 2016; Ramos, 2015; Zaidan and Carreira, 2008). Our results showed several differences among the studied species from the genera *Herbertia* and *Kelissa* regarding their investments in seed traits (e.g. seed production, mass, morphology and viability) and ecophysiological requirements (e.g. optimal germination conditions) that may influence reproductive success in their natural habitats.

Plants with high seed production but low viability have already been noticed for congeneric perennial grasses (Peters, 2002). Since seed production has been proven to be a key factor favoring common species population maintenance (Münzbergová, 2005), perhaps the diploid *H. aff. lahue* may be more commonly found than hitherto recorded, due to the large investment in seed production verified. This pattern has already been documented for some native Cerrado grasslands where a high seed production investment acts as a compensatory mechanism for low germination potential (Aires, 2013). These results are in agreement with those of Ashman *et al.* (2004), suggesting that trade-offs between seed production and vital rates (e.g. germination success) may be related with a possible reproductive strategy adaptation in which species dedicate more resource on seed quantity than quality. Conversely, *K. brasiliensis*, hexaploid *H. lahue* and *H. pulchella* presented lower seed production where the latter two recorded the better final germination percentages. These three taxa also present narrower distributions than the octoploid *H. lahue*. Even knowing that seed production cannot explain alone species endemism, rarity or restriction in natural distribution (Gabrielová et al., 2013; Janišová et al., 2018; Münzbergová, 2005; Powell et al., 2011), rarer species often produce fewer seeds than their common relatives (Lavergne et al., 2004; Murray et al., 2002). As suggested by Paulů *et al.* (2017), higher proportions of germinated seeds in rare species could therefore be explained by lower production of seeds of higher quality. This seems to be the same scenario for the genera *Kelissa* and *Herbertia* congeners. In addition, seed mass, which is expected to be inversely proportional to the number of seeds produced (Moles and Westoby, 2004), was another almost fulfilled trade-off premise, with the octoploid *H. lahue* been the only exception (i.e. with a large production of heavy seeds).

Seed mass was one of the most variable seed traits among the Tigridaeae species evaluated. This was a surprising outcome, as seed mass is considered a rather evolutionarily stable seed trait where variation is more comprehensively expected for high taxonomic levels than between species from close related genera (Fenner, 2000). Similarly to other ecophysiological studies (Carta et al., 2016; Paulu et al., 2017), the species *H. lahue* (hexaploid and octoploid cytotypes) and *H. pulchella* that have heavier seeds where the ones with the best germination performances. In addition, those were also the species with larger seed sizes, although no correlation between mass and seed shape could be identified. This is also an expected result, because heavier and larger seeds have more storage and nutrient compounds than lighter ones (Jakobsson and Eriksson, 2000; Rees, 2001).

Another important finding from our study was the recognition of morphological seed traits differences among studied Tigridaeae species. Despite the fact that morphological seed traits are notoriously phylogenetically conserved and have a potential usage as characteristics for delimitation of taxa at different taxonomic levels (Jacobs et al., 2010; Karaismailoğlu, 2015; Vandeloos et al., 2018), seed morphology has been underexploited in the taxonomy of Iridaceae (Erol and Küçüker, 2003). Although it was not possible to verify a seed morphology variation pattern that enable a clear species distinction, thickness and umbilicus area, which varied greatly, could be potential features with considerable taxonomic significance since some taxa could be differentiated. As already noticed by Goldblatt *et al.* (1990, 1989) for other Iridoideae (tribe Sisyrinchieae and its allies), black colored umbilicoid seeds with a globose shape appear to be an apomorphic condition in *Sisyrinchium* L. and *Echthronema* Herb. representing their basic seed types. To our knowledge, no further systematic investigations have been done regarding umbilicus trait features in Iridaceae. Seed shapes and colors observed for the studied species varied from angular to barrel-shaped with colors ranging from dark brown to light brown. Both umbilicus area and seed shape traits presented significant interspecific variation for some of the studied species (Fig. 1). Recent studies have been proposing new methodologies to categorize seeds according to their morphology from optimized metric estimates (Cervantes et al., 2016). The development of an easy-to-implement methodology that standardizes the collection of morphological seed data and is applicable to representatives of Iridaceae and relatives, may allow advances in systematic studies for the group. Remarkably, comparing seed morphological traits across *H. lahue* ploidy levels, the diploid *Herbertia* aff. *lahue* can be easily distinguished due to its smaller

dimensions. However, all *H. lahue* ploidy levels conserved a similar shape. Interestingly, the hexaploid *H. lahue* is the ploidy level with bigger seed dimensions, instead of the octoploid. This result is somewhat counterintuitive since positive relationships between genome size and seed mass are often verified (Beaulieu et al., 2007; Bretagnolle et al., 1995; Carta et al., 2014). As suggested by Jakobsson and Eriksson (2000), species with intermediately sized seeds are able to maximise the number of places (i.e. environments with variable natural conditions) where they are competitively superior during seedling recruitment. In view of this, that reinforces the reason why the octoploids are more widely distributed and frequently found than other *H. lahue* cytotypes. Furthermore, this can also be cautiously interpreted as a side effect from a current micro-speciation due to polyploidization (Carta et al., 2014; Münzbergová et al., 2015; Uysal et al., 2009).

Generally, the imbibition test showed that seeds from *Kelissa* and *Herbertia* species studied had water permeability and did not present physical dormancy. However, these seeds probably have some mechanism of germinative control linked to physiological dormancy, since no germination was observed in any laboratory using germination experiment before four weeks of incubation. Those Iridaceae genera are adapted to open grassland environments and in their natural habitats the species are subjected to a marked dry summer season. Thus, it is predictable that seeds present some dormancy mechanism to avoid germination during summer, which would expose seedlings to unfavourable conditions that threaten their survival (Carta et al., 2014; Copete et al., 2011; Herranz et al., 2013). This has been identified as a typical spring geophytes germination behaviour, where seeds germinate in autumn after dormancy break in summer and seedlings emerge in early spring (Newton et al., 2015; Vandeloos et al., 2012). To precisely classify seed dormancy, further tests taking into account embryo growth on future *in situ* germination experiments should be conducted (Baskin and Baskin, 2004; Carta et al., 2014). So far, we can only confirm that seeds from *H. lahue*, *H. pulchella* and *K. brasiliensis* present at least a non-deep physiological dormancy (Baskin and Baskin, 2003b) which is indeed a novelty for the Pampa biome Iridaceae.

The most important contribution from our study was the recognition of some seed ecophysiological requirements responsible to regulate germination timing in Tigridiaceae species. The laboratory germination experiments performed allowed us to elucidate that dry storage favors the germinative performance of seeds that were stored for longer periods. 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 period of six months of dry storage, there was a clearly promotion of germination. Even for *H. aff. lahue*, octoploid *H. lahue*, which indeed presented the weakest responses to the dry storage treatment, they responded positively to the six months dry storage when compared with the other time spans under summer season (SS) conditions. It is important to highlight that *K. brasiliensis* achieved its best germination result under the SS6 treatment. This information can be especially important due to the endemism of this species, because knowing its life cycle better (e.g. latter seedling emergence and establishment in nature) could assist in the elaboration of conservation strategies for their populations which can be more vulnerable to habitat loss (Copete et al., 2011; Galmés et al., 2006; Saatkamp et al., 2018).

As shown in the move-along experiment the seeds were favored when submitted to a temperature transition sequence. Seeds that were initially incubated at SS and then exposed to autumn season (AS) conditions reached higher percentages of germination. Thus, indicating that seeds physiological dormancy can be overcome through this treatment (Baskin and Baskin, 2003a). This was an expected result, since this temperature variation sequence simulates the natural condition experienced by grassland species in the Pampa biome. Intriguingly, *H. pulchella* seeds germinated substantially earlier under AS1 than AT, despite not having achieved an expressive FGP when directly placed under lower temperature conditions. This greater low temperature sensitivity can be interpreted as a mechanism to rapidly colonize available space promptly after the recognition of suitable temperature conditions to avoid competition (Carta et al., 2016; Zhang et al., 2014).

A very interesting finding from the germination tests performed in our study was that seeds from Tigridaeae species obtained faster and more efficient germinative responses in the photoinhibition experiments. Due to the scarcity of data on seed germination to *Herbertia* species (Forgiarini et al., 2017; Kew, 2017; Schiappacasse et al., 2005) and *K. brasiliensis* (Barroso, 2006), it was only known 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 Tigridaeae tribe representatives. Recently, studies have shown that negative photoblastic seeds are common among other geophyte species in Iridaceae and close related families (Carta et al., 2017, 2014; Copete et al., 2014; Newton et al., 2015; Vandeloock et al., 2018). In addition to the improvement in the germinative performance of all Tigridaeae

species, which started to germinate much earlier in the absence of light, they also demonstrated different preferences for incubation temperatures.

Under photoinhibition, hexaploid and octoploid *H. lahue*, which have wider distributions with heavier and larger seeds, presented better germination performances at elevated temperatures while diploid *H. aff. lahue*, *H. pulchella* and *K. brasiliensis*, which have narrower distributions with lighter and smaller seeds, showed 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 exposition, presenting greater plasticity to environment condition fluctuations during colonization and establishment in diverse open grassland habitats (Carta et al., 2014; Limón and Peco, 2016; Skourti and Thanos, 2015; Vandeloos et al., 2018; Westoby et al., 1992; Zhang et al., 2014). Taking into account hexaploid and octoploid *H. lahue* seed traits in combination with milder germinative requirements (i.e. temperature and light), it makes sense to recognize that these species colonize open grassland environments more easily and, therefore, being more commonly found due to their greater probability of survival to adverse natural conditions.

The opposite scenario can also be interpreted for diploid *H. aff. lahue*, *H. pulchella* and *K. brasiliensis*, only been found in habitats with more restrict conditions which favor the establishment of plants with small-sized, lighter and more photosensible seeds. Diploid *H. aff. lahue* populations were found in open grasslands quite occupied by forage species, indicating that its germinative success may depend more on soils with higher nutritional values and foraged areas that can maintain cooler temperatures and lower luminosity, due to its greater susceptibility to dryness by having seeds with lower reserves and greater affinity to photoinhibition (Limón and Peco, 2016; Skourti and Thanos, 2015; Ten Brink et al., 2013).

In the case of *H. pulchella*, the species inhabits open grasslands with rocky outcrops frequently found on hilltops within scarce vegetation cover. Populations are often associated with moss cushions on thin layers of soil with humus whose moisture can be shortly maintained after precipitations. These extreme habitat conditions may explain this rapid and early germination behavior from *H. pulchella* to ensure their survival by either avoiding competition with or getting a head-start over other species by increasing temporal niche differentiation (Zhang et al., 2014), since its seeds seems to germinate readily after their minimum physiological needs for cold temperatures and photoinhibition are reached.

Lastly, *K. brasiliensis* populations are typical from open and dryer grasslands with grainy substrate and low vegetation cover. Other species with restricted distribution areas are also known to occupy habitats where edaphic constraints are severe, while biotic interactions are reduced because of low vegetation cover (Imbert et al., 2012). These recognized limitations in their environment are also in accordance with the physiological preferences identified for *K. brasiliensis* seeds, whose smaller dimensions facilitate penetration into lower soil layers and photoinhibition may be ecologically advantageous for species growing in dry habitats, ensuring below-surface germination and hence protecting seedlings from dehydration and exposure to high temperatures (Ten Brink et al., 2013; Thanos et al., 1991; Vandeloos et al., 2018).

Ultimately, cold stratification experiments demonstrated that such exposure to cold temperature is not a prerequisite to alleviate seed dormancy of the species tested. However, these tests indicate that these species probably possess only a non-deep physiological dormancy instead of a combinational morphophysiological dormancy (Baskin and Baskin, 2003b). Moreover, they reinforced that the species tested have distinct temperature and photoblastic preferences, as equally addressed in the photoinhibition tests. Notwithstanding, in combination with the results from the dry storage assays performed, these experiments also suggest that Tigridae species may hold an afterripening requirement (Skourti and Thanos, 2015), meaning that their seeds need to undergo a process of gradual release of primary physiological dormancy, which starts during seed maturation and continues as a post maturation event at dry and warm conditions. To confirm these hypotheses, further germination tests which contemplate seed embryo development aspects must be performed.

Complementarily, we confirmed through flow cytometry that the morphological floral variations between cytotypes prescribed by Stiehl-Alves *et al.* (2016) for *H. lahue* can be effectively used to distinguish their populations in the field. It is also important to highlight that this is the first record of diploid *H. aff. lahue* populations since Winge (1959), which mistakenly identified diploid specimens as *Alophia pulchella* (Sweet) Kuntze, taxon later recognized as belonging to the genus *Herbertia* (Goldblatt, 1977, 1975). These floral characters, coupled with the differences we identified among seed traits and ecophysiological behavior for *H. lahue* ploidy, suggest that a further survey which expand diploid *H. aff. lahue* sampling, confirms its cytotype by chromosome counting and also evaluates genetic aspects related to their populations maintenance must be conducted to investigate its

boundaries as a different species among the genus *Herbertia* (Moraes et al., 2015; Stiehl-Alves et al., 2017, 2016).

Seeds from Tigridaeae species tested holds a non-deep physiological dormancy or at least an afterripening requirement that can be achieved at dry storage under warm condition. Germination tests showed that seeds benefits from photoinhibition as a strategy to possibly avoid unstable environmental conditions at the soil surface which could jeopardize seedling survival. Taken together, trade-offs between seed production, dimensions, biomass, and germination requirements, such as temperature affinities and photosensitivity, were interpreted as explanatory adaptations influencing species habitat adaptation hence in their natural distribution. Additionally, we confirmed through flow cytometry that ploidy levels from *Herbertia lahue* can be distinguished by floral characters and have remarkable differences among their seed traits and ecophysiology. Besides, our study is recording for the first time diploid populations from *H. lahue*. We recommend that future cytogenetic studies contemplates these populations to befittingly confirm this ploidy cytotype. Furthermore, a reassessment of species boundaries through a population genetic approach within *H. lahue* cytotypes which recognises distinct gene pools supported by seed traits and ecophysiological adaptations as diagnostic characters, could reveal a recent speciation process. We hope that our findings serve as an incentive for further investigations willing to reveal how ecophysiology could influence Pampa biome grassland species adaptation in their conquest of natural environments. Finally, a better understanding of the reproductive ecology and evolutionary history of those species are a required knowledge for the elaboration of effective management plans and restoration strategies that promote the Pampa biome conservation.

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Figures legends

Figure 1. Mean values of seed set, mass, length, width, thickness, umbilicus area, shape and water uptake capacity of studied species. Kb: *Kelissa brasiliensis*; Hal: diploid *Herbertia* aff. *lahue*; H16: hexaploid *H. lahue*; H18: octoploid *H. lahue*; Hp: *H. pulchella*. Different letters indicate significant differences (one-way ANOVA with Tukey post-hoc method, $p < 0.05$).

Figure 2. 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.

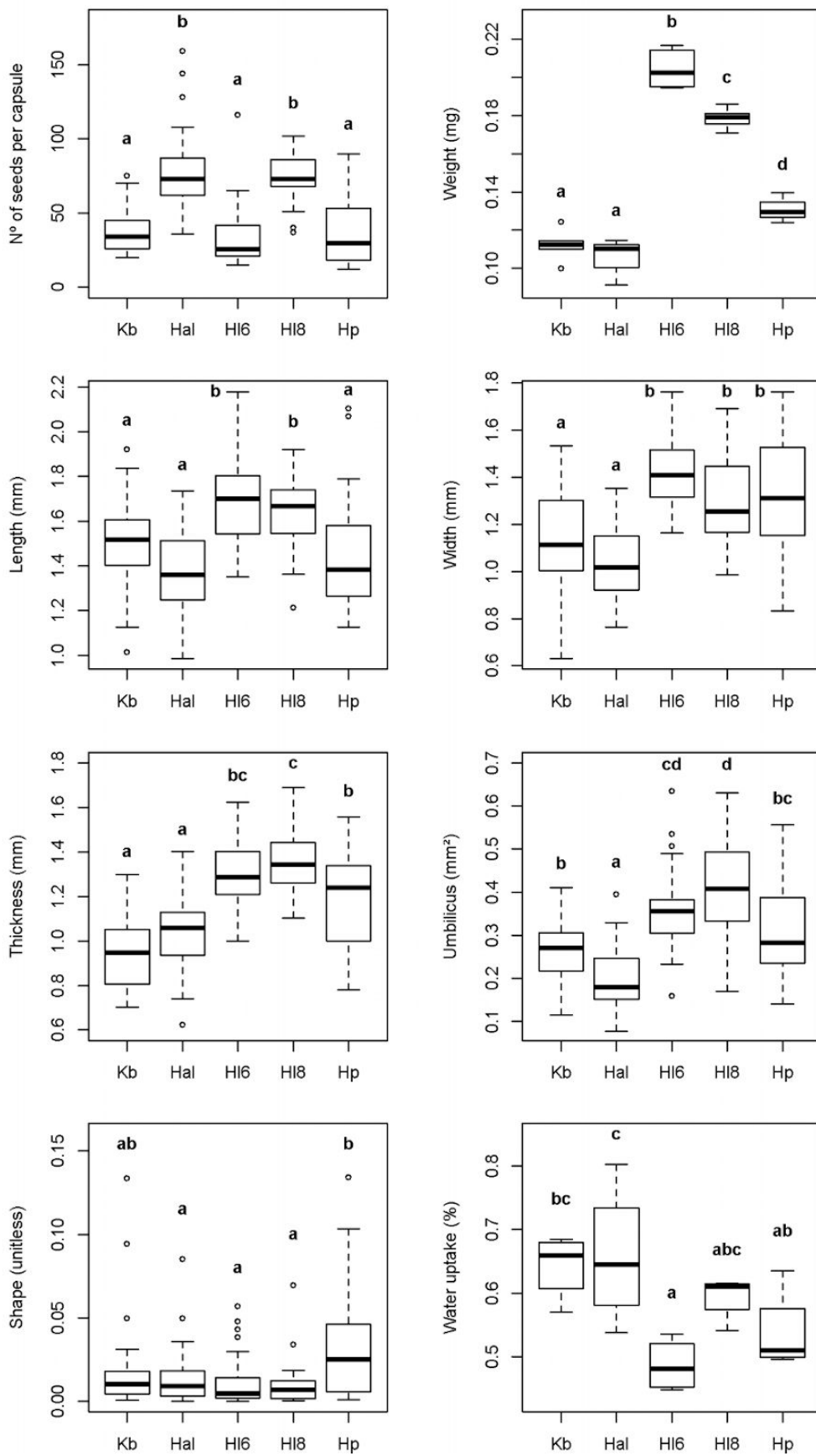


Figure 1. Mean values of seed traits.

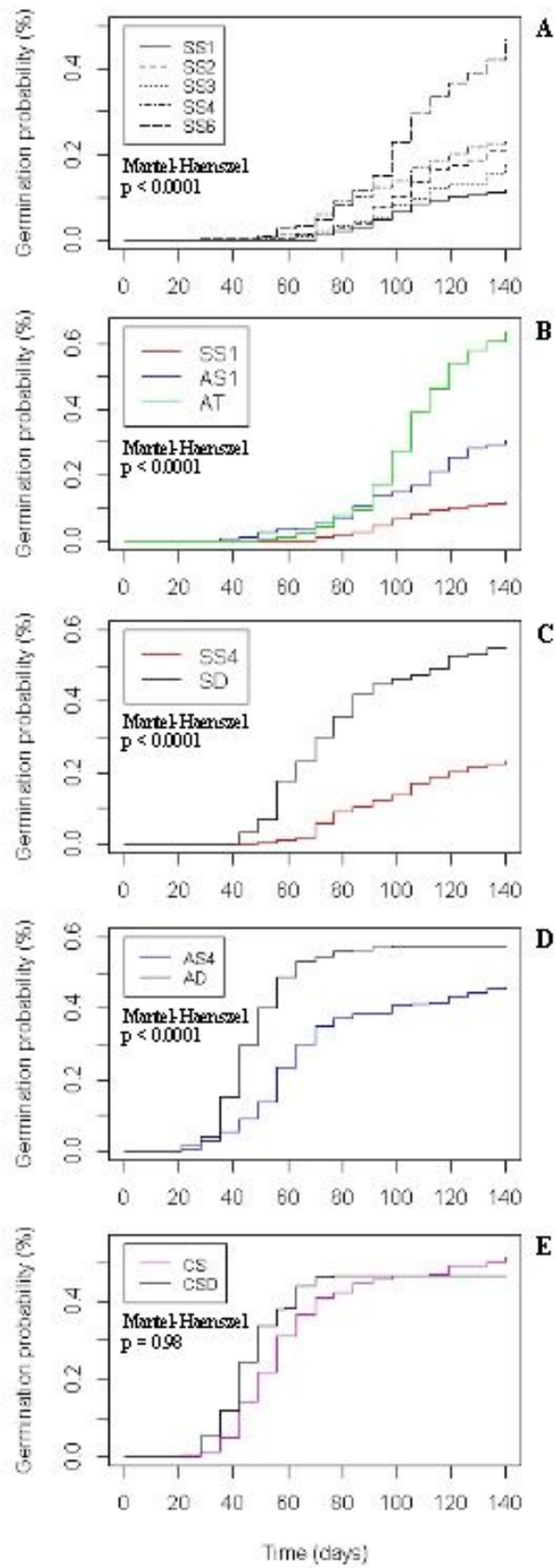


Figure 2. Survival curves.

Tables legends

Table 1. Collection sites of all sampled species populations.

Table 2. Tested germination treatments and measurements with seeds. SS: Summer season; AS: Autumn season; AT: Alternating temperature from summer to autumn season; SD: Summer season under darkness; AD: Autumn season under darkness; CS: Cold stratification before autumn season; CSD: Cold stratification before autumn season under darkness; IGD: Initial germination date; FGD: Final germination date; FGP: Final germination percent; MTG: Mean time germination.

Table 3. Germination treatments measurements and viability tests results (mean \pm SE). IGD: Initial germination date; FGD: Final germination date; MTG: Mean time germination; FGP: Final germination percent; CT: Cut test; TZ: Tetrazolium test.

Table 1. Collection sites.

Species	Ploidy level	Municipality	Longitude	Latitude	Elevation (m)	Voucher ¹
<i>Herbertia aff. lahue</i>	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
<i>Herbertia lahue</i>	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
	<i>Herbertia pulchella</i>		Caçapava do Sul	-30.69351	-53.39317	142
		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
<i>Kelissa brasiliensis</i>		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..

Table 2. Tested germination treatments.

Germination treatment conditions			Pre-treatments		N	Duration (days)	Germination measurements
Acronyms ¹	Season simulation	Photoperiod	Dry storage (months)	Cold stratification (weeks)			
SS0	Summer (15/25 °C)	Light (12/12 h)	0 (18-30 °C)		100	140	IGD
SS1	Summer	Light	1				FGD
AS1	Autumn (10/20 °C)	Light	1				FGP
AT	Summer to Autumn ²	Light	1				MTG ³
SS2	Summer	Light	2				
SS3	Summer	Light	3				
SS4	Summer	Light	4				
SD	Summer	Darkness	4				
AS4	Autumn	Light	4				
AD	Autumn	Darkness	4				
CS	Autumn	Light	4	1 (5 °C)			
CSD	Autumn	Darkness	4	1			
SS6	Summer	Light	6				

¹ Numbers (0 to 6) presented aside germination treatments acronyms represent the storage time experienced by seed lots in months before incubation.

² Temperature condition was alternated after 2 months of incubation.

³ MTG was calculated according to the following equation: $MGT = \sum Dn / \sum n$ (Ranal et al., 2009), where n is the number of seeds germinated on day D, and D is the number of days counted from the beginning of germination.

Table 3. Germination treatments measurements.

Species	Ploidy level	Germination treatment	IGD (days)	FGD (days)	MGT (days)	FGP (%)	CT (%)	TZ (%)
<i>Herbertia aff. lahue</i>	2x	SS1	-	-	-	-	94	100
		AS1	-	-	-	-	86	100
		AT	98 ± 1.48	131.3 ± 2.42	114 ± 1.77	35	88	100
		SS2	-	-	-	-	82	100
		SS3	127.8 ± 1.92	133 ± 0	130.4 ± 0.96	6	92	100
		SS4	108.5 ± 0.9	108.5 ± 0.9	108.5 ± 0.9	2	60	100
		SD	75.3 ± 5.46	126 ± 1.48	96.1 ± 2.82	33	87	100
		AS4	43.8 ± 1.22	120.8 ± 3.02	78.1 ± 2.75	22	96	100
		AD	33.3 ± 1.22	75.3 ± 2.18	52.1 ± 0.29	47	94	100
		CS	43.8 ± 1.92	98 ± 6.26	64.2 ± 1.66	35	92	100
		CSD	35 ± 1.48	61.3 ± 1.22	48.3 ± 0.66	46	89	100
		SS6	81.7 ± 9.07	137.7 ± 0.74	112.6 ± 3.84	15	90	86
		<i>Herbertia lahue</i>	6x	SS1	80.5 ± 0.74	126 ± 3.13	102.2 ± 0.88	33
AS1	77 ± 2.33			126 ± 2.95	100.9 ± 3.09	68	82	59
AT	68.3 ± 2.83			120.8 ± 1.22	91.5 ± 1.94	89	89	-
SS2	80.5 ± 1.28			129.5 ± 1.65	104.9 ± 1.99	61	86	96
SS3	66.5 ± 0.74			138.3 ± 0.64	107.3 ± 2.14	46	81	100
SS4	66.5 ± 1.65			129.5 ± 1.28	96.1 ± 1.53	67	92	100
SD	47.3 ± 1.22			66.5 ± 1.65	54.9 ± 1.3	90	93	100
AS4	45.5 ± 0.74			89.3 ± 1.92	64.6 ± 1.28	65	91	100
AD	36.8 ± 0.64			68.3 ± 2.83	47 ± 0.55	51	99	100
CS	36.8 ± 0.64			99.8 ± 4.35	54.6 ± 1.28	89	99	100
CSD	40.3 ± 3.02			57.8 ± 1.92	48.9 ± 2.2	28	92	100
SS6	68.3 ± 3.02			117.3 ± 2.83	90.4 ± 1.37	91	94	100
8x	SS1			101.5 ± 4.52	122.5 ± 0.9	112 ± 1.81	3	84
	AS1		112 ± 2.33	129.5 ± 1.65	120.5 ± 0.68	16	79	100
	AT		87.5 ± 3.97	138.3 ± 0.64	116.1 ± 0.85	56	74	60
	SS2		101.5 ± 2.71	126 ± 3.61	116.8 ± 1.24	9	97	84
	SS3		133 ± 0	133 ± 0	133 ± 0	2	92	100
	SS4		113.8 ± 1.92	115.5 ± 1.65	114.6 ± 1.76	6	55	100
	SD		54.3 ± 1.22	115.5 ± 4.84	84.2 ± 2.78	80	92	100
	AS4		49 ± 1.04	115.5 ± 3.97	81.2 ± 2.43	39	85	100
	CS		57.8 ± 1.61	129.5 ± 2.21	92.9 ± 3.58	37	76	100
	CSD		40.3 ± 0.64	70 ± 3.76	51.7 ± 1.48	41	94	100
SS6	91 ± 3.76		133 ± 1.81	119 ± 0.78	32	97	92	

Table 3. Continued.

Species	Ploidy level	Germination treatment	IGD (days)	FGD (days)	MGT (days)	FGP (%)	CT (%)	TZ (%)
<i>Herbertia pulchella</i>		SS1	89.3 ± 2.42	119 ± 3.13	97.8 ± 2.99	14	81	71
		AS1	49 ± 2.09	127.8 ± 1.61	86 ± 3.22	42	85	79
		AT	73.5 ± 3.97	131.3 ± 1.61	105.5 ± 2.06	61	82	56
		SS2	61.3 ± 0.64	99.8 ± 6.46	78.2 ± 2.77	18	76	67
		SS3	75.3 ± 3.52	96.3 ± 3.36	87.2 ± 3.1	13	86	100
		SS4	56 ± 1.04	122.5 ± 4.24	86.1 ± 2.66	34	67	100
		SD	57.8 ± 1.92	113.8 ± 3.82	87.4 ± 1.89	50	92	100
		AS4	26.3 ± 0.64	105 ± 4.04	50.7 ± 0.41	77	99	100
		AD	22.8 ± 0.64	63 ± 1.81	40.8 ± 0.5	91	96	100
		CS	29.8 ± 0.64	87.5 ± 3.97	51.4 ± 0.51	67	75	100
		CSD	26.3 ± 1.22	68.3 ± 0.64	42.2 ± 0.58	84	95	100
		SS6	52.5 ± 2.66	134.8 ± 1.22	100.7 ± 1.31	47	93	77
<i>Kelissa brasiliensis</i>		SS1	115.5 ± 2.71	136.5 ± 0.9	126 ± 1.81	4	90	100
		AS1	85.8 ± 0.64	101.5 ± 3.83	92.5 ± 2.11	7	90	100
		AT	85.8 ± 2.83	133 ± 1.48	108.8 ± 0.56	37	93	100
		SS2	101.5 ± 4.72	113.8 ± 4.82	106.8 ± 4.22	9	84	83
		SS3	91 ± 1.48	126 ± 2.33	109.5 ± 1.49	17	93	100
		SS4	98 ± 6.6	108.5 ± 6.72	103.3 ± 6.38	5	78	100
		SD	61.3 ± 1.22	101.5 ± 3.83	79.2 ± 1.05	22	92	100
		AS4	42 ± 1.81	120.8 ± 4.59	78.6 ± 2.49	20	87	100
		AD	38.5 ± 1.28	78.8 ± 5.75	55.1 ± 2.54	40	88	100
		CS	42 ± 1.81	96.3 ± 4.59	65.1 ± 1.3	24	88	100
		CSD	38.5 ± 1.28	61.3 ± 2.18	50.3 ± 1.41	35	99	100
		SS6	78.8 ± 2.18	140 ± 0	113.5 ± 1.12	51	93	77

Appendix

Supporting information to the paper Cristante et al. How do ecophysiological differences contribute to grassland species habitat adaptation? A case study with three species of the tribe Tigridieae (Iridaceae) from South Brazilian grasslands. *Flora*.

Appendix A. Tables S1 and S2. Seed traits measurements and flow cytometry analysis results.

Table S1. Ploidy level and estimated 2C results (mean \pm SE) obtained for *Herbertia* species through flow cytometry analysis (N = 15). MFI: Mean fluorescence intensity; CV: Coefficient variation.

Table S2. Average seed set, length, width, thickness, shape (mean \pm SE of 30 seeds), mass (mean \pm SE of six replicates of 100 seeds each) and water uptake capacity (mean \pm SE of four replicates of 25 seeds each) of studied species.

Table S1. Ploidy level.

Species	Ploidy level	Nuclei	Internal standard G1		Target species G1		DNA index ¹	2C (pg) ²	2C (Mbp) ³
			MFI	CV (%)	MFI	CV (%)			
<i>Herbertia aff. lahue</i>	2x	9402.8 ± 597.2	528.2 ± 6.7	4.3 ± 0.03	1122.4 ± 11.3	4.62 ± 0.27	2.125 ± 0.006	4.165 ± 0.012	4.074 ± 0.011
<i>Herbertia lahue</i>	6x	6865 ± 1427.9	1913.4 ± 367.9	4.18 ± 0.06	3800.4 ± 735.7	4.28 ± 0.04	1.985 ± 0.008	10.778 ± 0.045	10.541 ± 0.044
	8x	7542.4 ± 1620	4496 ± 1220.1	4.26 ± 0.09	13239.2 ± 3577.9	4.14 ± 0.09	2.951 ± 0.051	16.025 ± 0.276	15.672 ± 0.27

¹ DNA index was calculated using the formula: Target species G1 MFI / Internal standard G1 MFI (Doležel et al. 2007).

² 2C (pg) was calculated using the formula: DNA index x Internal standard 2C-value (Doležel et al. 2007).

³ 2C (Mbp) was calculated using the conversion factor 1 pg DNA = 978 Mbp (Doležel et al. 2007).

Table S2. Average seed traits.

Species	Ploidy level	Seed set	Length (mm)	Width (mm)	Thickness (mm)	Shape (unitless)	Umbilicus (mm ²)	Mass (mg)	Water uptake (%)
<i>Herbertia</i> aff. <i>lahue</i>	2x	77.9 ± 5.118	1.3513 ± 0.0342	1.0265 ± 0.0285	1.0377 ± 0.0302	0.0148 ± 0.0033	0.2005 ± 0.0133	0.1065 ± 0.0037	0.6576 ± 0.0553
<i>Herbertia lahue</i>	6x	33.733 ± 3.673	1.703 ± 0.0403	1.4181 ± 0.0262	1.3121 ± 0.0277	0.0118 ± 0.0028	0.3569 ± 0.0178	0.2043 ± 0.0042	0.4864 ± 0.0206
	8x	74.733 ± 2.9	1.6372 ± 0.0282	1.2964 ± 0.0342	1.3468 ± 0.0234	0.0097 ± 0.0025	0.4157 ± 0.02	0.1787 ± 0.0021	0.5943 ± 0.0179
<i>Herbertia pulchella</i>		37.633 ± 4.078	1.4328 ± 0.0446	1.3242 ± 0.0465	1.1957 ± 0.0395	0.0349 ± 0.0063	0.3109 ± 0.0195	0.1306 ± 0.0024	0.5378 ± 0.0327
<i>Kelissa brasiliensis</i>		37.833 ± 2.526	1.4903 ± 0.0343	1.1342 ± 0.0387	0.9494 ± 0.0295	0.0188 ± 0.0052	0.268 ± 0.0138	0.1121 ± 0.0032	0.6435 ± 0.0258

Appendix B. Histograms of relative fluorescence intensities (relative nuclear DNA contents) obtained after the analysis of isolated plant nuclei with *Herbertia lahue*.

Figure S1. Histograms of DNA content. A: diploid *Herbertia* aff. *lahue* with *Solanum lycopersicum* as internal standard; B: hexaploid *H. lahue* with *Zea mays* as internal standard; C: octoploid *H. lahue* with *Zea mays* as internal standard; IS: Internal standard peak; G1: target species peak.

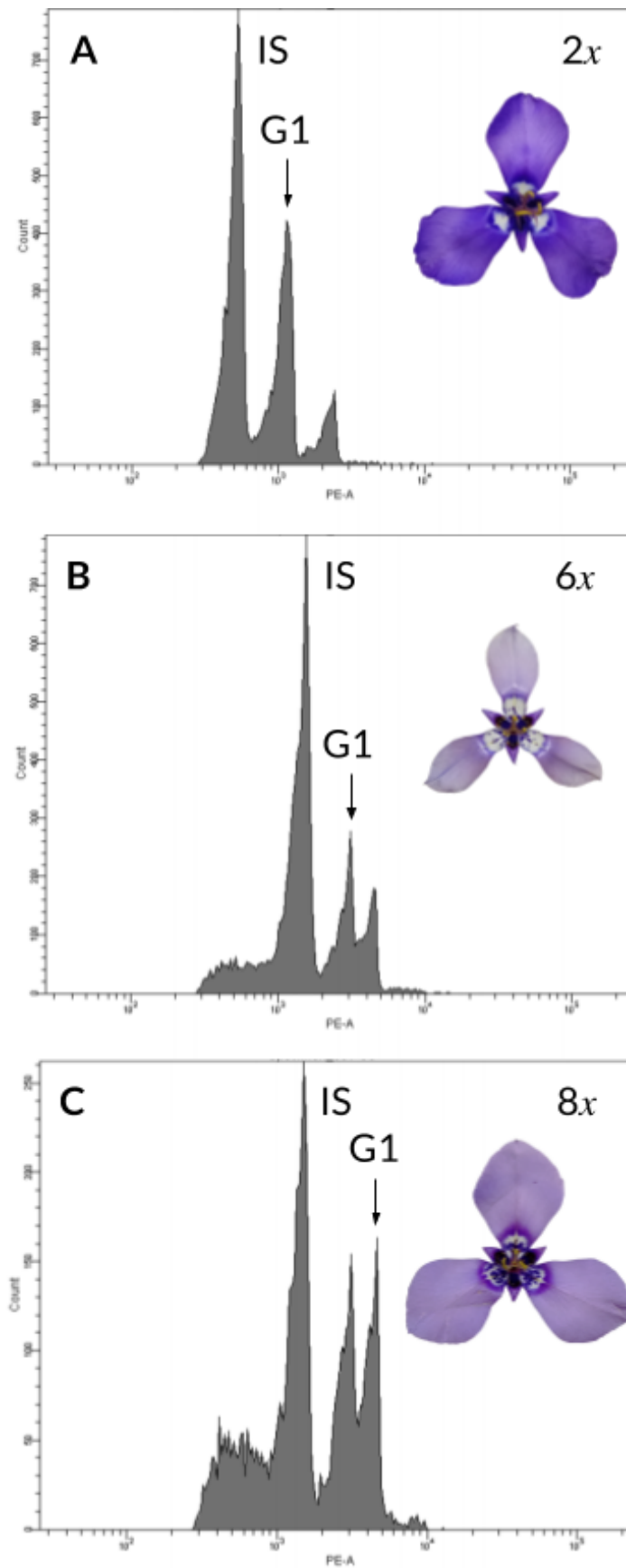


Figure S1. Histograms of DNA content.

Appendix C. Survival curves pairwise comparisons per species / cytotype assessed with Log-Rank tests (with Benjamini & Hochberg method for p values adjustment).

Appendix S1. Pairwise comparisons using Log-Rank test for dry storage treatment. spp=1: *Kelissa brasiliensis*; spp=2: diploid *Herbertia* aff. *lahue*; spp=3: hexaploid *H. lahue*; spp=4: octoploid *H. lahue*; spp=5: *H. pulchella*; begin=1: SS1; begin=2: SS2; begin=3: SS3; begin=4: SS4; begin=6: SS6.

Appendix S2. Pairwise comparisons using Log-Rank test for move along treatment. spp=1: *Kelissa brasiliensis*; spp=2: diploid *Herbertia* aff. *lahue*; spp=3: hexaploid *H. lahue*; spp=4: octoploid *H. lahue*; spp=5: *H. pulchella*; treat=1: SS1; treat=2: AS1; treat=3: AT.

Appendix S3. Pairwise comparisons using Log-Rank test for photoinhibition treatment under summer condition. spp=1: *Kelissa brasiliensis*; spp=2: diploid *Herbertia* aff. *lahue*; spp=3: hexaploid *H. lahue*; spp=4: octoploid *H. lahue*; spp=5: *H. pulchella*; treat=4: SS4; treat=5: SD.

Appendix S4. Pairwise comparisons using Log-Rank test for photoinhibition treatment under autumn condition. spp=1: *Kelissa brasiliensis*; spp=2: diploid *Herbertia* aff. *lahue*; spp=3: hexaploid *H. lahue*; spp=4: octoploid *H. lahue*; spp=5: *H. pulchella*; treat=6: AS4; treat=7: AD.

Appendix S5. Pairwise comparisons using Log-Rank test for cold stratification treatment. spp=1: *Kelissa brasiliensis*; spp=2: diploid *Herbertia* aff. *lahue*; spp=3: hexaploid *H. lahue*; spp=4: octoploid *H. lahue*; spp=5: *H. pulchella*; treat=8: CS; treat=9: CSD.

Appendix S1. Pairwise comparisons using Log-Rank test for dry storage treatment.

	spp=1, begin=1	spp=1, begin=2	spp=1, begin=3	spp=1, begin=4	spp=1, begin=6	spp=2, begin=1
spp=1, begin=2	0.15642	-	-	-	-	-
spp=1, begin=3	0.00399	0.15714	-	-	-	-
spp=1, begin=4	0.77188	0.25998	0.00958	-	-	-
spp=1, begin=6	3.0e-13	2.1e-09	2.8e-06	1.9e-12	-	-
spp=2, begin=1	0.06556	0.00361	5.1e-05	0.03909	2.9e-15	-
spp=2, begin=2	0.08129	0.00560	0.00012	0.05025	5.5e-14	1.00000
spp=2, begin=3	0.55638	0.38176	0.01816	0.79456	3.6e-12	0.02195
spp=2, begin=4	0.44715	0.03376	0.00052	0.29212	1.9e-14	0.20053
spp=2, begin=6	0.08631	0.79716	0.22300	0.15997	2.1e-09	0.00170
spp=3, begin=1	2.9e-08	2.4e-05	0.00330	1.2e-07	0.17386	2.9e-10
spp=3, begin=2	< 2e-16	1.2e-14	4.1e-11	< 2e-16	0.01647	< 2e-16
spp=3, begin=3	4.4e-12	1.4e-08	8.5e-06	2.1e-11	0.98067	4.4e-14
spp=3, begin=4	< 2e-16	< 2e-16	2.6e-13	< 2e-16	0.00062	< 2e-16
spp=3, begin=6	< 2e-16	< 2e-16	< 2e-16	< 2e-16	< 2e-16	< 2e-16
spp=4, begin=1	0.88265	0.13786	0.00473	0.65283	7.7e-12	0.08881
spp=4, begin=2	0.18438	0.90918	0.10667	0.30716	2.4e-10	0.00451
spp=4, begin=3	0.44982	0.03404	0.00056	0.29271	2.0e-14	0.19610
spp=4, begin=4	0.55452	0.39414	0.01890	0.78294	7.4e-12	0.02329
spp=4, begin=6	8.5e-07	0.00042	0.03732	2.8e-06	0.00465	5.8e-09
spp=5, begin=1	0.00941	0.22300	0.90255	0.02126	5.4e-06	0.00013
spp=5, begin=2	0.00029	0.02180	0.31514	0.00080	0.00154	2.8e-06
spp=5, begin=3	0.02397	0.39375	0.58087	0.04758	4.0e-07	0.00042
spp=5, begin=4	1.0e-07	3.9e-05	0.00469	3.4e-07	0.13543	1.1e-09
spp=5, begin=6	4.8e-12	1.3e-08	9.6e-06	2.1e-11	0.99423	5.6e-14

	spp=2, begin=2	spp=2, begin=3	spp=2, begin=4	spp=2, begin=6	spp=3, begin=1	spp=3, begin=2
spp=1, begin=2	-	-	-	-	-	-
spp=1, begin=3	-	-	-	-	-	-
spp=1, begin=4	-	-	-	-	-	-
spp=1, begin=6	-	-	-	-	-	-
spp=2, begin=1	-	-	-	-	-	-
spp=2, begin=2	-	-	-	-	-	-
spp=2, begin=3	0.02971	-	-	-	-	-
spp=2, begin=4	0.22300	0.18438	-	-	-	-
spp=2, begin=6	0.00298	0.24626	0.01668	-	-	-
spp=3, begin=1	2.1e-09	2.5e-07	2.1e-09	3.4e-05	-	-
spp=3, begin=2	< 2e-16	< 2e-16	< 2e-16	4.7e-15	0.00093	-
spp=3, begin=3	6.5e-13	4.7e-11	2.6e-13	1.5e-08	0.17521	0.03504
spp=3, begin=4	< 2e-16	< 2e-16	< 2e-16	< 2e-16	1.5e-05	0.20762
spp=3, begin=6	< 2e-16	< 2e-16	< 2e-16	< 2e-16	< 2e-16	6.7e-11
spp=4, begin=1	0.10669	0.47135	0.56869	0.07878	1.5e-07	< 2e-16
spp=4, begin=2	0.00700	0.45114	0.04192	0.69568	6.4e-06	3.1e-16
spp=4, begin=3	0.21945	0.18323	0.99720	0.01693	2.6e-09	< 2e-16
spp=4, begin=4	0.03126	0.98657	0.18438	0.25511	2.8e-07	< 2e-16
spp=4, begin=6	3.1e-08	8.3e-06	5.9e-08	0.00066	0.30499	3.0e-07
spp=5, begin=1	0.00029	0.03705	0.00130	0.33340	0.00474	6.3e-10
spp=5, begin=2	8.6e-06	0.00165	2.7e-05	0.03705	0.12142	2.2e-06
spp=5, begin=3	0.00081	0.08404	0.00381	0.55452	0.00089	1.2e-11
spp=5, begin=4	6.9e-09	8.6e-07	7.8e-09	7.7e-05	0.95250	0.00119
spp=5, begin=6	8.5e-13	4.6e-11	3.5e-13	1.5e-08	0.18757	0.03222

	spp=3, begin=3	spp=3, begin=4	spp=3, begin=6	spp=4, begin=1	spp=4, begin=2	spp=4, begin=3
spp=1, begin=2	-	-	-	-	-	-
spp=1, begin=3	-	-	-	-	-	-
spp=1, begin=4	-	-	-	-	-	-
spp=1, begin=6	-	-	-	-	-	-
spp=2, begin=1	-	-	-	-	-	-
spp=2, begin=2	-	-	-	-	-	-
spp=2, begin=3	-	-	-	-	-	-
spp=2, begin=4	-	-	-	-	-	-
spp=2, begin=6	-	-	-	-	-	-
spp=3, begin=1	-	-	-	-	-	-
spp=3, begin=2	-	-	-	-	-	-
spp=3, begin=3	-	-	-	-	-	-
spp=3, begin=4	0.00217	-	-	-	-	-
spp=3, begin=6	3.7e-14	1.8e-05	-	-	-	-
spp=4, begin=1	6.7e-11	< 2e-16	< 2e-16	-	-	-
spp=4, begin=2	2.1e-09	< 2e-16	< 2e-16	0.16368	-	-
spp=4, begin=3	3.1e-13	< 2e-16	< 2e-16	0.56869	0.04211	-
spp=4, begin=4	6.8e-11	< 2e-16	< 2e-16	0.46529	0.46529	0.18438
spp=4, begin=6	0.00993	3.5e-09	< 2e-16	2.6e-06	0.00014	6.5e-08
spp=5, begin=1	1.7e-05	9.9e-12	< 2e-16	0.01006	0.17804	0.00142
spp=5, begin=2	0.00298	5.9e-08	< 2e-16	0.00044	0.01345	3.2e-05
spp=5, begin=3	1.6e-06	1.6e-13	< 2e-16	0.02353	0.31514	0.00412
spp=5, begin=4	0.18226	6.2e-05	5.4e-14	4.0e-07	1.6e-05	9.7e-09
spp=5, begin=6	0.98651	0.00231	7.2e-15	6.8e-11	2.0e-09	3.5e-13

	spp=4, begin=4	spp=4, begin=6	spp=5, begin=1	spp=5, begin=2	spp=5, begin=3	spp=5, begin=4
spp=1, begin=2	-	-	-	-	-	-
spp=1, begin=3	-	-	-	-	-	-
spp=1, begin=4	-	-	-	-	-	-
spp=1, begin=6	-	-	-	-	-	-
spp=2, begin=1	-	-	-	-	-	-
spp=2, begin=2	-	-	-	-	-	-
spp=2, begin=3	-	-	-	-	-	-
spp=2, begin=4	-	-	-	-	-	-
spp=2, begin=6	-	-	-	-	-	-
spp=3, begin=1	-	-	-	-	-	-
spp=3, begin=2	-	-	-	-	-	-
spp=3, begin=3	-	-	-	-	-	-
spp=3, begin=4	-	-	-	-	-	-
spp=3, begin=6	-	-	-	-	-	-
spp=4, begin=1	-	-	-	-	-	-
spp=4, begin=2	-	-	-	-	-	-
spp=4, begin=3	-	-	-	-	-	-
spp=4, begin=4	-	-	-	-	-	-
spp=4, begin=6	9.5e-06	-	-	-	-	-
spp=5, begin=1	0.03732	0.03055	-	-	-	-
spp=5, begin=2	0.00160	0.35404	0.27740	-	-	-
spp=5, begin=3	0.08603	0.00893	0.71957	0.15188	-	-
spp=5, begin=4	9.4e-07	0.40000	0.00429	0.10430	0.00117	-
spp=5, begin=6	8.1e-11	0.00977	1.7e-05	0.00271	1.8e-06	0.15887

P value adjustment method: BH

Appendix S2. Pairwise comparisons using Log-Rank test for move along treatment.

	spp=1, treat=1	spp=1, treat=2	spp=1, treat=3	spp=2, treat=1	spp=2, treat=2	spp=2, treat=3
spp=1, treat=2	0.31373	-	-	-	-	-
spp=1, treat=3	1.6e-09	7.0e-07	-	-	-	-
spp=2, treat=1	0.05803	0.00866	1.6e-11	-	-	-
spp=2, treat=2	0.25066	0.04825	5.1e-10	0.31373	-	-
spp=2, treat=3	6.1e-09	2.2e-06	0.68718	4.9e-11	1.6e-09	-
spp=3, treat=1	1.9e-08	4.5e-06	0.89406	2.0e-10	5.0e-09	0.82234
spp=3, treat=2	< 2e-16	< 2e-16	8.4e-09	< 2e-16	< 2e-16	3.7e-10
spp=3, treat=3	< 2e-16	< 2e-16	< 2e-16	< 2e-16	< 2e-16	< 2e-16
spp=4, treat=1	0.86440	0.26691	1.2e-08	0.07806	0.32423	3.7e-08
spp=4, treat=2	0.00232	0.04752	0.00087	2.3e-05	0.00025	0.00264
spp=4, treat=3	< 2e-16	< 2e-16	0.00053	< 2e-16	< 2e-16	6.2e-05
spp=5, treat=1	0.00760	0.09052	0.00113	0.00010	0.00080	0.00267
spp=5, treat=2	2.8e-12	1.3e-09	0.14337	2.8e-14	1.6e-12	0.07619
spp=5, treat=3	< 2e-16	< 2e-16	3.6e-05	< 2e-16	< 2e-16	3.5e-06

	spp=3, treat=1	spp=3, treat=2	spp=3, treat=3	spp=4, treat=1	spp=4, treat=2	spp=4, treat=3
spp=1, treat=2	-	-	-	-	-	-
spp=1, treat=3	-	-	-	-	-	-
spp=2, treat=1	-	-	-	-	-	-
spp=2, treat=2	-	-	-	-	-	-
spp=2, treat=3	-	-	-	-	-	-
spp=3, treat=1	-	-	-	-	-	-
spp=3, treat=2	2.3e-08	-	-	-	-	-
spp=3, treat=3	< 2e-16	9.6e-09	-	-	-	-
spp=4, treat=1	1.0e-07	< 2e-16	< 2e-16	-	-	-
spp=4, treat=2	0.00285	< 2e-16	< 2e-16	0.00278	-	-
spp=4, treat=3	0.00055	0.00694	< 2e-16	< 2e-16	2.4e-12	-
spp=5, treat=1	0.00385	< 2e-16	< 2e-16	0.00820	0.81245	2.0e-11
spp=5, treat=2	0.09052	0.00035	8.4e-15	4.1e-11	9.5e-06	0.07009
spp=5, treat=3	0.00010	0.08414	7.2e-13	< 2e-16	7.8e-13	0.29140

	spp=5, treat=1	spp=5, treat=2
spp=1, treat=2	-	-
spp=1, treat=3	-	-
spp=2, treat=1	-	-
spp=2, treat=2	-	-
spp=2, treat=3	-	-
spp=3, treat=1	-	-
spp=3, treat=2	-	-
spp=3, treat=3	-	-
spp=4, treat=1	-	-
spp=4, treat=2	-	-
spp=4, treat=3	-	-
spp=5, treat=1	-	-
spp=5, treat=2	5.5e-06	-
spp=5, treat=3	3.6e-11	0.04210

P value adjustment method: BH

Appendix S3. Pairwise comparisons using Log-Rank test for photoinhibition treatment under summer condition.

	spp=1, treat=4	spp=1, treat=5	spp=2, treat=4	spp=2, treat=5	spp=3, treat=4	spp=3, treat=5
spp=1, treat=5	0.00050	-	-	-	-	-
spp=2, treat=4	0.26495	2.0e-05	-	-	-	-
spp=2, treat=5	6.4e-07	0.12860	1.5e-08	-	-	-
spp=3, treat=4	< 2e-16	3.4e-09	< 2e-16	4.2e-06	-	-
spp=3, treat=5	< 2e-16	< 2e-16	< 2e-16	< 2e-16	< 2e-16	-
spp=4, treat=4	0.75162	0.00112	0.16142	1.7e-06	< 2e-16	< 2e-16
spp=4, treat=5	< 2e-16	1.0e-15	< 2e-16	4.8e-12	0.00486	1.4e-11
spp=5, treat=4	2.3e-07	0.06761	4.8e-09	0.73637	4.1e-05	< 2e-16
spp=5, treat=5	1.3e-12	0.00015	1.7e-14	0.01796	0.04099	< 2e-16

	spp=4, treat=4	spp=4, treat=5	spp=5, treat=4
spp=1, treat=5	-	-	-
spp=2, treat=4	-	-	-
spp=2, treat=5	-	-	-
spp=3, treat=4	-	-	-
spp=3, treat=5	-	-	-
spp=4, treat=4	-	-	-
spp=4, treat=5	< 2e-16	-	-
spp=5, treat=4	6.3e-07	2.4e-10	-
spp=5, treat=5	3.2e-12	2.5e-06	0.05594

P value adjustment method: BH

Appendix S4. Pairwise comparisons using Log-Rank test for photoinhibition treatment under autumn condition.

	spp=1, treat=6	spp=1, treat=7	spp=2, treat=6	spp=2, treat=7	spp=3, treat=6	spp=3, treat=7
spp=1, treat=7	0.00141	-	-	-	-	-
spp=2, treat=6	0.68288	0.00487	-	-	-	-
spp=2, treat=7	2.2e-05	0.31093	0.00013	-	-	-
spp=3, treat=6	3.8e-11	0.00423	1.7e-09	0.14547	-	-
spp=3, treat=7	9.7e-07	0.05603	4.9e-06	0.33167	0.68288	-
spp=4, treat=6	0.00177	0.70108	0.00783	0.12903	0.00020	0.01879
spp=4, treat=7	1.8e-09	0.00398	1.5e-08	0.06624	0.52565	0.48983
spp=5, treat=6	< 2e-16	2.6e-09	< 2e-16	1.8e-06	0.00021	0.00023
spp=5, treat=7	< 2e-16	< 2e-16	< 2e-16	< 2e-16	< 2e-16	2.1e-11

	spp=4, treat=6	spp=4, treat=7	spp=5, treat=6
spp=1, treat=7	-	-	-
spp=2, treat=6	-	-	-
spp=2, treat=7	-	-	-
spp=3, treat=6	-	-	-
spp=3, treat=7	-	-	-
spp=4, treat=6	-	-	-
spp=4, treat=7	0.00046	-	-
spp=5, treat=6	1.0e-11	0.00370	-
spp=5, treat=7	< 2e-16	1.0e-10	0.00023

P value adjustment method: BH

Appendix S5. Pairwise comparisons using Log-Rank test for cold stratification treatment.

	spp=1, treat=8	spp=1, treat=9	spp=2, treat=8	spp=2, treat=9	spp=3, treat=8	spp=3, treat=9
spp=1, treat=9	0.07746	-	-	-	-	-
spp=2, treat=8	0.11525	0.76155	-	-	-	-
spp=2, treat=9	0.00074	0.16222	0.06910	-	-	-
spp=3, treat=8	< 2e-16	1.3e-13	< 2e-16	4.7e-09	-	-
spp=3, treat=9	0.41166	0.47011	0.53647	0.03488	2.8e-15	-
spp=4, treat=8	0.14657	0.68385	0.76159	0.04131	< 2e-16	0.53647
spp=4, treat=9	0.01009	0.50659	0.31806	0.48603	8.2e-12	0.15233
spp=5, treat=8	3.1e-12	1.7e-06	3.0e-08	0.00113	0.02750	5.3e-08
spp=5, treat=9	< 2e-16	2.8e-14	< 2e-16	1.7e-10	0.11525	2.8e-15

	spp=4, treat=8	spp=4, treat=9	spp=5, treat=8
spp=1, treat=9	-	-	-
spp=2, treat=8	-	-	-
spp=2, treat=9	-	-	-
spp=3, treat=8	-	-	-
spp=3, treat=9	-	-	-
spp=4, treat=8	-	-	-
spp=4, treat=9	0.22236	-	-
spp=5, treat=8	5.9e-10	3.3e-05	-
spp=5, treat=9	< 2e-16	6.6e-13	0.00104

P value adjustment method: BH

CAPÍTULO III

Considerações Finais

Este foi o primeiro estudo de caracterização ecofisiológica de espécies nativas campestres da família Iridaceae do bioma Pampa para a região do Rio Grande do Sul. De acordo com as características das sementes estudadas para *Herbertia lahue*, *H. pulchella* e *Kelissa brasiliensis*, detectamos diversas diferenças relacionadas a sua ecologia reprodutiva. Este conhecimento se mostra essencial para a elaboração de efetivas estratégias de conservação que visem a restauração do bioma Pampa através no manejo de sua flora nativa.

Mesmo sendo bastante conservados evolutivamente, todos os caracteres de sementes analisados neste trabalho demonstraram variações significativas entre as espécies analisadas. Apesar de não ter sido possível distinguí-las taxonomicamente através deles, caracteres de sementes potencialmente informativos foram reconhecidos. Futuras investigações com representantes da tribo Tigridieae que busquem contemplar os caracteres de sementes analisados em nosso estudo e combinação com diversos outros apontados na literatura (ex. categorias de formato de sementes, coloração, dimensões do embrião, micromorfologia da superfície de revestimento, etc) podem auxiliar a esclarecer melhor a história evolutiva do grupo.

Um dos resultados mais relevantes deste estudo foi o reconhecimento dos requerimentos e preferências ecofisiológicas das espécies observadas. De acordo com os testes de embebição e germinação de sementes pode-se revelar informações acerca dos mecanismos adaptativos desenvolvidos por estas espécies campestres que possivelmente garantem sua sobrevivência em seus ambientes de naturais. Registramos a ocorrência de dormência fisiológica nas sementes de todas as espécies investigadas. Ademais, a resposta germinativa de todas as sementes foi favorecida ao serem incubadas sob condições de fotoinibição. Apesar destas convergências, distintas preferências por condições de temperatura foram verificadas. Estes requisitos germinativos associados aos dados levantados sobre produção de sementes, suas dimensões e investimentos em biomassa, foram interpretados como adaptações que influenciam o *fitness* das espécies na conquista de seu habitat e, portanto, em sua distribuição natural.

Adicionalmente, confirmamos através de citometria de fluxo que os níveis de ploidia de *Herbertia lahue* podem ser distinguidos por caracteres florais e possuem diferenças notáveis entre suas características de sementes e ecofisiologia. De fato, nosso estudo está registrando pela primeira vez populações diploides de *Herbertia lahue*. Porém, estudos citogenéticos que contemplem essas populações precisam ser feitos para confirmar o citótipo.

Além disso, uma reavaliação dos limites das espécies através de uma abordagem de genética populacional dentre os citótipos de *Herbertia lahue*, que reconheça grupos genéticos distintos suportados por características de sementes e adaptações ecofisiológicas como caracteres diagnósticos, poderia revelar um processo de especiação recente.

Ao final, recomenda-se que a partir das informações adquiridas sobre o comportamento ecofisiológico destes representantes da tribo Tigridaeae, futuros estudos de germinação de médio e longo prazo com espécies de parentesco próximo aos gêneros *Herbertia* e *Kelissa* sejam realizados *in situ*. Deste modo, poderá se verificar se os dados até então obtidos podem ser utilizados na confecção de práticas de manejo em prol da restauração de áreas campestres do bioma Pampa.