GEOVANE DA SILVA DIAS

THE ROLE OF REPRODUTIVE AND VEGETATIVE FUNCTIONAL TRAITS ON ABIOTIC STRESS RESPONSES IN SPECIES FROM CAMPO RUPESTRE AND RESTINGA

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-graduação em Fisiologia Vegetal, área de concentração em Fisiologia Vegetal, para a obtenção do título de Doutor.

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O PAPEL DOS ATRIBUTOS FUNCIONAIS REPRODUTIVOS E VEGETATIVOS NAS RESPOSTAS AO ESTRESSE ABIÓTICO EM ESPÉCIES DO CAMPO RUPESTRE E DA RESTINGA

Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-graduação em Fisiologia Vegetal, área de concentração em Fisiologia Vegetal, para a obtenção do título de Doutor.

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DEDICATÓRIA

A todos apaixonados e entusiastas pela biologia, ecologia e fisiologia de sementes e em memória do meu avô Joãozinho Bandeira e de tia Maria do Carmo.

Dedico

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EPÍGRAFE

“Se não houver frutos,
valeu a beleza das flores;
se não houver flores,
valeu a sombra das folhas;
se não houver folhas,
valeu a intenção da semente.”

Henfil

RESUMO

As plantas estão constantemente sujeitas a condições estressantes, tendo que apresentar mecanismos de resistência aos mais diversos fatores estressores. Os atributos vegetativos e das sementes fornecem respostas de como as espécies conseguiram se adaptar aos mais diversos tipos de ambiente. Entender como as caraterísticas das plantas respondem ao ambiente fornece conhecimentos importantes para a conservação da biodiversidade, podendo ser utilizada para informar decisões sobre a preservação, conservação de espécies, populações ou comunidades, e em programas de restauração ecológica. No cenário atual climático as populações vegetais estão propensas a eventos de sazonalidade hídrica e aumento de temperatura. Tendo em vista este cenário climático, esse estudo teve como foco dois ecossistemas: o Campo rupestre e a Restinga. Ambientes esses que tem importância nos serviços ecossistêmicos que eles prestam e pelas constantes ameaças que vem sofrendo ao longo dos anos. Para isso quatro estudos foram desenvolvidos: i) o efeito dos ciclos de hidratação e desidratação (HD) em sementes de Campo rupestre; ii) extremos de alta temperatura e ciclos HD na germinação de três espécies de *Vellozia*; iii) a relação entre atributos vegetativos e das sementes em espécies de uma Restinga do sudeste brasileiro; iv) o estresse combinado de altas temperatura e hidratação descontínua em uma espécie característica de Restinga. É possível verificar que há uma relação entre atributos biométrico e germinativos das sementes de quatro espécies do Campo rupestre, e que eles podem ser utilizados na adição e escolha de espécies para a restauração de ambientes perturbados. No entanto, sua progressão pode ser prejudicial à germinação das sementes levando a morte e consequentemente impactando na regeneração e manutenção das populações naturais. Os ciclos HD melhoram os parâmetros germinativos e confere tolerância ao estresse pelo fogo em sementes de Vellozia sp. A tolerância ao fogo gerada pela passagem das sementes de Vellozia pelos ciclos HD é um processo de exaptação, induzindo maior acúmulo de prolina e ácido abscísico. A vegetação da Restinga apresenta estratégia ecológica estresse-tolerante, com requisitos germinativos de luz e temperatura diversos de suas sementes. Estudo de correlação mostrou que há relação positiva entre conteúdo de matéria seca foliar com os atributos biométricos e germinativos das sementes. E quando submetemos a espécie *Canavalia rosea* a eventos de HD e altas temperaturas, as suas sementes apresentam maior susceptibilidade aos estresses combinados do que isolados, reduzindo sua germinação e viabilidade. Entender a dinâmica de resposta das espécies do Campo rupestre e Restinga frente a estresse abiótico com o hídrico e o térmico, é fundamental na garantia da manutenção desses dois ecossistemas e dos serviços ecossistêmicos que eles prestam. Centrar esforços, recursos e pesquisas na ecofisiologia de sementes se faz necessário cada vez mais, uma vez que elas são responsáveis pela manutenção das populações naturais, na conquista de novos ambientes e recrutamento de plântulas.

Palavra-chave: Semente; germinação; ciclos hidratação-desidratação; alta temperatura.

ABSTRACT

Plants are constantly subjected to stressful conditions, requiring them to develop resistance mechanisms to a wide range of stressors. Vegetative and seed traits provide insights into how species have adapted to diverse environmental conditions. Understanding how plant characteristics respond to the environment offers crucial knowledge for biodiversity conservation, which can inform decisions on preservation, conservation of species, populations, or communities, and ecological restoration programs. In the current climate scenario, plant populations are prone to events of water seasonality and rising temperatures. Given this climatic context, this study focused on two ecosystems: the *Campo rupestre* and the *Restinga*. These environments are significant for the ecosystem services they provide and the constant threats they have faced over the years. To this end, four studies were conducted: i) the effect of hydration and dehydration (HD) cycles on *Campo rupestre* seeds; ii) extreme high temperatures and HD cycles on the germination of three *Vellozia* species; iii) the relationship between vegetative and seed traits in species from a *Restinga* in southeastern Brazil; and iv) the combined stress of high temperatures and discontinuous hydration on a characteristic *Restinga* species. It was observed that there is a relationship between biometric and germination traits in seeds of four *Campo rupestre* species, which can be used to select and add species for the restoration of disturbed environments. However, their progression may be detrimental to seed germination, leading to mortality and consequently impacting the regeneration and maintenance of natural populations. HD cycles improve germination parameters and confer fire stress tolerance in *Vellozia* sp. seeds. The fire tolerance generated by *Vellozia* seeds undergoing HD cycles is an exaptation process, inducing greater accumulation of proline and abscisic acid. The *Restinga* vegetation exhibits a stress-tolerant ecological strategy, with diverse light and temperature requirements for seed germination. A correlation study showed a positive relationship between leaf dry matter content and seed biometric and germination traits. When the species *Canavalia rosea* was subjected to HD events and high temperatures, its seeds showed greater susceptibility to combined stresses than to isolated ones, reducing germination and viability. Understanding the response dynamics of *Campo rupestre* and *Restinga* species to abiotic stresses, such as water and thermal stress, is essential for ensuring the maintenance of these two ecosystems and the ecosystem services they provide. Focusing efforts, resources, and research on seed ecophysiology is increasingly necessary, as seeds are responsible for the maintenance of natural populations, the colonization of new environments, and seedling recruitment.

Keywords: Seed; germination; hydration-dehydration cycles; high temperature.

INDICADORES DE IMPACTO

O Campo rupestre e a Restinga prestam importantes serviços ecossistêmicos de provisão, regulação, cultural e suporte. Nosso trabalho tem impacto a nível cultural, pois a sociedade que mora próximo a esses dois ecossistemas o utilizam para prática de esporte e lazer. No âmbito tecnológico a aplicação dos ciclos de hidratação-desidratação pode ser utilizada como técnica de *priming* para espécies nativas em projetos de restauração ecológica. Tendo como impacto social diminuir ou amenizar os efeitos das mudanças climáticas através de ações ambientais de manutenção e restauração dos ecossistemas naturais. Diminuir o impacto das ações antrópicas sobre esses dois ecossistemas é essencial para a manutenção e preservação deles. O presente trabalho pode ser classificado dentro das Política Nacional de Extensão com impacto na cultura, educação, meio ambiente e tecnologia e produção. Dentre os 17 Objetivos de Desenvolvimento Sustentável da Organização das Nações Unidas, os impactos da pesquisa estão alinhados com os eixos 13 (Ação Contra a Mudança Global do Clima) e 17 (Vida Terrestre). Em que o foco central da nossa pesquisa foi entender quais as respostas das espécies vegetais do Campo rupestre e Restinga, frente a estresses hídrico e térmico, que são ambientes com serviços ecossistêmicos e que são negligenciados e ameaçados constantemente.

IMPACT INDICATORS

The *Campo rupestre* and *Restinga* ecosystems provide essential ecosystem services, including provisioning, regulating, cultural, and supporting services. Our work has a cultural impact, as the communities living near these two ecosystems utilize them for sports and recreational activities. On a technological level, the application of hydration-dehydration (HD) cycles can be used as a priming technique for native species in ecological restoration projects. Socially, this research aims to mitigate the effects of climate change through environmental actions focused on the maintenance and restoration of natural ecosystems. Reducing the impact of anthropogenic activities on these two ecosystems is crucial for their preservation and sustainability. This study aligns with the National Extension Policy, impacting culture, education, the environment, technology, and production. Among the 17 United Nations Sustainable Development Goals (SDGs), the research impacts are aligned with Goal 13 (Climate Action) and Goal 15 (Life on Land). The central focus of our research was to understand how plant species from the *Campo rupestre* and *Restinga* respond to water and thermal stress, as these environments provide critical ecosystem services yet are often neglected and threatened. By addressing these challenges, our work contributes to the conservation of these ecosystems and supports global efforts to combat climate change and promote terrestrial biodiversity.

SUMÁRIO

FIRST PART
INTRODUCTION

Brazil is a megadiverse tropical country, encompassing a range of phytophysiognomies from wet to dry, including biodiverse fields and grasslands. In recent years, the majority of research has focused on forest plant species, leaving open ecosystems largely neglected. As a result, there is a significant gap in studies on non-forest ecosystems (GUERRA et al., 2020), as well as a disparity in research funding and investment when comparing forested and non-forested environments. Research on the ecological restoration of tropical ecosystems has also been disproportionately directed toward forested areas rather than non-forested ones (GUERRA et al., 2020). This lack of research and understanding of vegetation dynamics and environmental processes in non-forest ecosystems poses a critical limitation to the development and implementation of effective policies, laws, and practices for their restoration and management. Brazil is home to two globally recognized biodiversity hotspots, the Atlantic Forest (Mata Atlântica) and the *Cerrado* (MYERS et al., 2000; CEPF, 2022), underscoring the urgent need to address these research gaps to ensure the conservation and sustainable use of these unique and threatened ecosystems.

Despite harboring the highest biodiversity among savannas, open fields, and grasslands, which cover 27% of the country's territory, these ecosystems have historically been neglected in terms of conservation efforts and scientific research (OVERBECK et al., 2022). By 2019, Brazil had lost approximately 46% of the original distribution of its grassland and savanna ecosystems (OVERBECK et al., 2022). Although these phytogeographical regions exhibit high species richness and endemism, they are particularly vulnerable to anthropogenic impacts and climate change.

Since colonization, these environments have been—and continue to be—extensively exploited, from their occupation for the establishment of large urban centers to the expansion of agricultural frontiers and the extractive exploitation of their resources (mineral, animal, plant, cultural, among others) (BORGES, REZENDE & COELHO JÚNIOR, 2009; FERNANDES, 2016). These ecosystems are situated in areas of intense urbanization, industrialization, and agricultural activity, alongside anthropogenic actions that contribute to rising average temperatures, increased atmospheric CO_2 emissions, and alterations in rainfall patterns in tropical regions (SCARANO & CEOTTO, 2015; SOUZA et al., 2020; HOFMANN et al., 2021). Coupled with the lack of environmental legislation or non-compliance with laws protecting these ecosystems, these factors represent significant threats

to the risk of extinction for populations of native species. The weakening of laws protecting native vegetation (Law 12.651, amended by Law 12.727) could undermine soil and watershed protection, biodiversity conservation, and even agricultural productivity, with no clear benefit to the country.

The *Cerrado* encompasses forest, savanna, and grassland formations (such *as campo sujo*, *campo limpo*, and *campo rupestre*) (RIBEIRO & WALTER, 2008). The *Campo rupestre* is distributed across three major Brazilian biomes. It occurs in the westernmost part of the *Mata Atlântica* in the state of *Minas Gerais*, as well as in the *Cerrado* savannas to the north and west of *Minas Gerais*. In the state of Bahia, the *Campo rupestre* is found within the semi-arid *Caatinga* biome (ZAPPI et al., 2017). *Campo rupestre* is characterized as mountaintop vegetation, occurring in quartzite, sandstone, and ironstone formations, and is classified into quartzitic, granitic, and ferruginous *campo rupestre* (JACOBI & CARMO, 2008; SILVEIRA et al., 2016).

In the literature, there is considerable variation in nomenclature and confusion with other high-altitude vegetation formations, such as high-altitude grasslands (*campos de altitude*) (VASCONCELOS, 2011). Silveira et al. (2016) provide two definitions for *Campo rupestre*: the sensu lato, or simply *Campo rupestre*—a mosaic of mountainous, grassy-shrub vegetation, prone to fire, with quartzite, sandstone, or ferruginous rocky outcrops (ferruginous formations in bands, such as itabirites and ironstone known locally as *canga*), along with sandy, stony, and flooded grasslands. Within the *Campo rupestre* landscape, there are also patches of transitional vegetation, such as Cerrado, gallery forests, and mountaintop forests. The *Campo rupestre* sensu stricto comprises only the grassland mosaic and associated vegetation on rocky outcrops, excluding forests, gallery forests, and mountaintop forests (SILVEIRA et al., 2016). These ecosystems provide a range of ecosystem services, including water supply, hydroelectric power generation, ecotourism, medicinal plants, native forage production, and underground carbon storage (FERNANDES et al., 2020).

The *Campo rupestre* harbors the greatest biodiversity and degree of endemism among plant species in Brazil (GIULIETTI et al., 1988; SILVEIRA et al., 2016). Its flora comprises a mosaic of species with diverse adaptations and multiple biogeographical origins (SILVEIRA et al., 2016). *Campo rupestre* is characterized by nutrient-poor soils, water deficits, high irradiance, and elevated concentrations of trace elements (JACOBI & CARMO, 2008; OLIVEIRA et al., 2016). The *Campo rupestre* is the most biodiverse Brazilian ecosystem, with the highest number of endemic and rare species (Fig. 1). However, there are no strict public policies in place to protect this ecosystem (MIOLA et al., 2019). The *Campo rupestre*

is threatened by mining, annual anthropogenic fires, invasive species, overexploitation of ornamental plants, and uncontrolled urbanization (SILVEIRA et al., 2016). From a legislative perspective, the *Campo rupestre* is one of the most neglected ecosystems. First, there is no specific law to protect this environment. Second, the application of laws designed for other ecosystems, which are not analogous to the *Campo rupestre*, leaves it vulnerable to the overexploitation of its natural resources (MIOLA et al., 2019).

Figure 1: *Campo rupestre* of the *Serra da Calçada, Nova Lima-MG, Brazil.*

Source: From the author

The *Mata Atlântica* is composed of native forest formations and associated ecosystems, such as mangroves (*manguezais*), coastal sand dunes (*restingas*), high-altitude grasslands (*campos de altitude*), and inland swamps (*brejos interioranos*) (MMA, 2021). The *Restinga* ecosystem spans the entire Brazilian coastline, extending westward into the interior of the country (FALKENBERG, 1999). *Restinga* are sandy deposits parallel to the coastline, typically elongated in shape, formed by sedimentation processes and home to diverse plant communities influenced by marine conditions. They are considered edaphic communities because their development depends more on the nature of the substrate than on the climate (BRASIL, 2002). *Restinga* vegetation plays a crucial role in stabilizing sandy substrates and maintaining natural drainage, thereby preventing erosion. It comprises species of varying sizes, depending on the successional stage, including herbaceous, shrubby, and arboreal strata

(BRASIL, 2002). This vegetation provides food resources and nesting sites for both resident and migratory fauna (BRASIL, 1999; SCHERER et al., 2005).

The *Restinga* is a sandy deposit parallel to the coastline, characterized by a mosaic of physiognomically distinct plant communities influenced by marine conditions (Fig. 2). These communities exhibit unique edaphoclimatic characteristics, such as salinity, extreme temperatures, flooding, drought, constant wind, and nutrient-poor soils (BRASIL, 1996; BRASIL, 2002; SCARANO, 2002). The *Restinga* ecosystem boasts a high richness of plant species, with an estimated 1.598 species occurring along the Brazilian coast (RESTINGAS.NET, 2022).

Figure 2: *Restinga* of the *Parque Estadual Paulo César Vinha*, *Guarapari-ES, Brazil.*

Source: From the author

The *Restinga* ecosystem has been legally protected since the enactment of the old Forest Code (Law No. 4,771/1965), which classified it as a "dune stabilizer or mangrove stabilizer." This protection was reaffirmed by Law No. 12,651/2012 (Art. 4, Item VI), which governs the protection of native vegetation and designates *Restinga* as a Permanent Preservation Area (BRASIL, 2012). Additionally, the protection of Restinga is ensured by Law N^{o}. 11,428/2006 – the Atlantic Forest Law (Lei da Mata Atlântica), which "regulates the use and protection of native vegetation in the Atlantic Forest biome," including *Restinga* as one of its native forest formations and/or associated ecosystems (BRASIL, 2006).

The similarity between *Restinga* and *Campo rupestre* arises from the floristic connections between these two formations (GIULIETTI & PIRANI, 1988; FIASCHI & PIRANI, 2009). Studies of different species from *Campo rupestre* and *Restinga* by Alves, Cardin, and Kropf (2007) confirmed that 16% of the taxa occur in both phytophysiognomies. Due to their shared edaphoclimatic and floristic characteristics, these environments are ideal for studying the physiological differences among the species that inhabit them. This floristic similarity is likely due to dispersal by hydrochory (ALVES, CARDIN & KROPF, 2007).

From an evolutionary perspective, native species in these two ecosystems have adapted to specific stressors in their environments. The edaphoclimatic conditions act as ecological filters that species must overcome to establish, reproduce, and colonize these areas (LAMBERS & OLIVEIRA, 2019). Edaphoclimatic factors play a crucial role in shaping vegetation in ecosystems, and both *Campo rupestre* and *Restinga* share characteristics such as shallow, nutrient-poor soils, water deficits, high irradiance, and elevated temperatures.

Water deficit and high temperatures are the primary stressors affecting germination and seedling establishment. According to reports from the Intergovernmental Panel on Climate Change (IPCC, 2013, 2021) and the *Painel Brasileiro de Mudança do Clima* (PBMC, 2013), the projected climate change scenario predicts altered rainfall patterns, and a temperature increase of 1.5–3°C for these ecosystems. If this scenario materializes, seeds in tropical ecosystems will be more likely to experience hydration and drying cycles (discontinuous hydration) in their tissues (BASKIN & BASKIN, 1982; DUBROVSKY, 1996) and encounter high temperatures during their time in the soil seed bank.

Functional traits are defined as any characteristic that indirectly influences fitness through its effects on growth, reproduction, and survival. These traits can be morphological, biochemical, physiological, structural, phenological, or behavioral and are expressed in the phenotypes of individual organisms. They are considered relevant to an organism's response to the environment and/or its effects on ecosystem properties (VIOLLE et al., 2007; DÍAZ et al., 2013). Strategies such as competition, stress tolerance, and ruderalism represent different ways organisms adapt to their environment to optimize survival and reproduction (GRIME & PIERCE, 2012; PIERCE et al., 2017). Vegetative and seed traits are essential for understanding and maintaining natural ecosystems and for selecting species for ecological restoration. Integrating vegetative and seed functional traits provides insights into species tolerance or susceptibility during seedling recruitment and establishment, given their critical role in the occupancy, recruitment, and maintenance of natural populations (AUBIN et al.,

2016; DAILLING et al., 2020). There is a need to focus research efforts on dispersal, longevity, germination timing, and establishment, utilizing morphological, physiological, and biochemical seed characteristics (SAATKAMP et al., 2019).

Tropical non-forest ecosystems such as *Campo rupestre* and *Restinga* are characterized by high biodiversity, species endemism, and significant ecosystem services. However, their preservation and the legislation protecting these areas are either deficient or nearly non-existent. While *Restinga* is protected by resolutions and legal frameworks (BRASIL, 2002), *Campo rupestre* lacks specific laws ensuring its preservation (SILVEIRA et al., 2016; MIOLA et al., 2019). Additionally, proposed bills such as PL 3209/2021 and PL 4,444/2021, which aim to redefine priority areas of *Restinga* or restrict beach access, create gaps in its preservation.

Therefore, hypothesizing a current climate scenario of increased water deficit and rising temperatures, native plant species in diverse biodiverse phytophysiognomies exhibit physiological and metabolic adjustments to various environmental stresses. The passage of seeds through discontinuous hydration cycles may confer greater tolerance to water and thermal stress events. Given this context, this study aimed to (1) understand the germination of native species from *Campo rupestre* and *Restinga* under water availability and high-temperature stress, (2) investigate the biochemical and physiological responses of seeds to combined stress, and (3) explore the relationship between vegetative, biometric, and germinative traits of seeds from these two ecosystems.

This work, divided into four chapters, examined 19 species from *Campo rupestre* and *Restinga* regarding germination, hydration-dehydration cycles, increasing temperatures, and the relationships between seed and vegetative functional traits. The goal was to understand how these species might tolerate combined stress factors in the current climate scenario and in light of future climate change. The results could provide insights into seed persistence and plant tolerance to multiple stresses, contributing to the conservation and restoration of these ecosystems.

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SECOND PART

PAPER 1: DO THE FUNCTIONAL TRAITS OF THE SEEDS OF *CAMPO RUPESTRE* SPECIES CORRELATE WITH TOLERANCE TO HYDRATION-DEHYDRATION CYCLES?

The manuscript is written by *Planta* journal standards.

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MAIN CONCLUSION

Campo rupestre seeds can tolerate hydration-dehydration cycles. Seed size affects germination speed and synchronization, with larger seeds germinating more rapidly and synchronously. Smaller seeds have greater thickness and proline.

ABSTRACT

After dispersal, the seeds encounter a series of barriers and adverse conditions for germination and seedling establishment. In open seasonal environments such as *campo rupestre*, desiccation tolerance is an important trait to seeds' survival. The biometric attributes of seeds respond to the environmental conditions in which the seeds are produced. Still, few studies relate the biometric, germination, and biochemical attributes of seeds to their desiccation tolerance capacity. Therefore, this study aimed to investigate whether there is any relationship between the biometric, germination, and biochemical traits of the seeds of four species of *campo rupestre* when subjected to hydration-dehydration (HD) cycles. To this end, seeds of *Deluciris rupestrris*, *Kielmeyra coriacea*, *Lafoensia pacari*, and *Mimosa calodendron* were exposed to up to 2 HD cycles and their length, width, thickness, fresh weight, water content, germination percentage, speed, time and synchronization were assessed. In general, the cycles benefited the germination of all four species. One HD cycle promoted an increase in the speed and synchronization of germination, while the second HD cycle proved to be more harmful, reducing germination and leading to proline accumulation at the end of the cycle for the species *K. coriacea*. With the progression of HD cycles, germinative traits are related to biometric traits. Where length, weight, and fresh weight with *K. coriacea*, germination percentage, and speed with *L.pacari*, proline, and thickness with *M. calodendron* and time with *M. calodendron*. The combined use of germination, biochemical, and biometric attributes is a tool for selecting species in *campo rupestre* restoration programs.

Key-words: Seed functional trait, seed germination, proline, hydration-dehydration cycles.

INTRODUCTION

Seed biology research provides critical insights for biodiversity conservation and can inform decisions on in situ management of species, populations, or communities, *ex situ* seed conservation, ecological restoration (Saatkamp et al., 2019), and tolerance to abiotic stresses. Seed biometric traits reflect the environmental conditions experienced by the mother plants during seed development, influencing their size, weight, and shape (Peñaloza and Durán, 2015; Patrício and Trovão, 2020; Renzi et al., 2020). These traits are also key determinants of seed dispersal, longevity, and persistence in the soil seed bank (Bekker, Ozinga, and Thompson, 2003).

Seed functional traits are directly related to their survival and persistence in various environments. Seeds that tolerate desiccation (orthodox seeds) are typically small, lightweight, and have greater thickness (Baskin and Baskin, 2014). These seeds can survive low moisture and extreme temperatures, making them well-adapted to persist in the soil seed bank for extended periods (Tweddle et al., 2003). On the other hand, larger seeds (in length, width, and mass), heavier and with higher water content, tend to have a greater probability of survival under adverse conditions, such as water or thermal stress (Dantas et al., 2014; Saatkamp et al., 2019). These seeds generally possess greater nutritional reserves, which favor germination and seedling establishment in challenging environments (Westoby et al., 1996; Leishman et al., 2000).

In tropical environments, seed biometric traits are influenced by the environmental conditions experienced by the mother plant. In the *Cerrado*, for example, environmental conditions may act as cues signaling unfavorable conditions for seed germination (Sales, Pérez-García & Silveira, 2013). In semi-arid regions, species with larger seeds and greater nutritional reserves allow the embryo to germinate and survive longer without external nutrients (Patrício and Trovão, 2020). In more humid areas, species that produce large, high-biomass seeds exhibit slower initial development and depend on seed reserves. In contrast, smaller seeds are produced in larger quantities and have lower biomass (Polli et al., 2020).

When a plant is under stress, it increases the concentration of osmoprotectants (mainly sugars and amino acids) in its tissues, and this endogenous increase can be redirected to developing organs, such as seeds (Mattioli, Costantino, and Trovato, 2009; Wang, 2018). Seed thickness and metabolic adjustments are key mechanisms for maintaining longevity under water-limited conditions. These two characteristics positively correlate with seed viability and persistence in the soil seed bank (Davis et al., 2008; Long et al., 2015; Sano et al., 2016). Metabolites such as sugars and amino acids accumulate in plants in response to

various environmental stresses (Yamada and Osakabe, 2018; Batista-Silva et al., 2019; Rai, 2002). Plant species can produce seeds with greater mechanical resistance by developing more resistant seed coats and accumulating storage reserves, acquiring desiccation tolerance during the maturation phase (Radchuk and Borisjuk, 2014; Sripathy and Groot, 2023). The accumulation of proline during seed maturation is correlated with the induction of desiccation tolerance (Kavi Kishor and Sreenivasulu, 2014).

The relationship between seed biometric traits and metabolites has been documented for forest species, where seed traits and proline levels in seedling tissues are indicative of drought-tolerant species suitable for use in tree restoration programs (Kijowska-Oberc, Dylewski, and Ratajczak, 2023; Dias et al., 2024). However, for rupestrian fields (*campo rupestre*), species that naturally experience seasonal droughts, it is still not fully understood whether desiccation tolerance maintained over hydration-dehydration (HD) cycles can explain seed survival.

In climatically seasonal environments such as *campo rupestre*, seeds are exposed to seasonal water availability. The *campo rupestre* has two well-defined seasons: a dry winter and a rainy summer (Negreiros et al., 2014). Under the current climate change scenario, the frequency and intensity of seasonal water availability are escalating, leading to discontinuous hydration events in seed tissues, which may induce a priming effect (Dubrovisky, 1998; Santini et al., 2017). The ability of seeds to tolerate water deficit (or any stress) and recover the biochemical, physiological, and epigenetic information triggered during the germination is defined as "priming memory" (Dubrovisky, 1996; Chen and Arora, 2013; Louis, Dhanker and Puthur, 2023). However, the duration of hydration and the periodicity of HD cycles can cause hydration and drying damage to seeds, triggering physiological, biochemical, and cellular stress responses (Dias et al., 2024; Oliveira et al., 2024).

Correlating seed attributes such as dispersal, persistence, germination timing, and establishment is of paramount importance for the conservation of plant genetic resources (Saatkamp et al., 2019). A series of studies have linked seed attributes (biometric, physiological, and biochemical) to their survival and persistence in various environments. Our hypothesis is that the biometric characteristics of seeds from four *campo rupestre* species positively contribute to their germination and biochemical attributes after exposure to HD cycles. Our main objective is to understand whether there is a correlation between the biometric, germination, and biochemical (proline accumulation) functional traits of *campo rupestre* seeds when exposed to HD cycles.

MATERIAL AND METHODS

Seed collection

For this experiment, species were selected based on their seeds having no physiological dormancy and sufficient mass for biochemical analysis. Mature fruits were collected, and the seeds were processed in the laboratory and stored under refrigeration until the tests began. The experiment was conducted at the SEEDS Physiology Research Group laboratory at the Federal University of Lavras, Minas Gerais, Brazil.

The seeds were collected in Monumento Natural da Serra da Calçada (MONASC), Nova Lima, Minas Gerais, Brazil (20°05'55.2"S, 43°58'58.2"W). The local climate is classified as tropical with a dry winter (Cwb) according to the Köppen classification, with an average temperature of 20.7 °C, a low annual temperature range, and an average annual precipitation of 2,041.6 mm (Rios et al., 2023). The seeds used in the experiment were from the following species and collection periods: *Deluciris rupestris* (Ravenna) Lovo and A. Gil, collected in May 2021*; Kielmeyera coriacea* Mart. and Zucc., collected from July to 2022; *Lafoensia pacari* A.St.-Hil., collected in June 2021; and *Mimosa calodendron* Mart. ex Benth., collected in April 2021 (Fig. 1). The seeds were collected from 20 individuals per species and stored in Kraft paper at 4 °C until the start of the experiments, which were conducted in August 2024.

Seed functional traits

To assess the functional traits of the four species, 10 replicates of 10 seeds each were used to measure length (mm), width (mm), and thickness (mm) using a digital caliper, as well as seed mass (g) using an analytical balance with a precision of 0.001 g (Table 1). Seed moisture content was determined using 5 replicates of 20 seeds for *K. coriacea* and *L. pacari*, and 25 seeds for *D. rupestris* and *M. calodendron*. The initial fresh weight of the seeds was measured before and after drying in an oven at 105 °C for 24 hours (Brasil, 2009). The moisture content was calculated and expressed on a wet basis (Table 1).

Imbibition and drying curves

The imbibition curve was constructed using five replicates of 25 seeds for *K. coriacea* and *L. pacari*, and five replicates of 20 seeds for *D. rupestris* and *M. calodendron*. After measuring the initial fresh weight, the seeds were weighed on an analytical balance with a precision of 0.001 g. The seeds were weighed at one-hour intervals for the first 12 hours

under laboratory conditions. After 12 hours, the seeds were weighed every 12 hours until they reached 120 hours. Following this period, the seeds were weighed every 24 hours until radicle protrusion occurred (Supplementary Material Fig. S1).

For the drying curve, the seeds were soaked until they reached the midpoint of phase II for each species and then dried in silica gel at 25 °C until they returned to their initial fresh weight (Supplementary Material Fig. S2). To overcome physical dormancy, the seeds of *M. calodendron* were submerged in sulphuric acid for 5 minutes.

Hydration-dehydration (HD) cycles

Using the values from the imbibition curves, the imbibition and drying times were determined for each species, with the imbibition time corresponding to half of phase II of the imbibition curve and the drying time corresponding to the return of the initial fresh weight of the seeds. The imbibition and drying times, respectively, for each species were as follows: *D. rupestris* (120 and 24 h), *K. coriacea* (96 and 24 h), *L. pacari* (25 and 9 h), and *M. calodendron* (25 and 5 h)y. The control treatment (0HD) consisted of no hydration or drying, the 1HD seeds were imbibed in water for the time determined for each species and then dried according to the drying curve. The 2HD treatment was carried out the same way as 1HD, except the hydration and drying process was repeated twice (Fig. 2). Hydration and drying were conducted under controlled light conditions (12 hours of light and 12 hours of darkness) at a constant temperature of 25 °C in germination chambers.

Germination test

After applying the treatments, the seeds of the four species (5 replicates of 20 seeds each for *K. coriacea* and *L. pacari*, and 25 seeds each for *D. rupestris* and *M. calodendron*) were disinfected in a 1.0% sodium hypochlorite (NaClO) solution with two drops of commercial detergent for 10 minutes and then rinsed three times with deionized water. After disinfection, the seeds were placed in Petri dishes and randomly distributed in germination chambers under a 12-hour photoperiod with a light intensity of 40 μmol photons m^{-2} s^{-1}. The chambers were maintained at the optimal germination temperature of 25 °C for each species: *D. rupestris* (Freitas, 2023), *K. coriacea* (Ranieri et al., 2012), *L. pacari* (Oliveira and Lemes, 2014), and *M. calodendron* (Freitas, 2023). Germination was assessed daily, with root protrusion (<2 mm) used as the criterion for germination. At the end of the test, the following parameters were evaluated: germination percentage, germination speed index (Maguire,

1962), mean time to reach 50% germination (T_{50} - Farooq et al., 2005), and synchronization index (Z - Ranal and Santana, 2006).

Sample collection and proline analysis

The seeds of the four species were collected after hydration (considering the 1HD treatment) or rehydration (2HD treatment). Seeds that did not undergo HD cycles were collected at the midpoint of phase II of the imbibition curve for each species. Seeds subjected to HD cycles were collected at one-quarter of the time of phase II. Five replicates with 20 seeds (*K. coriacea* and *L. pacari*) and 25 seeds (*D. rupestris* and *M. calodendron*) were used, respectively. Each replicate was ground in liquid nitrogen, and the genetic pool was created using 0.1 g of fresh seeds (5 replicates with 20 seeds each for *K. coriacea* and *L. pacari*, and 25 seeds each for *D. rupestris* and *M. calodendron*).

Proline was determined according to Bates (1973), where the material was added to microtubes containing 2 mL of 3% sulfosalicylic acid. The material was centrifuged at 14,000 rpm for 5 minutes at room temperature. Endogenous proline levels were quantified by adding 500 µL of acidic ninhydrin solution (2.5 g of ninhydrin dissolved in 40 mL of 6M phosphoric acid and 60 mL of glacial acetic acid), 500 µL of glacial acetic acid, and 500 µL of the sample. The mixture was then vortexed and heated in a water bath at 100 °C for 1 hour. After cooling, the absorbance was measured at 520 nm using a spectrophotometer. A standard proline curve at 100 µg/mL was constructed to determine endogenous proline levels.

Statistical analysis

The experiment was conducted in a completely randomized design with three seed hydration conditions: control (0HD), one HD cycle (1HD), and two HD cycles (2HD). The Shapiro-Wilk and Levene tests were used to verify the normality and homogeneity of variances for the germination and biochemical data, respectively. This was followed by analysis of variance (ANOVA) and the Tukey test ($p < 0.05$). Calculations were performed using R version 4.3.1 (ExpDes.pt package). A Principal Component Analysis (PCA) was conducted to assess the effect of HD cycles on biometric, germination, and biochemical parameters. The following R packages were used: cluster, corrplot, factoextra, FactoMineR, ggplot2, ggsci, mice, PCAmixdata, readxl, sn, and PerformanceAnalytics.

RESULTS

Campo rupestre seeds show differences in biometric traits

The seeds of the four species differed in terms of their functional traits. *K. coriacea* and *L. pacari* exhibited the highest values for length (34.35 ± 1.9 mm and 22.00 ± 0.62 mm, respectively), width (13.46 ± 0.6 mm and 8.08 ± 0.19 mm, respectively), and fresh weight (0.60 ± 0.02 g and 0.19 ± 0.002 g, respectively). In terms of thickness, *M. calodendron* had the highest recorded value (1.52 ± 0.05 mm). Meanwhile, *D. rupestris* showed a higher moisture content in its seeds (10.67 ± 1.11%) (Fig. 1, Table 1).

Seed germination responses followed HD cycles

The four species responded differently to HD cycles. For *D. rupestris*, the cycles did not significantly affect the germination percentage (Fig. 3A, Table 2). However, the Germination Speed Index (GSI) showed significant differences ($F = 17.362$, $df = 2$, $p < 0.01$; Fig. 3B, Table 2), with a mean value of 2.22 ± 0.04 for the 1HD treatment. For T_{50}, the 1HD cycle reduced the time to 10.66 ± 0.37 days, with a significant effect observed for this variable ($F = 13.716$, $df = 2$, $p < 0.01$; Fig. 3C, Table 2). Regarding synchronization (Z), the passage of seeds through the cycles also yielded significant results ($F = 6.2982$, $df = 2$, $p < 0.05$; Fig. 3D, Table 2). However, both the 1HD and 2HD treatments reduced synchronization values. Therefore, for *D. rupestris*, the 1HD cycle was more effective in increasing germination speed but did not improve germination percentage or synchronization.

The species *K. coriacea* benefited from the passage of its seeds through the HD cycles. The germination percentage remained unchanged between 0HD and 1HD, but the 2HD treatment reduced seed germination by 79 ± 2.19% ($F = 12.4$, $df = 2$, $p < 0.01$; Fig. 4A, Table 2). However, for the other variables, the HD cycles showed improvements. The GSI was higher in the 1HD treatment compared to 0HD and 2HD ($F = 144.08$, $df = 2$, $p < 0.01$; Fig. 4B, Table 2). The 2HD treatment reduced T_{50} ($F = 168.65$, $df = 2$, $p < 0.01$; Fig. 4C, Table 2). Additionally, both 1HD and 2HD treatments were more effective than the control in synchronizing germination ($F = 8.5635$, $df = 2$, $p < 0.01$; Fig. 4D, Table 2).

The germination of *L. pacari* was also affected by HD cycles, although there were no significant differences between treatments in terms of germination percentage (Fig. 5A, Table 2). The 2HD treatment increased GSI ($F = 13.012$, $df = 2$, $p < 0.01$; Fig. 5B, Table 2) and reduced T_{50} ($F = 8.0798$, $df = 2$, $p < 0.01$; Fig. 5C, Table 2). In contrast, synchronization (Z) was negatively affected by the cycles, with higher values observed in the 0HD treatment (0.43 ± 0.03; $F = 17.63$, $df = 2$, $p < 0.01$; Fig. 5D, Table 2).

For *M. calodendron*, the 2HD treatment increased the germination percentage (F = [value], df = [value], p = [value]; Fig. 6A, Table 2) and decreased T_{50} (F = 5.025, df = 2, p < 0.01; Fig. 6B, Table 2). Meanwhile, for GSI (F = 13.012, df = 2, p < 0.01; Fig. 6C, Table 2) and synchronization (Z) (F = 10.44, df = 2, p < 0.01; Fig. 6D, Table 2), the 1HD cycle showed higher values (2.67 ± 0.12 and 0.22 ± 0.01, respectively) compared to the 0HD treatment.

Seed biometric and germination traits correlate throughout the HD cycles

The multivariate PCA analysis showed that, as the cycles progressed, the biometric traits of the seeds were correlated with germination parameters and endogenous proline levels. The cumulative variance explained by the first two principal components across the cycles (or no cycle) was equal to or greater than 70% (Fig. 7). Seed thickness was positively correlated with proline content in *M. calodendron*, regardless of the HD cycle (Fig. 7B). For larger seeds, such as those of *K. coriacea*, fresh weight, length, and width remained consistent regardless of the cycles (Fig. 7A). The application of the 2HD cycle divided the species into four distinct groups: the first group included parameters such as length, width, and fresh weight (associated *with K. coriacea*); the second group was characterized by thickness and proline content, which correlated more strongly with M. calodendron; the third group included moisture content, germination percentage, and GSI for *L. pacari*; and the last group consisted solely of T_{50} for *D. rupestris* (Fig. 7C).

DISCUSSION

The effect of the HD cycles on the four species had a natural hydropriming effect, increasing, standardizing, and accelerating germination (Paparella et al., 2015; Wojtyla et al., 2016; Farooq et al., 2019). This beneficial effect was observed from the 1HD cycle onwards for all species. The positive impact of HD cycles has already been documented in various species from diverse ecosystems (Dubrovsky, 1996; Contreras-Quiroz et al., 2016; Santini et al., 2017; Gonçalves et al., 2020). This hydropriming effect can mitigate the damage caused by water stress. HD cycles can help rescue the ‘priming memory’ of seeds, aiding in the transfer of "stress memory" to seedlings and their offspring in response to reduced water availability (Saha et al., 2011; Dias et al., 2024).

However, the intensity, duration, and frequency of droughts can lead to more recurrent hydration and dehydration cycles, which may prevent complete tissue hydration (Müller and Bahn, 2022; Dias et al., 2024). This can have a detrimental effect on germination, as observed

in our study with *K. coriacea*, where the progression of HD cycles reduced the germination percentage and increased proline content in response to the cycles (Fig. 4A and 4E). A similar effect was reported by Ren and Tao (2003), who observed that undergoing three HD cycles reduced germination in five species of the genus *Calligonum*. This indicates that not all species benefit from HD cycles, as their tolerance is often expressed during a single episode of water scarcity in the soil, making them susceptible to recurrent droughts. In the UPGMA analysis, *M. calodendron* and *D. rupestris* were grouped as the most tolerant to the 1HD cycle, while *L. pacari* showed intermediate tolerance, and *K. coriacea* was the most sensitive, particularly during the second HD cycle (Fig. 7).

Interestingly, winged seeds (*K. coriacea* and *L. pacari*) exhibited higher values for traits related to anemochory dispersal (Matlack, 1987; Westoby et al., 1996), showing high GSI values after undergoing the cycles. This is an advantageous mechanism for pioneer species to colonize new sites. Faster germination can be particularly beneficial for species that experience only short periods of suitable climatic conditions for germination and seedling establishment (Kadereit, Newton, and Vandelook, 2017). The increase in germination speed induced by HD cycles allows for more efficient use of water—a scarce resource—during germination and seedling establishment (Correa et al., 2020).

Seed thickness is a trait that modulates the interaction between internal structures and the external environment, and its composition influences species persistence under different environmental conditions (Souza and Marcos-Filho, 2001; Johri, Ambegaokar, and Srivastava, 2013). Under stressful conditions, during seed formation, thickness and rigidity increase (Noodén, Blakley and Grzybowski, 1985; Coen and Magnani, 2018), giving the seed greater protection against mechanical damage, predators and abiotic and biotic stresses after dispersal (Nonogaki, Bassel and Bewley, 2010; Baskin and Baskin, 2014). This was observed in *D. rupestris* and *M. calodendron*, which already have higher seed thickness (Table 1).

In *M. calodendron*, proline levels decreased with the progression of cycles, reflecting its tolerance to stress caused by HD cycles. This species already has high endogenous proline levels in its seeds (Fig. 6E), which may contribute to its prolonged persistence in the soil seed bank. In species with physical dormancy, such as those in the Fabaceae family, the impermeability of the seed coat to water allows seeds to remain in the seed bank for extended periods (Ooi et al., 2014; Matheus et al., 2017; Zhang et al., 2020). Species within the Fabaceae family exhibit varied responses to HD cycles, with some showing beneficial effects (Lima et al., 2018), no effect (Lima and Meiado, 2018), or even harmful effects on

germination and seedling establishment (Lima, Oliveira, and Meiado, 2018). Lima and Meiado (2024) observed that seedlings from Senna spectabilis var. excelsa seeds that underwent HD cycles had higher proline levels in their leaves when exposed to water deficit.

The increase in proline levels, even in non-Fabaceae species, may indicate tolerance to desiccation or water stress. Dias et al. (2024) found that when *Tabebuia heterophylla* seeds underwent HD cycles at different imbibition times, both seeds and seedlings increased their proline levels. Similarly, the progression of cycles increased endogenous proline levels in seeds of *Sarcomphalus joazeiro* (Oliveira et al., 2024). This differential increase in proline in both seeds and seedlings may represent a survival mechanism, with seeds dispersing with high proline levels and synthesizing more in response to stress. Thus, in this study, the capacity to accumulate proline over HD cycles may prepare seeds for future stresses, serving as a way to rescue the 'priming memory.'

D. rupestris also exhibited considerable proline levels in its seeds, which did not decrease with the progression of cycles. Proline serves as an important source of nitrogen, carbon, and energy (Matysik et al., 2002). This high proline content ensured seed survival during the cycles without altering the germination percentage. Like *M. calodendron*, *D. rupestris* seeds are dispersed with high proline levels. Notably, seeds dispersed with advantageous biometric attributes that remain in the seed bank and increase proline content may have greater chances of survival, persistence, and tolerance.

Biometric seed traits are important tools for understanding environmental filters, dispersal, longevity, and functional strategies adopted by species in their habitats (Patrício and Trovão, 2020; Silva et al., 2024). When correlated with germination under stressful conditions—such as the HD cycles in this study—they provide insights into seed tolerance and survival after dispersal and during seedling establishment (Chen and Arora, 2013; Saha et al., 2022). There is a growing need for plant studies to deepen our understanding of seed characteristics and their role in plant plasticity, adaptation, evolution, distribution, and dynamics. This knowledge is essential to anticipate how biodiversity will respond to human impacts and to preserve natural ecosystems (Saatkamp et al., 2019). This work provides insights for future research on seed traits and persistence: (i) proline content and its relationship with seed persistence; (ii) biometric and biochemical seed traits in *campo rupestre* species; (iii) field studies to understand the survival of metabolically prepared seedlings; and (iv) using these tools to inform revegetation programs in *campo rupestre*.

CONCLUSION

We concluded that germination and biochemical characteristics contributed more significantly than biometric characteristics to tolerance to HD cycles. The application of stresses - which the seeds are likely to encounter in their natural environment - during germination provides a comprehensive view of their sensitivity or tolerance. The use of HD cycles has proven effective in improving germination parameters, increasing germination speed, reducing germination time, and normalizing germination. These cycles not only improve germination but also prepare the seeds metabolically for future stresses. Furthermore, the combination of biometric, germination, and biochemical attributes serves as an excellent tool for selecting species for the restoration of seasonally dry environments, such as the *campo rupestre*.

It is important to note that germination parameters alone may not be enough to fully understand the seed journey and seedling recruitment in the soil seed bank. However, HD cycles can be seen as a valuable tool for selecting seeds and seedlings that are better prepared for future environmental challenges. HD cycles can help identify seeds with greater tolerance to water deficit and similar conditions, leading to the formation of more vigorous seedlings characterized by a higher content of proline and other beneficial metabolic components.

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AUTHOR CONTRIBUTION STATEMENT

GSD, EMB conceived and designed the research; GSD, JKT carried out all the experimental work; GSD performed the statistical analysis; GSD, JKT, EMB contributed to data interpretation; GSD, EMB wrote the manuscript. All the authors contributed to data discussion, and conclusions, and reviewed and approved the final manuscript.

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Figure 1

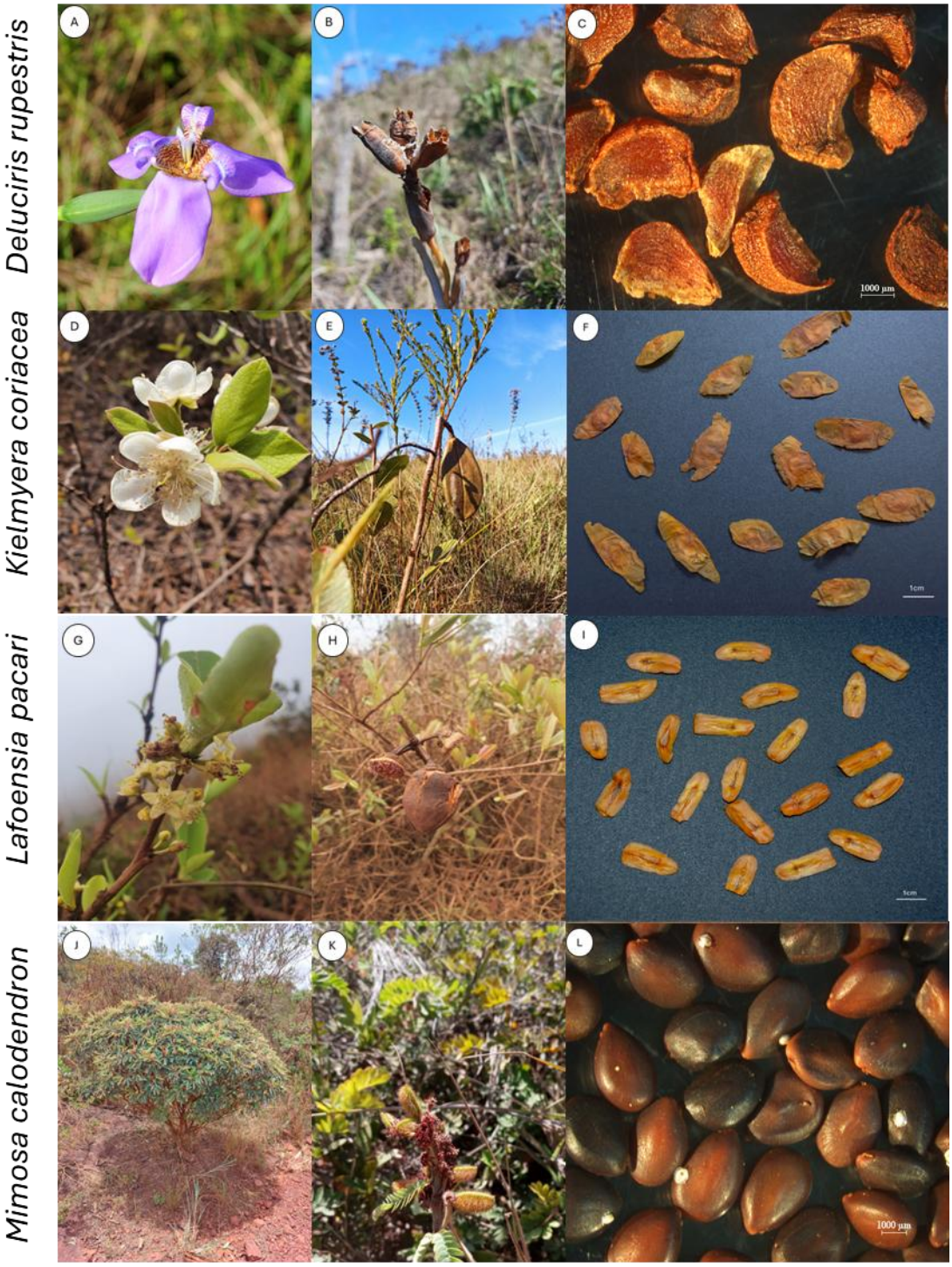

Figure 1: Flowering, fruiting, and seeds of *Deluciris rupestris* (A, B, and C), *Kielmeyera coriacea* (D, E, and F), *Lafoensia pacari* (G, H, and I), and *Mimosa calodendron* (J, K, and L), respectively. Photos by Páulo Pimentel (B, D, E, G, H, J), Milena Teles (K), and Pablo Burkowski Meyer (A).

Figure 2

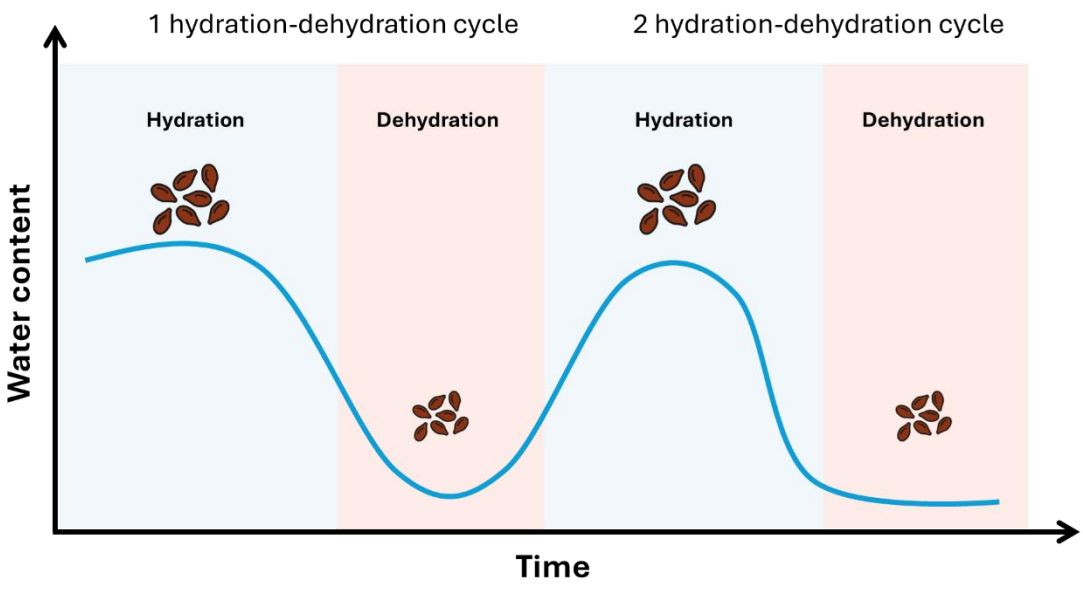

Figure 2: Schematic model of applying hydration and dehydration cycles in the seeds of *campo rupestre* species.

Table 1: Seed functional traits length, width, thickness, fresh weight, and moisture content of four species *campo rupestr*

Species	Family	Length (mm)	Width (mm)	Thickness (mm)	Fresh weight (g)	Moisture content (%)
Kielmeyera coriaceae	Calophylaceae	34.3539±1.9	13.4689±0.6	1.4386±0.17	0.6093±0.02	6.79±1.88
Mimosa calodendron	Fabaceae	3.4755±0.08	2.5008±0.06	1.5275±0.05	0.0895±0.001	4.53±0.19
Deluciris rupestris	Iridaceae	3.4328±0.12	2.1066±0.1	0.9589±0.08	0.052±0.001	10.67±1.11
Lafoensia pacari	Lecythidaceae	22.0081±0.62	8.0879±0.19	0.6169±0.04	0.1985±0.002	8.38±0.15

Figure 3

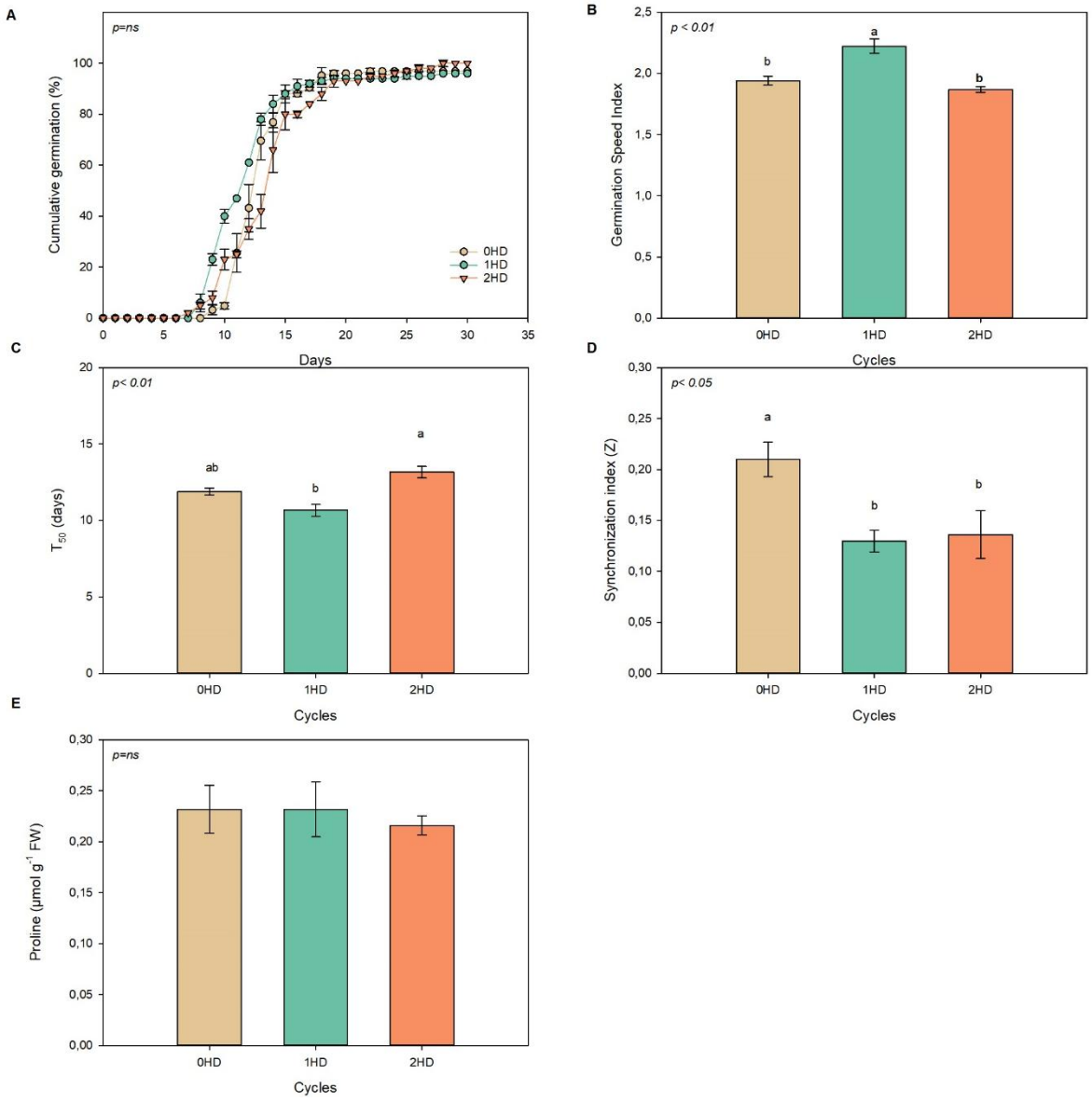

Figure 3: Cumulative germination (A), germination speed index -GSI (B), T_{50} (C), synchronization index (D), and proline (E) in *Deluciris rupestris* seeds subjected to hydration and dehydration cycles. Averages followed by the same letters do not differ significantly by Tukey's test at 5% probability, ns = not significant.

Figure 4

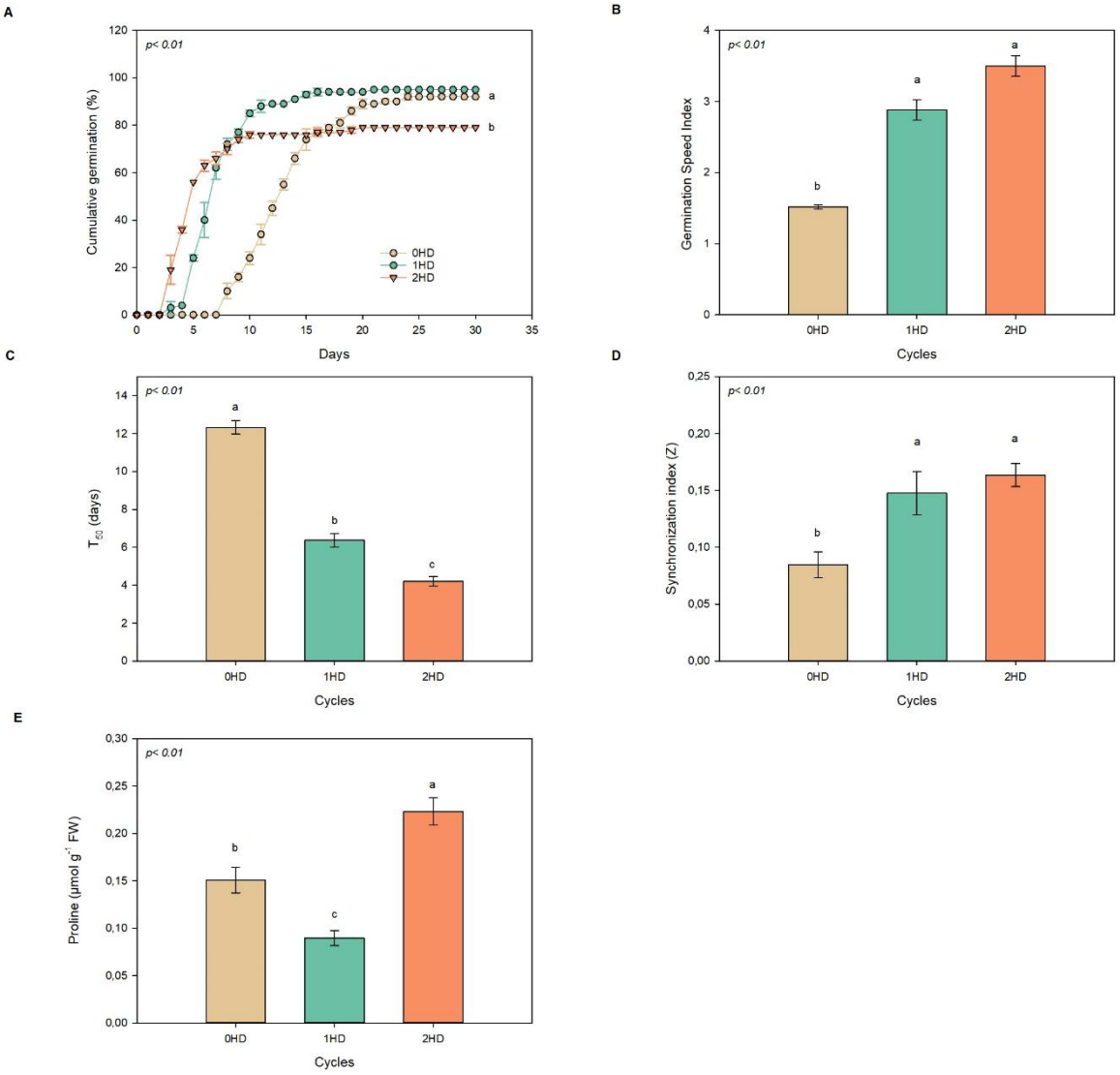

Figure 4: Cumulative germination (A), germination speed index -GSI (B), T_{50} (C), synchronization index (D), and proline (E) in *Kielmeyera coriacea* seeds subjected to hydration and dehydration cycles. Averages followed by the same letters do not differ significantly by Tukey's test at 5% probability, ns = not significant.

Figure 5

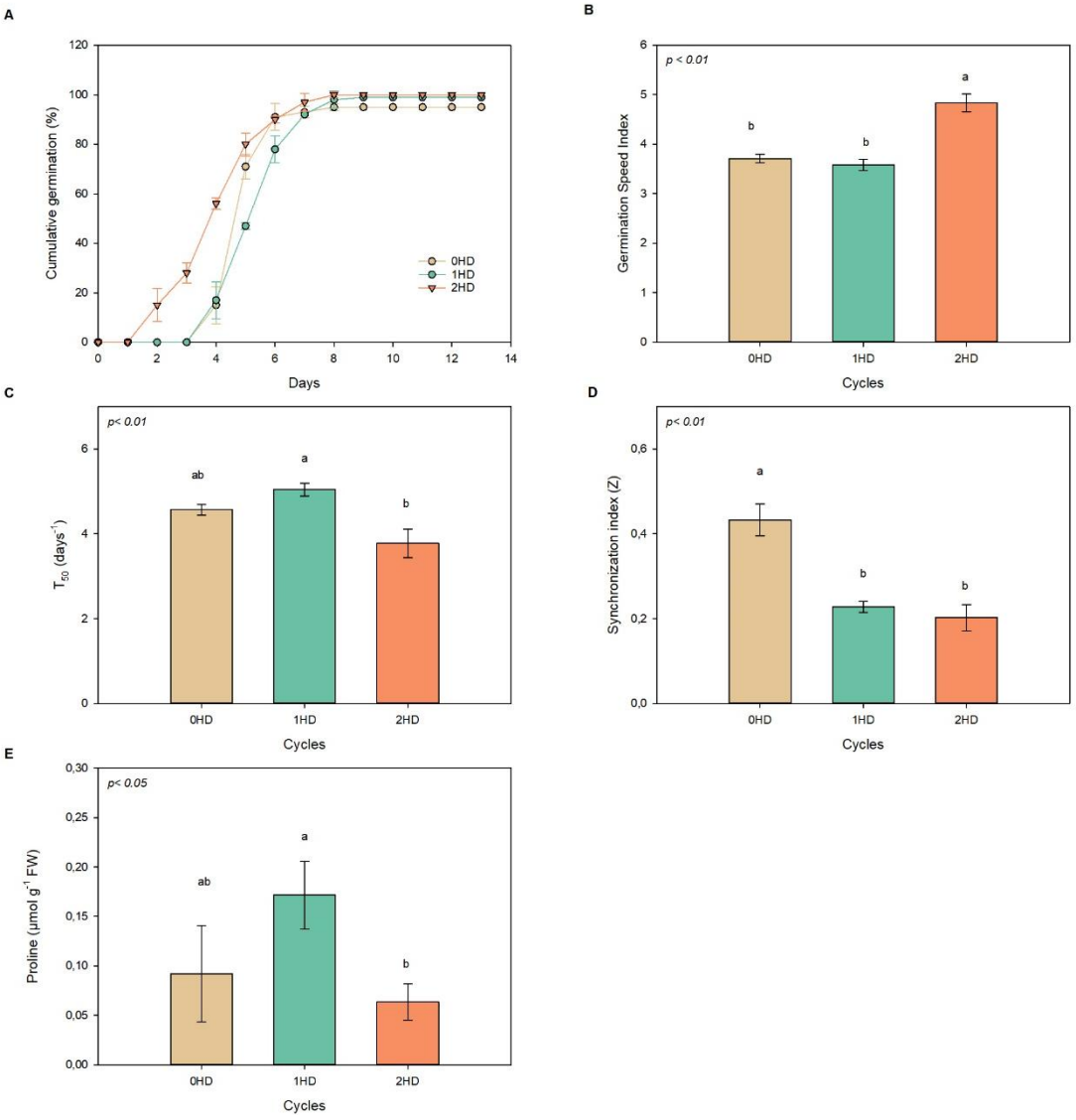

Figure 5: Cumulative germination (A), germination speed index -GSI (B), T_{50} (C), synchronization index (D), and proline (E) in *Lafoensia pacari* seeds subjected to hydration and dehydration cycles. Averages followed by the same letters do not differ significantly by Tukey's test at 5% probability, ns = not significant.

Figure 6

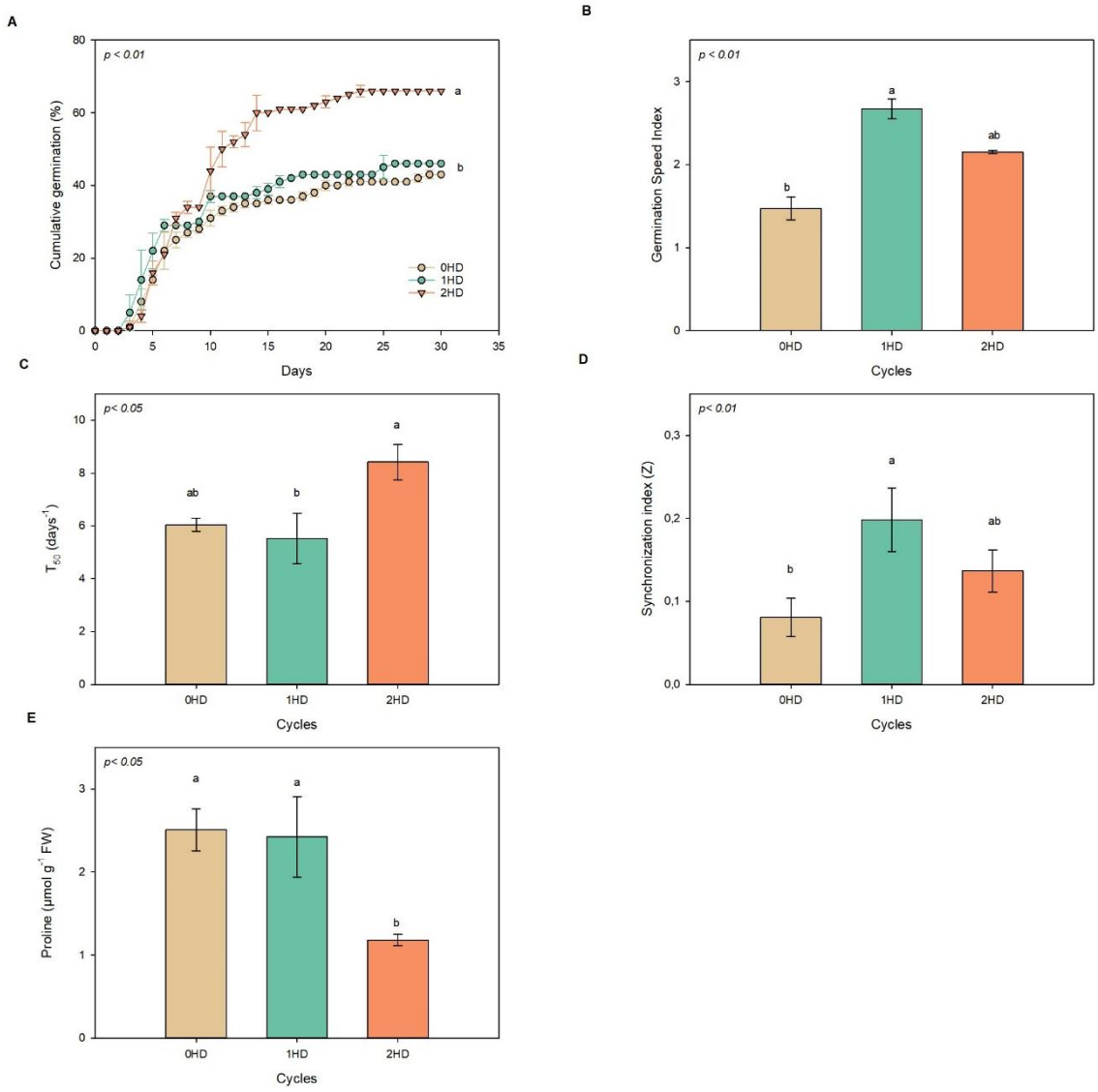

Figure 6: Cumulative germination (A), germination speed index -GSI (B), T_{50} (C), synchronization index (D), and proline (E) in *Mimosa calodendron* seeds subjected to hydration and dehydration cycles. Averages followed by the same letters do not differ significantly by Tukey's test at 5% probability, ns = not significant.

Table 2. Results of the statistical analysis (ANOVA and Tukey's test) to evaluate the germination parameters of seeds from *campo rupestre* species exposed to hydration-dehydration cycles.

	F	***df***	***p***		***F***	***df***	***p***
Deluciris rupestris				*Lafoensia pacari*			
Germination	*	*	*	Germination	*	*	*
Germination Speed Index	17.362	2	0.00081448	Germination Speed Index	31.017	2	$9.1722x10^{-5}$
T_{50}	13.716	2	0.00079432	T_{50}	8.0798	2	0.0059886
Z	6.2982	2	0.013485	Z	17.63	2	0.00026799
Proline	0.20006	2	0.82135	Proline	6.8161	2	0.01577
Kielmeyera coriacea				*Mimosa calodendron*			
Germination	12.4	2	0.0012023	Germination	12.435	2	0.0011885
Germination Speed Index	144.08	2	$1.4643x10^{-7}$	Germination Speed Index	13.012	2	0.0065769
T_{50}	168.65	2	$1.6438x10^{-9}$	T_{50}	5.025	2	0.025979
Z	8.5635	2	0.0048901	Z	10.44	2	0.011122
Proline	29.55	2	0.0001109	Proline	7.0635	2	0.014307

*****The repetitions presented the same values, it was decided not to perform the statistical test.

Figure 7

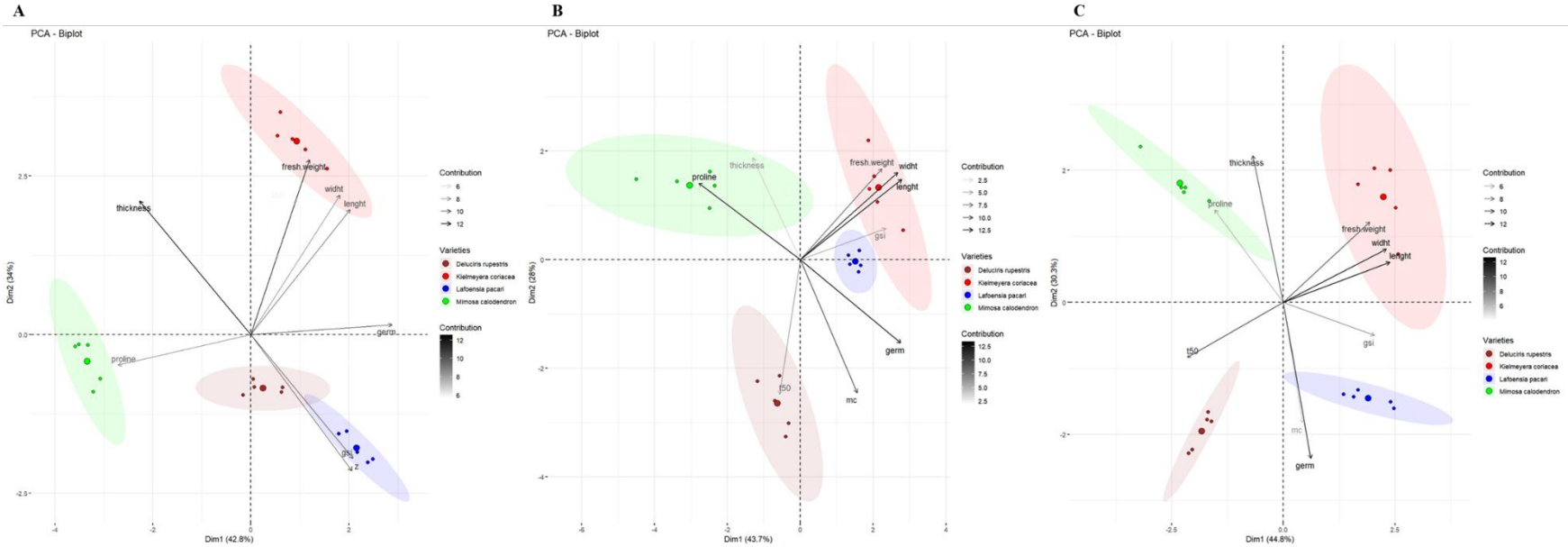

Figure 7: Multivariate principal component analysis (PCA) of the biometric, germination, and biochemical traits of *Deluciris rupestris*, *Kielmeyera coriacea*, *Lafoensia pacari*, and *Mimosa calodendron* subjected to 0HD (A), 1HD (B), and 2HD (C) cycles. Abbreviations: germ – germination percentage, gsi – germination speed index, z – synchronization index, t50 – time to 50% of seed population germinate, mc- moisture content, length, width, fresh weight, and proline.

Supplementary Material

Figure S1

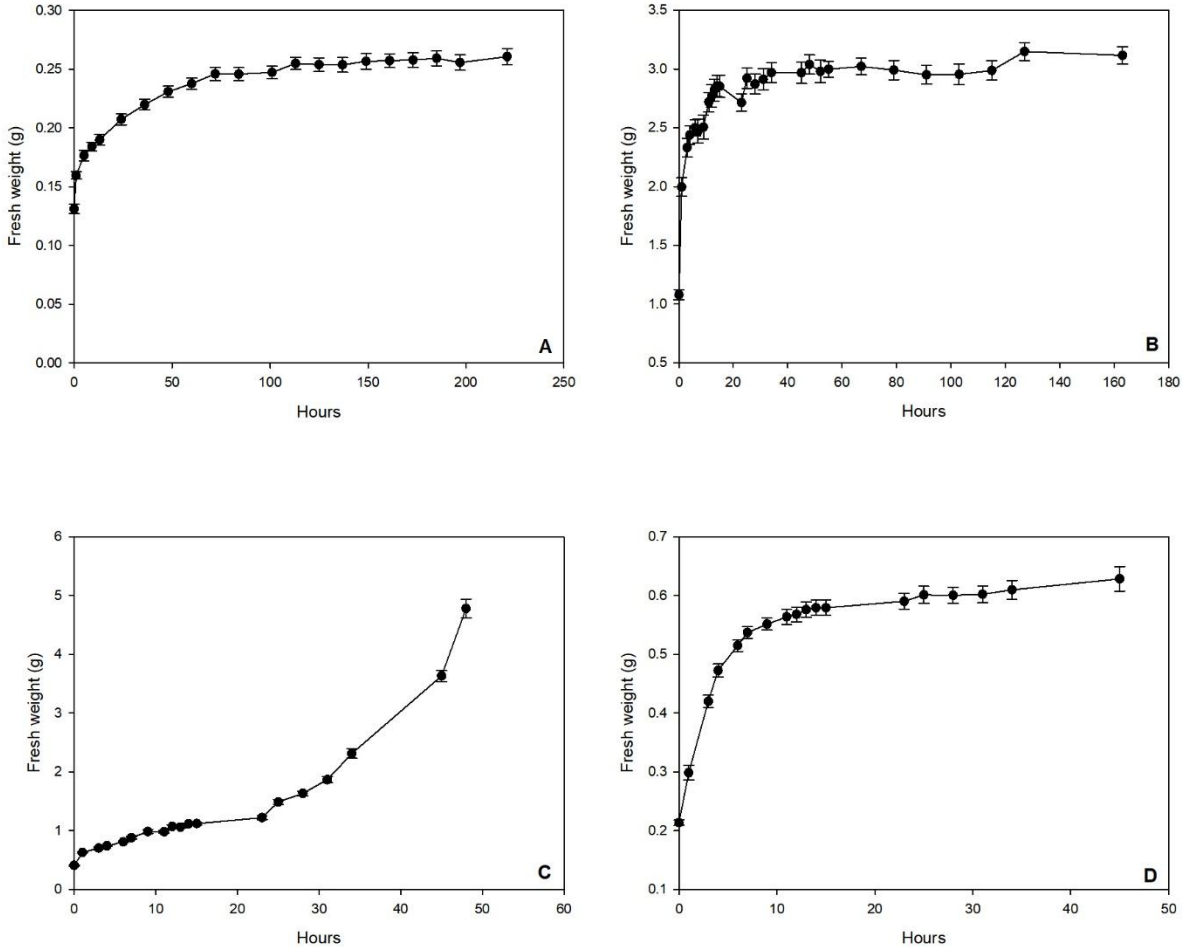

Figure S1: Imbibition curve of seeds of *Deluciris rupestris* (A), *Kielmeyera coriacea* (B), *Lafoensia pacari* (C) and *Mimosa calodendron* (D). Bars are standard error (N=100 for *K.coriacea* and *L. pacari* and N=125 for *D. rupestris* and *M. calodendron*).

Figure S2

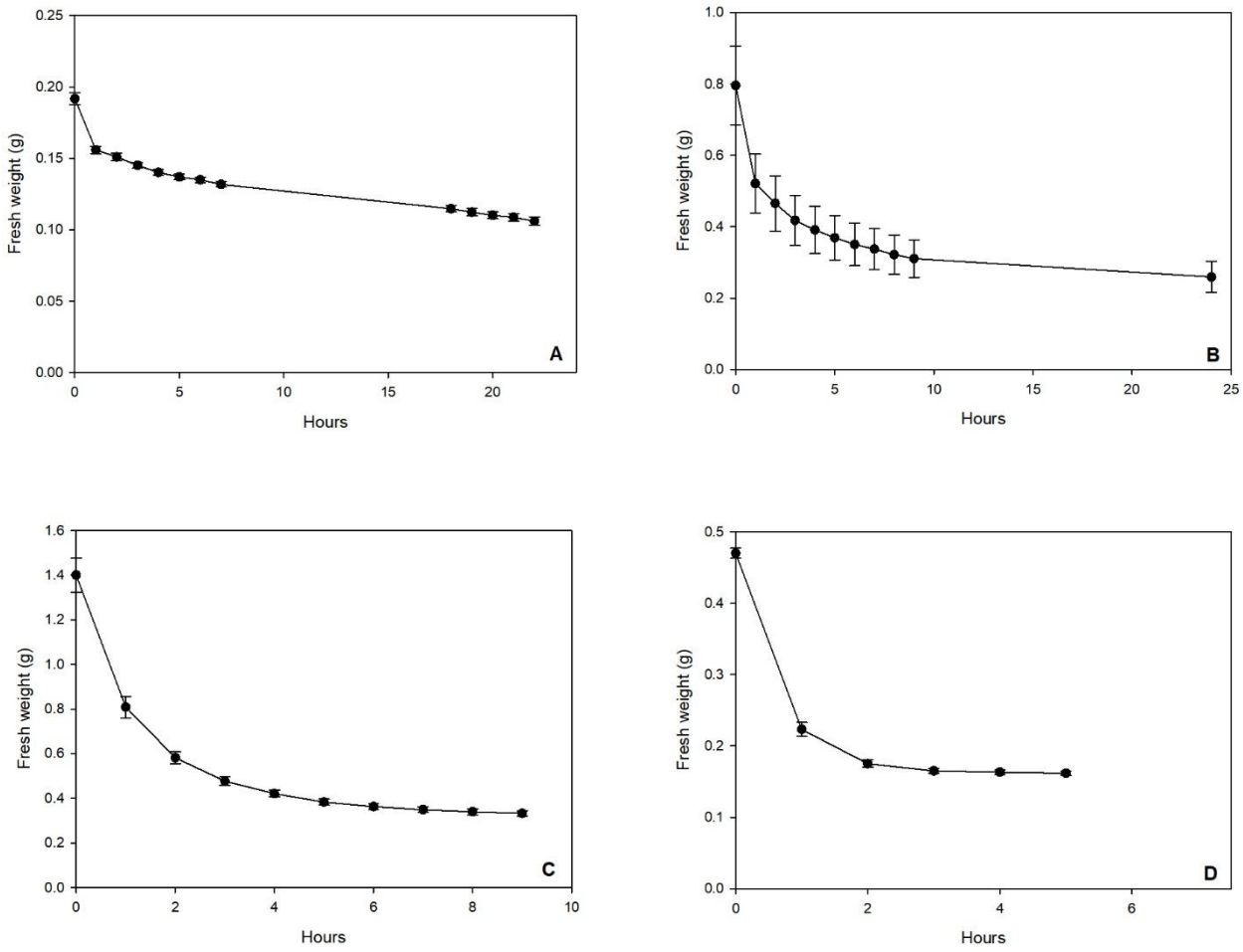

Figure S2: Drying curve of seeds of *Deluciris rupestris* (A), *Kielmeyera coriacea* (B), *Lafoensia pacari* (C) and *Mimosa calodendron* (D). Bars are standard error (N=100 for *K.coriacea* and *L. pacari* and N=125 for *D. rupestris* and *M. calodendron*).

PAPER 2: AN ETERNAL FLAME? WET-DRY CYCLING INDUCE HEAT TOLERANCE IN *VELLOZIA* SPECIES

Manuscript written according to the Annals of Botany guidelines for the call for papers State of the Global Flora (Cerrado/Tropical Grasslands)

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ABSTRACT

The Velloziaceae family is distributed in the *campos rupestres*, a region characterized by two distinct seasons—a dry winter and a rainy summer—as well as natural fires. *Vellozia* species disperse their seeds between the wet and dry seasons, and the duration of seed persistence in the soil seed bank is determined by abiotic conditions, such as wet-dry (WD) cycles. To better understand the germination and tolerance of *Vellozia* species to different abiotic stresses, seeds of three *Vellozia* species (*Vellozia caruncularis*, *Vellozia compacta*, and *Vellozia nanuzae*) were exposed to four treatments per species: control (no WD cycle and a temperature of 25°C); +100°C (heat shock without WD cycles); WD (seeds subjected to a wet-dry cycle without heat shock); WD+100°C (WD cycle followed by heat shock). The three *Vellozia* species responded differently to the treatments. *V. caruncularis* was the most sensitive to heat stress (+100°C and WD+100°C), showing no priming effect from the WD cycle. This species exhibited higher levels of abscisic acid and proline, along with reduced germination speed, under the WD+100°C treatment. In contrast, *V. compacta* demonstrated tolerance to heat stress when combined with the WD cycle (WD+100°C), which increased germination speed after heat shock exposure and elevated endogenous proline levels. *V. nanuzae*, on the other hand, benefited from heat exposure (+100°C), with the WD cycle further enhancing germination under heat shock conditions (WD+100°C). The seeds of *V. nanuzae* exhibited tolerance to high temperatures, likely due to their already high endogenous proline levels (observed in both the control and WD treatments). In conclusion, the seeds of these *Vellozia* species displayed varying responses to heat shock, ranging from sensitivity to tolerance. The WD cycle played a role in increasing endogenous proline levels in some species, while others relied on their inherent proline reserves to cope with stress.

Keywords: Velloziaceae; natural hydropriming; proline; abscisic acid; heat shock.

INTRODUCTION

Campos rupestres are ancient and megadiverse ecosystems that occur at high altitudes, characterized by nutrient-poor soils, high solar irradiance, constant winds, seasonal water availability, and the occurrence of natural fires (Jacobi and Carmo, 2008; Oliveira et al., 2016; Silveira et al., 2016). Found on quartzite, sandstone, or ferritic substrates at altitudes above

900 meters, it is estimated that the *campos rupestres* cover approximately 66,450 km² and are home to more than five thousand known species (Silveira et al., 2016). The vegetation of the *campos rupestres* is highly adapted to the unique edaphoclimatic conditions of this ecosystem (Alcântara et al., 2015).

Velloziaceae is an amphitropical family composed of five genera (*Acanthochlamys*, *Barbacenia*, *Barbaceniopsis*, *Vellozia*, and *Xerophyta*) and comprises approximately 265 species, of which about 230 are endemic to the Neotropical region, being highly representative of the *campos rupestres* (Giulietti and Pirani, 1988; Mello-Silva, 1995; Mello-Silva, 2018). In Brazil, two genera occur, *Barbacenia* and *Vellozia*, with a total of 231 species (Mello-Silva, 2015; Flora e Funga do Brasil, 2025). The genus *Vellozia* comprises about 125 species, of which 123 are endemic (Flora e Funga do Brasil, 2025). Populations of *Vellozia caruncularis* are distributed in ferruginous *campos rupestres*—specifically in *canga couraçada* (Viana and Lombardi, 2007; Vicent and Maguro, 2008). *Vellozia compacta* is found in ferruginous *campos rupestres—canga nodular*, where soils are rocky and temperatures can reach extreme highs (Vicent and Maguro, 2008). *Vellozia nanuzae* is a microendemic species, restricted to a few rock crevices in limited geographical areas (Mello-Silva, 2015; Cunha-Blum et al., 2020).

Seed dispersal in *Vellozia* species occurs at the end of the rainy season and extends into the late dry season (Le Stradic et al., 2018; Freitas, 2023; Ordóñez-Parra et al., 2024). During this period, seeds are exposed to a wide range of environmental stresses (Garcia and Diniz, 2003). Species found in *campos rupestres*, whose seeds persist in the soil seed bank, are exposed to various environmental stresses, including high solar radiation, wide temperature fluctuations, and prolonged drought (Garcia et al., 2020). Occasional rainfall or fluctuations in soil humidity during the dry season are often accompanied by the passage of fire in *campos rupestres*. Germination of *Vellozia* seeds occurs under a wide range of environmental factors, such as light intensity, temperature, and seasonal water deficit (Bicalho et al., 2018; Garcia et al., 2020). As these species inhabit environments with unique climatic conditions, their seeds have evolved mechanisms to either tolerate or escape these stresses.

While residing in the soil, seeds undergo tissue imbibition. Before becoming fully hydrated, they may dry out again, a process known as the wet-dry cycle (WD) (Dubrovisky, 1998; Long et al., 2011). WD cycles act as a form of natural hydropriming for seeds (Santini, Rojas-Aréchiga, and Morales, 2017; Dias et al., 2024), enhancing germination and metabolic adjustment (Paparella et al., 2015; Lutts et al., 2016). This process helps mitigate the effects

of various abiotic stresses, such as water deficit, salinity, and extreme temperatures (Casenave and Toselli, 2010; Yusefi-Tanha et al., 2019; Nicolau et al., 2020; Nascimento et al., 2021; Pereira et al., 2023; Oliveira et al., 2024).

During the dry season, *campos rupestres* vegetation is prone to natural fires. Fire is a natural disturbance in these ecosystems (Veldman et al., 2015; Fernandes et al., 2016). Some species are adapted to occasional fires and even require fire to induce flowering (Richardson and Wagenius, 2022)—examples include *Vellozia pyrantha* (Conceição, 2018) and *Vellozia sincorana* (Conceição and Orr, 2012). Fire can break seed dormancy (Pausas and Lamont, 2022) and improve germination parameters (Ramos et al., 2019). Certain *Vellozia* species, such as *V. variabilis* and *V. pyrantha*, are particularly fire-prone due to the presence of flammable resins in their leaves (Martins and Paiva, 2016; Santos et al., 2021). In the current context of climate change, the increasing intensity and duration of high temperatures (heat waves), combined with reduced rainfall, create favorable conditions for more frequent and severe fires (Lamont, 2022). Additionally, anthropogenic activities are exacerbating the frequency and spread of fires at various scales.

Seeds of *Vellozia* species exhibit long-term persistence in soil seed banks (Garcia et al., 2017), and it is expected that they can endure heat shock and WD cycles. Therefore, we hypothesize that seeds of three *Vellozia* species (*Vellozia caruncularis, Vellozia compacta*, and *Vellozia nanuzae*) that undergo WD cycles develop tolerance to heat shock stress. This study aims to uncover the physiological mechanisms developed by *Vellozia* seeds in response to combined heat shock and WD treatments. It provides new insights into how *Vellozia* seeds persist in soil seed banks, considering the variability in heat shock and water availability.

MATERIAL AND METHODS

Seed Collection

Seeds were collected from 20 individuals in Serra da Calçada, Nova Lima - MG, Brazil (20°05'55.2"S, 43°58'58.2"W) between 2019 and 2022. The region has a mesothermal Cwb climate (mountain climate with rainy summers and dry winters) according to the Köppen classification (Kottek et al., 2006; Beck et al., 2018). The experiment used seeds of *Vellozia caruncularis* Mart. ex Seub. collected between February and May 2019, *Vellozia compacta* Mart. ex Schult. & Schult.f. collected between late 2021 and early 2022, and *Vellozia nanuzae* L.B.Sm. & Ayensu collected in April 2022 (Fig. 1). Moisture content was determined using the oven method at 105 ± 2°C (Brasil, 2009). The values were expressed on a dry basis, using

five replicates of 25 seeds each for *V. nanuzae* and 0.0125 g of seeds for *V. compacta* and V. *caruncularis*. The moisture content of the seeds was 14.57 ± 2.23%, 18.08 ± 2.10%, and 11.75 ± 1.78% for *V. caruncularis*, *V. compacta*, and *V. nanuzae*, respectively.

Imbibition and Drying Curves

Five replicates of 50, 25, and 20 seeds of *V. caruncularis*, *V. compacta*, and *V. nanuzae*, respectively, were used to construct the imbibition curve. After measuring the initial fresh weight, the seeds were weighed on a 0.001 g analytical balance at intervals of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 24, 48, 72, 96, and 120 hours (Fig. 2). For the drying curve, the seeds were imbibed for 72 hours and then dried in silica gel at 25°C until they returned to their initial fresh weight (Fig. 2).

Heat Shock and Wet-Dry Cycle Treatments

Based on the literature (Zirondi et al., 2019; Fernandes et al., 2021) and preliminary tests, the three species were exposed to 100°C for 5 minutes in an oven. The wet-dry (WD) cycle was applied based on the timing of the imbibition curve (72 hours) and the drying curve (1 hour). Therefore, seeds were imbibed for 72 hours, followed by 1 hour of drying in silica gel at 25°C. Four treatments were applied to each species: control: (no WD cycle and maintained at 25°C); +100°C (heat shock without WD cycle); WD (wet-dry cycle without heat shock) and WD+100°C (wet-dry cycle followed by heat shock) (Fig. 3).

Germination Test

After treatment application, seeds of the three *Vellozia* species (five replicates of 20 seeds each for *V. compacta* and *V. nanuzae*, and 25 seeds for *V. caruncularis*) were disinfected in a 1.0% sodium hypochlorite (NaClO) solution with two drops of commercial detergent for 10 minutes, followed by three rinses with deionized water. After disinfection, the seeds were placed in Petri dishes and randomly distributed in germination chambers under a 12-hour photoperiod with 40 μmol photons m^{-2} s^{-1} at the optimal germination temperature of 25°C for each species: *V. caruncularis* (Le Stradic et al., 2015), *V. compacta* (Bicalho et al., 2018), and *V. nanuzae* (Freitas, 2023). At the end of the test, germination percentage and germination speed index (Maguire, 1962) were evaluated.

Abscisic Acid (ABA) Determination

Seeds of the three *Vellozia* species were collected 72 hours after hydration (or rehydration for WD treatments). To obtain 0.2 g of fresh mass, 300 seeds of *V. nanuzae*, 400

seeds of *V. compacta*, and 600 seeds of *V. caruncularis* were used per replicate (due to differences in seed size and weight among the species). For ABA quantification, 0.1 g of seeds was macerated in liquid nitrogen, and 1 mL of extraction buffer was added, containing 80% methanol, 20 mL/L^{-1} acetic acid, and 10 mg/L^{-1} butylhydroxytoluene (Weiler, 1982; Bicalho et al., 2015; Bicalho et al., 2018). The samples were centrifuged at 12,000 g for 15 minutes at 4°C (the extraction was performed twice, yielding a total of 2 mL of extract). ABA quantification was performed using Phytodetek® ABA test kits (Agdia Incorporated, Indiana, USA) according to the manufacturer's instructions.

Proline Quantification

Proline was quantified according to Bates (1973). Fresh seeds (0.1 g) of *Vellozia spp.* were macerated with 2 mL of 3% salicylic acid and centrifuged at 14,000 rpm for 5 minutes at room temperature. Endogenous proline was quantified by adding 500 µL of acidic ninhydrin solution (2.5 g ninhydrin dissolved in 40 mL of 6 M phosphoric acid and 60 mL of glacial acetic acid), 500 µL of glacial acetic acid, and 500 µL of the sample. The mixture was vortexed, heated in a water bath at 100°C for 1 hour, and then cooled. Absorbance was measured at 520 nm using a spectrophotometer. A standard curve of proline at 100 µg/mL was used to determine endogenous proline levels.

Statistical Analysis

The experiment was conducted in a completely randomized design with a 2 × 2 factorial arrangement [two hydration conditions (control and one wet-dry cycle) and two temperature conditions (25°C and 100°C heat shock)]. Five replicates were used for the germination test, and three replicates were used for biochemical analyses. Normality and homogeneity of variances were assessed using the Shapiro-Wilk and Levene tests, respectively. Data were subjected to two-way ANOVA, and when statistically significant (F-test at 5% significance), means were compared using Tukey's post-hoc test at 5% significance. Analyses were performed in R version 4.3.1 (ExpDes.pt package). A Principal Component Analysis (PCA) was conducted using data on germination percentage, germination speed index, proline, and ABA. The following R packages were used: cluster, corrplot, factoextra, FactoMineR, ggplot2, ggsci, mice, PCAmixdata, readxl, sn, and PerformanceAnalytics. An unweighted pair group method with arithmetic mean (UPGMA) based on Euclidean distance was constructed using the same variables.

RESULTS

The wet-dry cycle and heat shock have different effects on the germination of the three Vellozia species

The three *Vellozia* species responded differently to wet-dry (WD) cycles and heat shock exposure. *V. caruncularis* seeds showed an 11% reduction in germination when exposed only to heat shock (+100°C) and a 12% reduction when exposed to WD+100°C compared to the control ($F = 14.6944$, $df = 1$, $P < 0.01$; Fig. 4A, Table 1). The germination speed index (GSI) was negatively influenced by heat shock ($F = 11.387$, $df = 1$, $P < 0.01$; Fig. 4D, Table 1) and the interaction between the WD cycle and heat shock ($F = 47.402$, $df = 1$, $P < 0.01$; Fig. 4D, Table 1). Seeds exposed to the WD cycle alone showed higher GSI values compared to the control and WD+100°C treatments.

The germination percentage of *V. compacta* was not influenced by any treatment ($F = 0.37120$, $df = 1$, $P =$ ns; Fig. 4B, Table 1). However, the GSI showed a significant interaction between the WD cycle ($F = 5.8535$, $df = 1$, $P < 0.05$; Fig. 4E, Table 1) and heat shock ($F = 14.0399$, $df = 1$, $P < 0.01$; Fig. 4E, Table 1). The highest GSI values were observed in seeds that underwent the WD cycle (2.40 ± 0.06), while heat shock alone also increased the GSI.

The germination percentage of *V. nanuzae* was only affected by heat shock ($F = 7.4926$, $df = 1$, $P < 0.05$; Fig. 4C, Table 1). It showed an increase in germination percentage when seeds were exposed to heat shock, either after undergoing the WD cycle (85% ± 0.001) or without it (79.62% ± 1.85). The GSI of *V. nanuzae* showed a significant interaction between the WD cycle and heat shock ($F = 6.3194$, $df = 1$, $P < 0.05$; Fig. 4F, Table 1), with the +100°C treatment showing the lowest GSI value compared to the other treatments.

Proline is a metabolite that accumulated in tissues in response to water and heat stress.

Proline accumulation responded significantly to both the wet-dry (WD) cycle and heat shock, as well as to the interaction between these factors. For the species *V. caruncularis*, proline levels showed significant effects for heat shock ($F = 15.2687$, $df = 1$, $P < 0.01$; Fig. 5A, Table 1) and for the interaction between the WD cycle and heat shock ($F = 16.8555$, $df = 1$, $P < 0.01$; Fig. 5A, Table 1). For ABA levels, there was a significant difference for the WD cycle ($F = 12.0672$, $df = 1$, $P < 0.01$; Fig. 5D, Table 1) and for the interaction of the two factors ($F = 5.7012$, $df = 1$, $P < 0.05$; Fig. 5D, Table 1). The highest proline (0.4318 ± 0.0497 μmol proline g^{-1} FW) and ABA (4.5539 ± 0.1687 pmol/mg) values were observed in the WD+100°C treatment.

The species *V. compacta* showed greater proline accumulation after undergoing the WD cycle ($F = 18.0433$, $df = 1$, $P < 0.01$; Fig. 5B, Table 1), with no significant difference related to heat shock. In contrast, *V. nanuzae* exhibited higher proline accumulation at 25°C, regardless of heat shock exposure ($F = 5.7616$, $df = 1$, $P < 0.05$; Fig. 5C, Table 1). The ABA content in the seeds of *V. compacta* and *V. caruncularis* did not show significant differences between treatments.

Principal Component Analysis (PCA) revealed that the tolerance or sensitivity of *Vellozia* species to heat shock may be linked to the accumulation of proline or ABA in their tissues, as well as changes in germination parameters. The sum of the main components, PC1 and PC2, accounted for 69.7% of the variance, showing a clear separation among the three species based on germination and biochemical data. The GSI and ABA values were the most strongly correlated factors in distinguishing *V. caruncularis* from the other two species, while germination and proline levels grouped *V. compacta* and *V. nanuzae* together.

DISCUSSION

The seed germination of the three *Vellozia* species responded in contrasting ways to both the wet-dry (WD) cycle and heat shock, whether applied separately or together. In this study, the germination of *V. nanuzae* was favored by WD cycles and heat shock. Previous literature has reported that heat shock enhances the germination of this species (Fernandes et al., 2021). Combining the WD cycle and heat shock improved germination speed compared to seeds subjected to heat shock alone. In other words, *V. nanuzae* seeds germinate more quickly when they undergo imbibition and drying followed by exposure to high temperatures, likely due to the priming effect of the WD cycle. In contrast, the germination of *V. compacta* was unaffected by WD cycles or heat shock, although the germination speed index (GSI) increased after exposure to 100°C (Fig. 4E). This aligns with findings by Zirondi et al. (2019), who observed that *V. compacta* seeds tolerate exposure to 100°C without a reduction in germination percentage. On the other hand, *V. caruncularis* showed a decline in germination after heat shock, with no positive effect from the WD cycle (Fig. 4C). Fernandes et al. (2021) reported a reduction in germination for *V. caruncularis* at temperatures above 80°C, indicating its sensitivity to heat stress. In this species, the priming effect of the WD cycle was insufficient to confer tolerance to high temperatures.

In addition to enhancing germination parameters in *V. nanuzae*, the WD cycle also improved the tolerance of *V. compacta* to heat shock. It has been observed that undergoing a

WD cycle induces the accumulation of osmoprotective substances (e.g., soluble sugars, amino acids, proline, glycine-betaine, gamma-aminobutyric acid, polyols, and polyamines), which confer greater tolerance to a range of environmental stresses (Ghosh et al., 2021). In this study, *V. compacta* accumulated proline after the WD cycle, a response previously documented in various species under abiotic stress (Dias et al., 2024; Pereira et al., 2024; Oliveira et al., 2024). This differential accumulation of proline, facilitated by the WD cycle, may have served as a protective mechanism, as evidenced by the increased GSI in the WD+100°C treatment compared to +100°C alone. The role of proline in inducing thermotolerance has been well-documented in agricultural species (Alamri et al., 2019; Tonhati et al., 2020) and appears to be an important mechanism for native species like *V. compacta.* This suggests a tolerance mechanism that enables the species to resist the effects of heat shock (Nascimento et al., 2021).

The seeds of *V. nanuzae* exhibited high proline concentrations under natural conditions, which decreased only slightly after exposure to high temperatures. This likely explains their tolerance to both fire and water deficit (Hayat et al., 2012; Kishor et al., 2022). Endogenous proline content is influenced by thermal and precipitation conditions during seed formation (Kijowska-Oberc et al., 2020). Proline acts as an environmental signaling molecule, accumulating in plant tissues in response to environmental conditions. In this study, proline accumulation was observed in response to both heat shock and WD cycles, though the response varied among the Vellozia species.

Many stress signaling pathways in plants, such as those involving Ca^{2+}, light, MAP kinase, and phytohormones, are linked to the biosynthesis and accumulation of ABA (Kumar et al., 2019). In this work, the increase in ABA levels in the WD+100°C treatment for *V. caruncularis* (Fig. 5D) indicated that the WD cycle alone was insufficient to mitigate the effects of heat shock. This was reflected in the reduced germination percentage under the WD+100°C treatment. Proline accumulation in plants can be influenced by both ABA-dependent and ABA-independent signaling pathways (Mahajan and Tuteja, 2005). Our experiments revealed that V. caruncularis seeds accumulated higher ABA levels after undergoing the WD cycle and heat shock, suggesting increased proline biosynthesis. ABA induces proline biosynthesis, which helps reduce reactive oxygen species (ROS) as part of a stress recovery mechanism (Cao et al., 2020; Shrestha et al., 2021). However, the proline accumulation was insufficient to mitigate the effects of high temperatures on *V. caruncularis*,

as evidenced by the reduced germination percentage and speed after the WD cycle and heat shock.

The three *Vellozia* species exhibited distinct responses to the WD cycle and heat shock. V. caruncularis did not benefit from the WD cycle as a mitigator of heat shock effects. Instead, it accumulated ABA and proline in response to stress, resulting in reduced germinability. Populations of *V. caruncularis* are typically found in *canga couraçada* and in regions with deep sandy soils or intermediate soil conditions (Viana and Lombardi, 2007; Vicent and Meguro, 2008; Alcantara et al., 2015), where WD cycles occur naturally, but fires are less frequent. In contrast, *V. compacta* seeds demonstrated tolerance to heat shock after undergoing the WD cycle, as evidenced by increased proline levels and GSI compared to seeds exposed to heat shock alone. *V. nanuzae* exhibited high germination rates when exposed to heat shock alone and benefited further from the WD cycle when combined with heat shock, without significant ABA or proline accumulation. This suggests that the WD cycle enhances the tolerance of *V. compacta* and *V. nanuzae* to high temperatures.

V. compacta is found in n*odular cangas*, where soils with exposed rocks can reach high temperatures (Vicent and Maguro, 2008). In contrast, *V. nanuzae* is a microendemic species restricted to a few rock crevices in limited geographical areas (Mello-Silva, 2015; Cunha-Blum et al., 2020). The occurrence of these species in fire-prone areas and the high proline content in their seeds may explain their resistance to abiotic stress. The grouping analysis likely reflects the microclimates and microhabitats where these species occur. Our germination results support this interpretation and align with findings by Fernandes et al. (2021) for *V. nanuzae*. However, *V. compacta* and *V. caruncularis* did not exhibit the same characteristics. *V. caruncularis* is particularly sensitive to heat shock (Fernandes et al., 2021), and the WD cycle did not improve its tolerance to high temperatures (Fig. 6).

CONCLUSION

The WD cycle favors germination and promotes heat shock tolerance in *V. compacta* and *V. nanuzae*. However, this tolerance effect is not observed in *V. caruncularis*. Depending on the intensity, frequency, and severity of fires, natural populations of *V. caruncularis* may experience a reduction in the recruitment of new individuals. In contrast, *V. compacta* and *V. nanuzae* exhibit tolerance to both water and thermal stress (whether isolated or combined) and may be suitable for the restoration of affected areas characterized by climatic seasonality and the occurrence of natural fires.

The responses of *Vellozia* seeds observed in this study reflect the microhabitat conditions of the *campos rupestres* where they are found and provide insights into the future persistence of these species in the soil seed bank. In a climate change scenario marked by increased fire incidence and more frequent dry environments, it is crucial to take measures to prevent disruptions in population dynamics in the field.

ACKNOWLEDGMENTS

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Figure 1

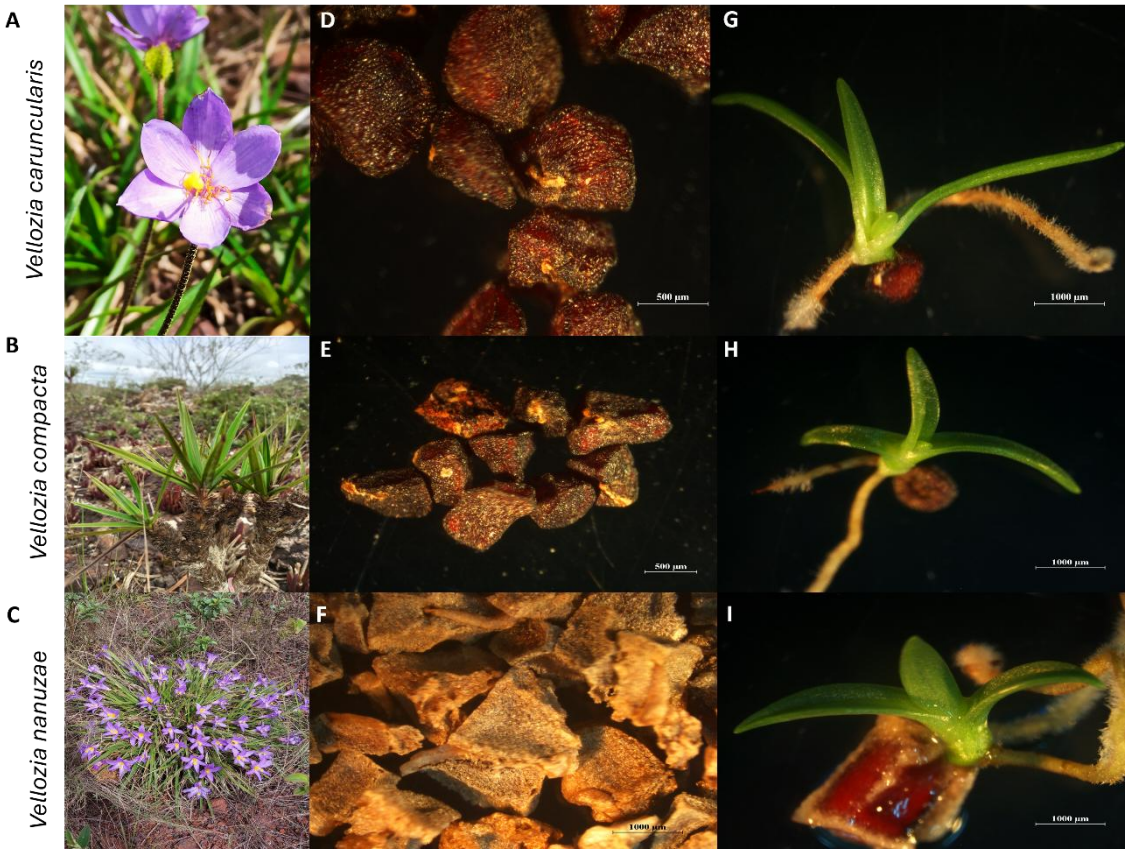

Fig. 1: Individuals in a reproductive stage in the collection area, seeds and seedlings of *Vellozia caruncularis* (A, D, G), *Vellozia compacta* (B, E, H), and *Vellozia nanuzae* (C, F, I), respectively. Photography's by Paulo Pimentel (A, B, and C).

Figure 2

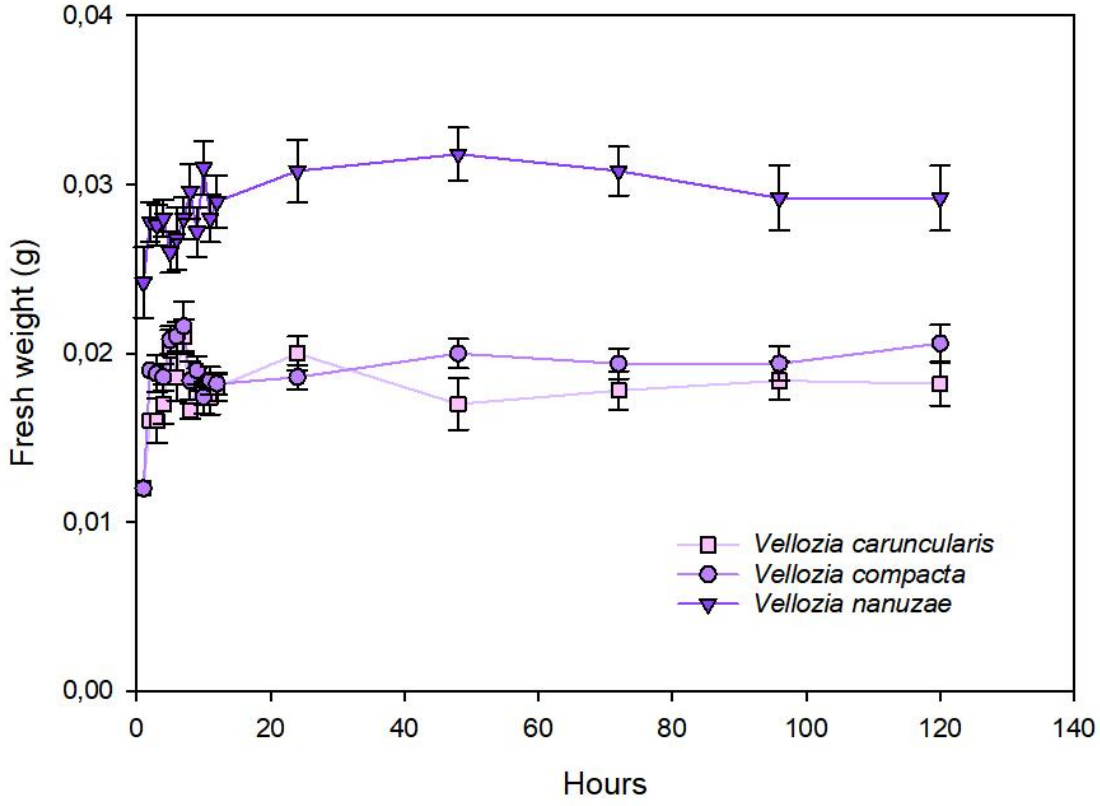

Fig. 2: Seed imbibition curves of *Vellozia caruncularis*, *Vellozia compacta* and *Vellozia nanuzae*.

Figure 3

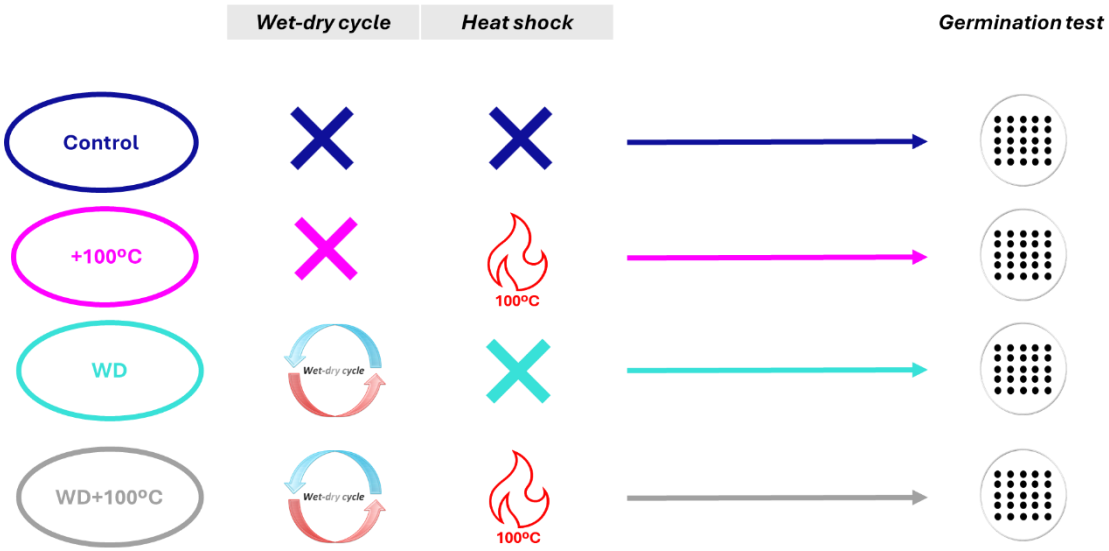

Fig. 3: Schematic model of WD-cycling and heat-shock treatments applied to seeds of three *Vellozia* species.

Figure 4

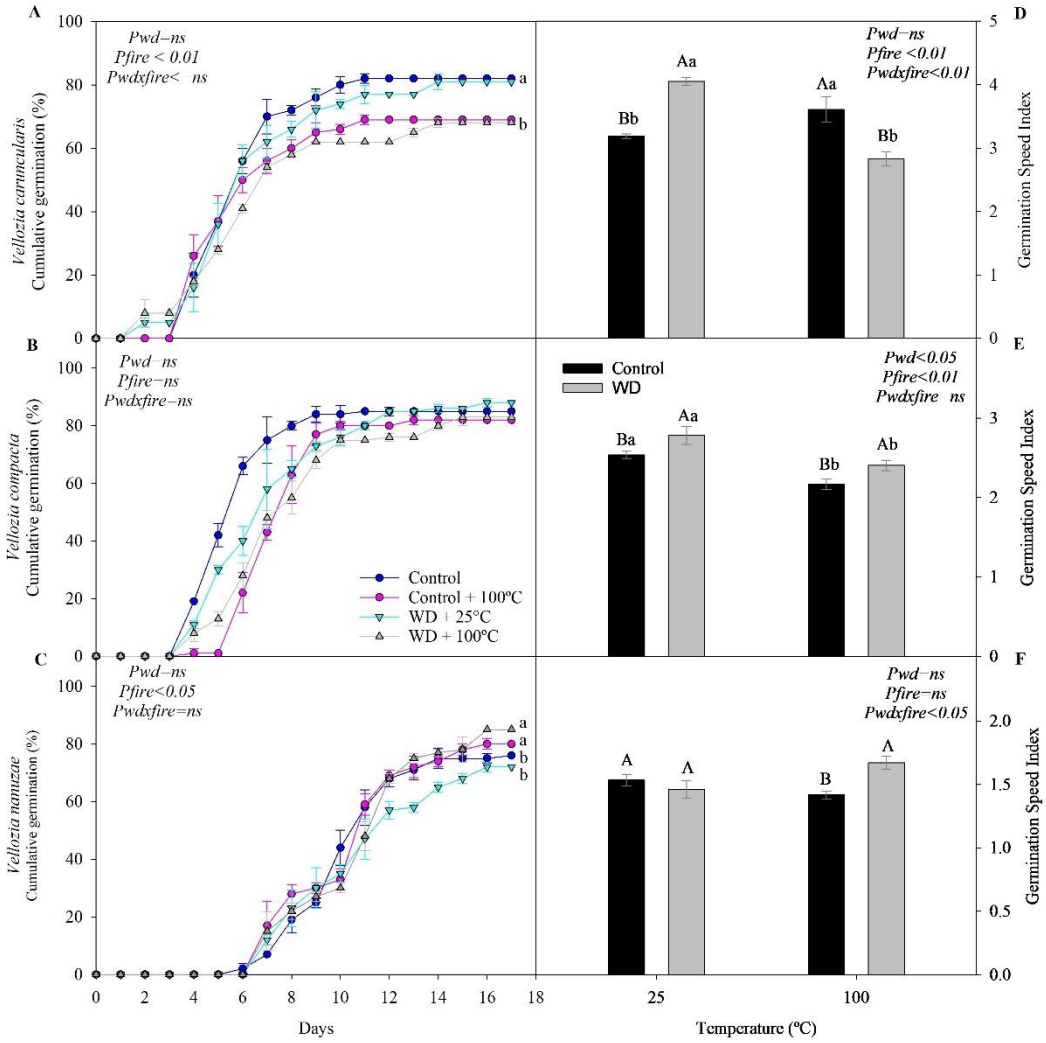

Fig. 4: Cumulative germination and germination speed index - GSI of *Vellozia caruncularis* (A, D), *Vellozia compacta* (B, E), and *Vellozia nanuzae* (C, F) subjected to a wet-dry cycle and heat shock, respectively. Bars are means ±SE. Capital letters compare statistical results in each WD cycle. Lowercase letters compare each heat shock. Averages followed by the same letters do not differ significantly by Tukey's test at 5% probability, ns = not significant; pt = P value between WD cycles; pfire = P value between thermal shocks; pwdxfire = P value of the interaction between WD cycles and thermal shocks.

Table 1. Results of statistical analysis (ANOVA and Tukey test) for evaluations of germination and biochemical parameters of *Vellozia spp*. seeds exposed to wet-dry cycle and heat shock.

Specie		*F*	*df*	*p*		*F*	*df*	*p*
Vellozia caruncularis	**Germination**				**Proline**			
	Wet-dry cycle	0.6944	1	0.42883	Wet-dry cycle	4.7647	1	0.060595
	Heat shock	14.6944	1	0.00499	Heat shock	15.2687	1	0.004497
	Wet-dry cycle*Heat shock	0.2500	1	0.63054	Wet-dry cycle*Heat shock	16.5585	1	0.003587
	Germination Speed Index				**ABA**			
	Wet-dry cycle	0.132	1	0.72623	Wet-dry cycle	12.0672	1	0.008396
	Heat shock	11.387	1	0.00972	Heat shock	2.4218	1	0.158273
	Wet-dry cycle*Heat shock	47.402	1	0.00013	Wet-dry cycle*Heat shock	5.7012	1	0.044012
Vellozia compacta	**Germination**				**Proline**			
	Wet-dry cycle	0.37120	1	0.55925	Wet-dry cycle	18.0433	1	0.00281
	Heat shock	2.55125	1	0.14887	Heat shock	3.9886	1	0.08087
	Wet-dry cycle*Heat shock	0.07836	1	0.78663	Wet-dry cycle*Heat shock	0.9670	1	0.35423
	Germination Speed Index				**ABA**			
	Wet-dry cycle	5.8535	1	0.04189	Wet-dry cycle	0.0215	1	0.88706
	Heat shock	14.0399	1	0.00565	Heat shock	1.5314	1	0.25099
	Wet-dry cycle*Heat shock	0.0020	1	0.96524	Wet-dry cycle*Heat shock	1.3906	1	0.27218
Vellozia nanuzae	**Germination**				**Proline**			
	Wet-dry cycle	0.0213	1	0.88761	Wet-dry cycle	2.4518	1	0.156025
	Heat shock	7.4926	1	0.02556	Heat shock	5.7616	1	0.043154
	Wet-dry cycle*Heat shock	2.5656	1	0.14788	Wet-dry cycle*Heat shock	3.7139	1	0.090116
	Germination Speed Index				**ABA**			
	Wet-dry cycle	1.8792	1	0.20764	Wet-dry cycle	2.58997	1	0.14621
	Heat shock	0.5423	1	0.48251	Heat shock	0.20782	1	0.66060
	Wet-dry cycle*Heat shock	6.3194	1	0.03615	Wet-dry cycle*Heat shock	0.00000	1	0.99914

Figure 5

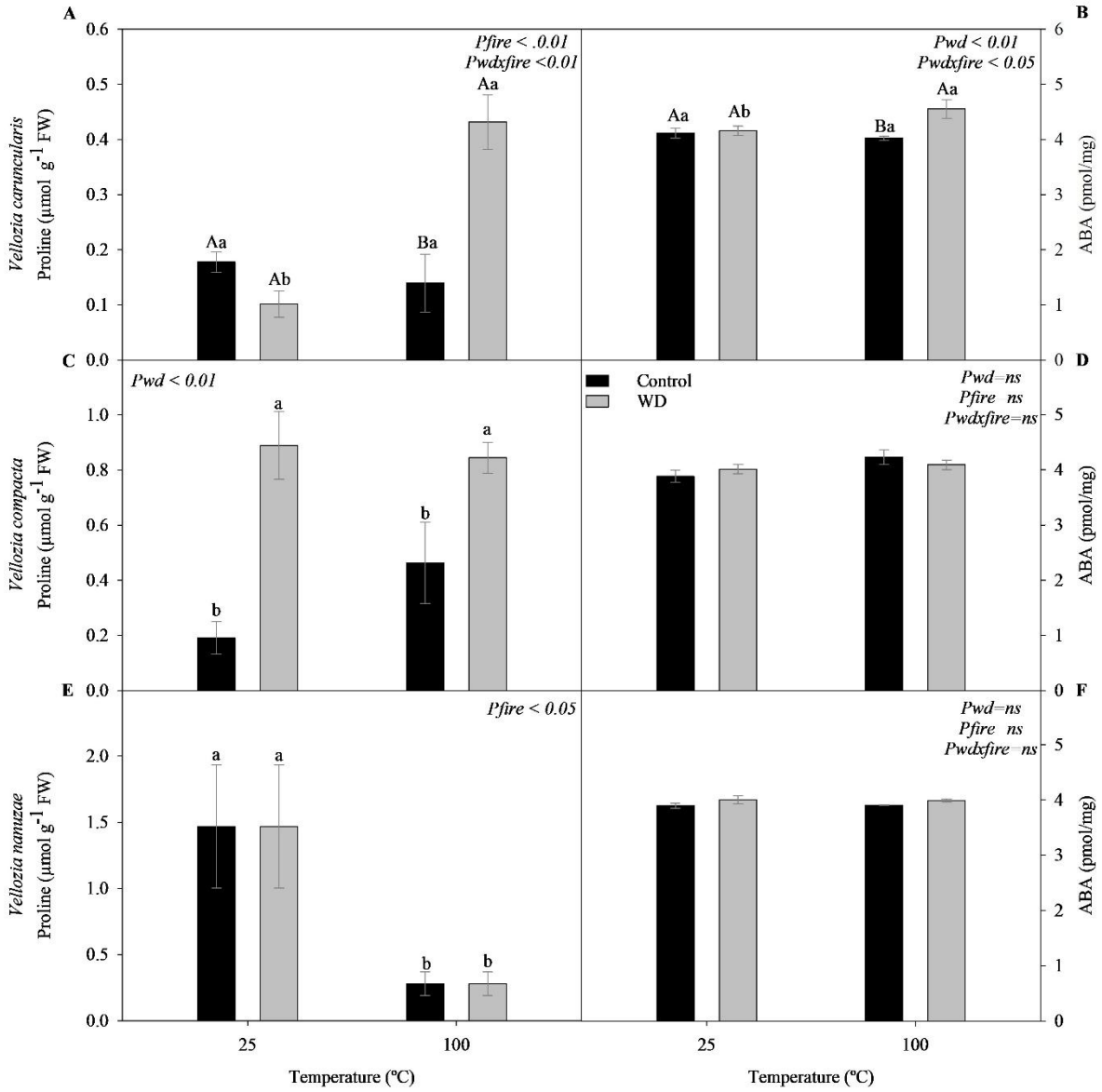

Fig. 5: Endogenous levels of proline and abscisic acid (ABA) in seeds of *Vellozia caruncularis* (A, D), *Vellozia compacta* (B, E), and *Vellozia nanuzae* (C, F) subjected to a wet-dry (WD) cycle and heat shock, respectively. Bars are means ±SE. Capital letters compare statistical results in each WD cycle. Lowercase letters compare each heat shock. Averages followed by the same letters do not differ significantly by Tukey's test at 5% probability, ns = not significant; pt = P value between WD cycles; pfire = P value between thermal shocks; pwdxfire = P value of the interaction between WD cycles and thermal shocks.

Figure 6

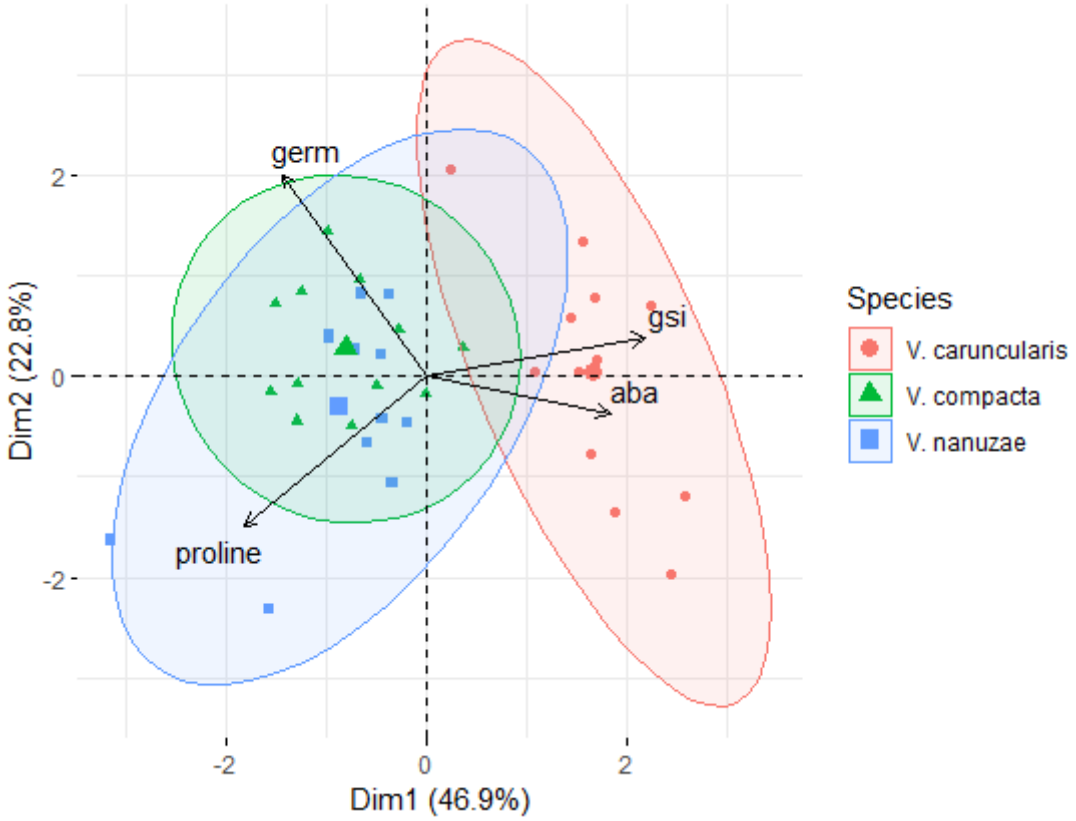

Fig. 6: Principal Component Analysis (PCA) based on germination and biochemical characteristics of *Vellozia caruncularis*, *Vellozia compacta*, and *Vellozia nanuzae* seeds after wet-dry cycle and heat shock.

PAPER 3: FUNCTIONAL TRAITS OF SEEDS CONTRIBUTE TO PLANT ECOLOGICAL STRATEGIES? A CASE STUDY IN A SOUTHEAST BRAZILIAN RESTINGA

Manuscript written according to the Functional Plant Biology guidelines

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ABSTRACT

Coastal ecosystems provide vital services to the species that inhabit them and their surrounding areas. These species are exposed to stress factors such as salinity, light intensity, heat stress, and pollution, which are exacerbated by human activities and climate change. This study investigated the relationship between vegetative and seed traits and their role in understanding the ecological strategies adopted by *Restinga* species. An integrative functional approach, incorporating vegetative, biometric, and seed germination trait, was used to study 12 species across *Restinga* phytophysiognomies. *Restinga* vegetation exhibits a stress tolerance strategy. A dissimilarity analysis grouped the species based on their germination responses and biometric traits. Leaf dry matter content (LDMC) showed positive correlations with functional biometric characteristics, germination percentage, speed, and synchrony. Seed moisture content, however, only correlated positively with T_{50} (the time required for 50% of seeds to germinate*).* *Restinga* species are tolerant to stress and have seeds with varied germination responses in terms of percentage, time, speed, and synchrony of germination. Vegetative functional traits alone explain the tolerance and survival of adult individuals, while reproductive functional traits provide insights into seed dispersal, germination, longevity, and seedling establishment. These characteristics are crucial in understanding the responses of Restinga vegetation to environmental pressures.

Keywords: Vegetative traits, seed traits, germination parameters, sandy coastal.

INTRODUCTION

Coastal environments are important in providing ecosystem services of provisioning (supply of sand and minerals, water collection and purification), regulation (coastal and storm protection, erosion control, prevention of seawater intrusion into the mainland), cultural (tourism, education and research, health benefits), and supporting (providing food and nesting sites for resident and migratory species) (Brasil, 1999; Brasil, 2002; Scherer et al., 2005; Lakshmi, 2021). *Restinga* (Brazilian coastal plains) exhibits a vegetation gradient that increases as one moves away from the sea, distributed across different vegetation zones or mesohabitats (Araujo and Oliveira, 1988; Falkenberg, 1999; Rocha et al., 2004). *Restinga* vegetation comprises open areas covered with halophytic/psammophilous-reptant species up

to trees that form a thin canopy, primarily composed of scattered shrubs (Assumpção and Nascimento, 2000). *The Restinga* are characterized by a mosaic of distinct vegetation types, including beach communities, open scrublands, dune fields, and coastal plain forests, each with unique physiognomies (Araujo and Pereira 2009). The characteristics of *Restinga* vegetation create conditions of high irradiance, temperature, and low humidity (Pireda et al., 2019).

Restinga is a resource-poor environment with stressful environmental characteristics such as high luminosity, salinity, high temperatures, and low water and nutrient availability (Scarano, 2002). To establish themselves in these locations, plants need to develop morphological, anatomical, physiological, and/or ecological strategies that allow them to tolerate or escape these environmental conditions. Competition, stress tolerance, and ruderalism (CSR) are strategies that organisms adopt depending on their environment. Competitive species (C) invest in continuous vegetative growth and rapid development to secure resources in stable and productive habitats. Stress-tolerant species (S) protect their metabolic performance in variable and resource-poor environments by focusing on resource retention and repair of cellular components in dense and persistent tissues. The R strategy, or ruderalism, involves investing resources in propagules that allow the population to regenerate in response to repeated events of biomass destruction or disturbances (Grime and Pierce, 2012; Pierce et al., 2017).

These strategies represent different ways in which organisms adapt to their surroundings and optimize their chances of survival and reproduction (Grime and Pierce, 2012). The CSR ecological strategy defines a triangular space between extreme strategies that maximize resource acquisition in productive environments (competitive strategy), survival in stressful conditions (stress-tolerant strategy), and short life expectancy where disturbances are frequent (ruderal strategy) (Silva et al., 2017).

To determine CSR strategies, it is essential to consider universal traits capable of representing the extremes of a trade-off between larger size and a conservative economy, in contrast to a more acquisitive economy (Pierce et al., 2017). Traits related to leaf size and economics are widely available, applicable to diverse plant life forms, and highly representative of plant functional trade-offs (Pierce et al., 2017). Plants that produce larger seeds may have fewer resources available for the development of large leaves, while plants with smaller seeds may invest more in leaf area to maximize light capture (Moles et al., 2005). Studies have shown that species with larger seeds tend to have thicker leaves and

higher leaf mass per area (LMA), which may be linked to a resource-conservation strategy in stressful environments (Wright et al., 2004). Variation at the leaf level reflects a significant portion of the overall functional variation in plants (Díaz et al., 2016).

Seed functional traits provide insights into dispersal, longevity, germination, and seedling establishment (Saatkamp et al., 2019). The interaction between germination physiology and seed morphological characteristics reflects the pressures to avoid abiotic stresses (Fernandez-Pascual, 2020). Seeds that invest in size increase germination time and early seedling performance (Suárez-Vidal et al., 2017). Research involving seed traits often focuses only on biometric attributes, neglecting aspects such as germination, dormancy, germination requirements, and seedling establishment, using only biometric characteristics (Saatkamp et al., 2019). However, it is important to highlight that seeds play a crucial role in the occupation, recruitment, and maintenance of natural populations (Dailling et al., 2020). To gain a comprehensive understanding, it is necessary to focus research on dispersal, longevity, germination time, and establishment. This requires the use of morphological, physiological, and biochemical characteristics of seeds (Saatkamp et al., 2019).

The relationship between seed and vegetative functional traits has been widely studied in plant ecophysiology, as both are closely linked to the survival, reproduction, and adaptation strategies of plants to their environments (Leishman et al., 2000; Westoby et al., 2002; Wright, et al., 2004; Moles, et al., 2005; Poorter, Poorter & Villar, 2009; Pierce et al., 2014; Díaz, et al., 2016; Santini et al., 2017). Plant functional traits may be correlated, as they reflect plant responses to environmental pressures and their evolutionary histories (Violle et al., 2007).

Studies have shown that reproductive traits are correlated with vegetative traits (leaves), making them necessary/effective in understanding the functionality of natural ecosystems. Using reproductive and vegetative traits, Pierce et al. (2017) observed that seed mass and other traits related to seed production are correlated with traits associated with plant size and C (competitive) strategy. Seed mass is another attribute that correlates positively with vegetative attributes, where species with larger seeds have higher leaf dry matter content (LDMC) (Simpson et al., 2021). Leaf area is another attribute that correlates positively with seed mass and is conserved among all life forms. And there is a triangular relationship between seed mass and leaf area that is driven by habitat variation and soil productivity (Santini et al., 2017). However, in studies with alpine species, Hoyle et al. (2015) found that

germination traits correlate with other aspects of seed ecology but form an independent axis relative to vegetative traits.

Plants adopt different ecological strategies to overcome ecological filters and survive various types of stress (Pierce et al., 2017). Understanding how climate acclimatization is perceived and how it can help identify potential stakeholder tensions when implementing adaptation strategies in biodiversity conservation contexts is crucial (Spencer et al., 2024). The conservation and restoration of these areas are vital for maintaining ecosystem services, such as those provided by *Restinga*. Despite being protected by law (Law No. 4.771/1965; National Environment Council Resolution (CONAMA) No. 302/2002; Law No. 12.651/2012 (Art. 4, Item VI); Law No. 11.428/2006 - Atlantic Forest Law; CONAMA No. 417/2009), real estate speculation, anthropogenic actions (pollution), and the climate emergency scenario have reduced their original distribution (Borges et al., 2009; Overbeck et al., 2022).

The combination of vegetation and seed functional traits can be a tool in ecophysiology and ecological restoration studies, as reproductive success depends on seed functional traits (Milla et al., 2009; Laughlin et al., 2020). Understanding how plants respond (or will respond) to this new scenario of pollution, greater intensity, frequency, and duration of stress is a key factor in maintaining ecosystems. With this in mind, our hypothesis was to identify the ecological strategies adopted by *Restinga* plants and to determine whether the biometric and germination traits of seeds correlate with each other and with vegetative traits. Our research aimed to understand how vegetative and reproductive traits – seeds – correlate and contribute to the dynamics of Restinga vegetation.

METHODOLOGY

Collection site and species

The collections were made between February 3 and 4, 2023 at *Paulo César Vinha State Park*, *Guarapari*, *Espírito Santo*, Brazil (Fig. 1). The species were selected according to the dispersal time of their seeds, distributed in the different Restinga phytophysiognomies. The species used for the functional attributes and their respective families and geographical locations are detailed in Table 1 below.

Vegetative functional traits

The only species that were not used for the vegetative attributes listed in Table 1 were *Bromelia antiacantha*, *Jacquinia armilaris*, and *Pilosocereus arrabidae.* The ecological

strategy CSR (competition, stress-tolerant and ruderal) was identified through the collection of branches from mature and healthy individuals. The CSR values were calculated for each species following the methodology proposed by Pierce et al. (2017). The three functional leaf characteristics employed were leaf area (LA, mm^2), leaf dry mass (LDW, mg) and leaf fresh mass (LFW, mg). The sampling of these characteristics was conducted following the protocol proposed by Perez-Harguindeguy et al. (2016).

The collected branches were placed in plastic bags, saturated with water, and transported to the laboratory for further analysis. In the laboratory, the plant samples were kept in water for a period of five hours until they reached full turgidity. Three leaves were then selected from each individual for measurement. The leaf area was obtained using a mobile phone camera, with the measurements subsequently taken using Image J software. The fresh weight of the leaves was obtained from turgid leaves, while the dry weight was determined using an analytical balance (precision of 0.001 g) after 96 hours of drying in an oven at 60°C.

Seed functional traits

The species selected for analysis of the biometric and germination functional parameters of the seeds were the same as those used for the vegetative functional attributes (Table 1), with the exception of *Aechmea lingulata* and *A. nudicaulis*, which were not in their seed dispersal period.

The biometric variables assessed were length (mm), width (mm), thickness (mm), using a digital caliper, and mass (g) of the seeds on an analytical balance (0.001g). We used 10 replicates of 10 seeds each for each variable measured and species, except for *P. arrabidae*, for which we used 5 replicates of 100 seeds each for the fresh weight, and 5 replicates of 40 seeds for other parameters. The moisture content of the seeds was determined using 5 replicates of 10 seeds for each species (except for *P. arabidae*, we used 30 seeds for each replicate), with the initial fresh weight recorded for each seed. Following 24 hours in an oven maintained at 105°C (as recommended by Brasil, 2009), the seeds were weighed again, and the moisture content was expressed on a wet basis (Table 2).

Germination test

Seeds of the 10 species (5 replicates of 20 seeds each for *B. antiacantha*, *C. virginianum*, and *S. tomentosa*; 25 for *C. myrsinites*, *G. brasiliensis*; *M. obtusifolia* and *M.*

splendens; 4 replicates of 25 seeds each for *C. rosea* and *J. brasiliensis*) were disinfected in a solution of 1.0% sodium hypochlorite (NaClO) plus two drops of commercial detergent for 10 min and rinsed three times in deionized water. Following disinfestation, the seeds were placed in Petri dishes randomly distributed in germination chambers under a 12 h photoperiod and 40 μmol photons m^{-2} s^{-1} at the optimum germination temperature for each species. Germination was assessed daily for 30 or 60 days (depending on the species) and the criterion used was radicle protrusion <2mm. The germination parameters assessed were the final germination percentage, the germination speed index (GSI) as defined by Maguire (1962), the average time for germination to reach 50% (T_{50}) as defined by Farooq et al. (2005), and the synchronization index (Z) as defined by Ranal & Santana (2006). The species *C. rosea*, *C. virginianum*, and *S. tomentosa*, which exhibited physical dormancy, were subjected to abrasion with sandpaper No. 80 and immersion in concentrated sulfuric acid for 30 minutes, respectively (Table 2).

STATISTICAL ANALYSES

The data was entered into the 'StrateFy' Excel table, where each species' ecological strategy percentage was calculated. The values for leaf area (LA), specific leaf area (SLA), and leaf dry matter content (LDMC) were entered into the table to calculate the proportion of C, S, and R selection for each CSR strategy (Pierce et al., 2017). A UPGMA was constructed for the seeds' biometric and germination traits, and a Pearson correlation was made with the species that possessed both vegetative and seed traits.

RESULTS

The species collected in the *Restinga* exhibit characteristics of stress-tolerant species, with 80% of their attributes classifying them under this ecological strategy. *Monteverdia obtusifolia* and *Centrosema virginianum* were the only species with significant values for both competition and stress-tolerant strategies, leading to their classification as S/CS (Fig. 2).

UPGMA analysis of the species, based on their biometric and germination characteristics, grouped the species according to their responses and germination requirements. The isolation of *C. rosea* was attributed to its higher biometric values and its high and rapid germination rate. *M. obtusifolia*, *S. tomentosa*, and *P. arribadae* formed a group due to their similar germination responses in terms of speed, time, and synchronization. The species *C. myrsinites*, *C. virginianum*, *B. antiacantha*, and *J. armilaris* were grouped

together because they share similar moisture content levels. Meanwhile, *G. brasiliensis* and *M. splendens* were grouped due to their longer germination times (Fig. 3).

The correlation analysis between vegetative, biometric, and germination parameters of the seeds revealed a positive correlation between LA (leaf area) and LDMC (leaf dry matter content) with seed length, width, thickness, fresh weight, germination percentage, GSI (germination speed index), and synchronization. Conversely, a negative correlation was observed with moisture content, biometric seed traits, and T_{50} (time to 50% germination) (Fig. 4).

DISCUSSION

The CSR values of the *Restinga* plants studied show that the species qualify as stress-tolerant. The species present in these environments need to invest in mechanisms to tolerate or escape these stressors (Scarano, 2002; Ciccarelli, 2015). Natural stressful conditions plus anthropogenic disturbances play a role in the ecological dynamics of sandy coastal plains (Ciccarelli, 2015). Evaluating CSR ecological strategies in Brazilian coastal plains, Silva et al., (2018) and Costa et al., (2020) showed a prevalence of S/CS strategies. Studies in coastal environments with high anthropogenic pressures and disturbances have shown a prevalence of competition and ruderalism strategies (Lee et al., 2020).

Seed functional traits provide insight into seed dispersal, longevity, germination, and seedling establishment (Saatkamp et al., 2019). The interaction between germination physiology and seed morphological characteristics further reflects the pressures to avoid abiotic stresses (Donohue et al., 2010). Seeds that invest in size increase germination time and early seedling performance (Suárez-Vidal et al., 2017). This is the case with *C. rosea* species which showed the highest values for biometric and germination parameters. This is explained by the phytophysiognomies they occupy: *C. rosea* has large seeds and high tolerance to salinity and high temperature, occupying the reptant psammophilous formation (Moreno-Cassasola et al., 1994; Mendoza-González, Martínez & Lithgow, 2014).

The species *M. obtusifolia*, *P. aribadae*, and *S. tomentosa* were grouped in the same grade due to their shared germination response. This grouping can possibly be explained by the fact that both species occupy areas of shrubby *Restinga* (Carvalho et al., 2018). Although *Restingas* are highly stressful ecosystems, in open shrubby *Restinga*, shrubs can form patches separated by areas of exposed sandy soil or form a continuous thicket (closed shrubby), creating a microclimate conducive to the germination and establishment of new species with

different germination requirements (light, temperature, moisture, and salinity) in these ecosystems due to the improvement of microclimatic factors (Shumway, 2000; Zaluar and Scarano, 2000). Another characteristic that may explain this grouping is the facilitation process by the hemicryptophyte palm (*Allagoptera arenaria*), since isolated adults of *P. arrabidae* are associated with individuals of *A. arenaria*, and are rarely seen on bare sand in coastal sandy plains (Zaluar and Scarano, 2000). The germination and establishment of *P. arrabidae* in microsites of relief or under the shade of other plants are conditions favorable to high germination and greater recruitment due to the temperature, light, and humidity conditions. These light and humidity conditions create a better germination environment (Martins et al., 2012).

The species that shared the same moisture content (*C. virginianum*, *C. myrsinites*, *B. antiacantha*, and *J. armilaris*) were grouped in the same grade. The relationship between seed water content and tolerance to abiotic stress in *Restinga* ecosystems is a crucial aspect for understanding the ecology of germination and plant establishment in these environments. Seeds with higher water content tend to exhibit greater physiological plasticity, which can confer greater resistance to adverse conditions such as high temperatures, salinity, and water deficit, which are common in *Restinga* (Suárez-Vidal et al., 2017; Saatkamp et al., 2019). The water inside the seeds acts as a metabolic regulator, enabling essential biochemical processes, such as reserve mobilization and protein synthesis, to occur even under stressful conditions (Bewley et al., 2013). This characteristic is particularly important in coastal ecosystems, where microclimatic variation and soil heterogeneity impose significant challenges to seedling survival.

The ability to retain water and maintain metabolic activity under adverse conditions allows seeds to germinate and establish even in highly stressful environments, contributing to the maintenance of biodiversity and the resilience of these coastal ecosystems (Huang et al., 2020; Zhang, Zhao, and Zhu, 2020). This relationship highlights the importance of physiological and ecological studies for the conservation and restoration of *Restinga* areas, especially in scenarios of climate change and increasing anthropogenic pressures.

In stressful environments, such as *Restinga*, the mean germination time (T_{50}) is a crucial parameter that reflects the establishment strategy of species. Seeds with a prolonged T_{50} may indicate an adaptation to adverse conditions (Saatkamp et al., 2019), such as high salinity, extreme temperatures, and water deficit, which are common in these ecosystems. The delay in germination may be an evolutionary response to avoid exposing seedlings to critical

periods of abiotic stress, such as prolonged droughts or high temperatures, which can compromise initial survival (Donohue, 2005; Fenner and Thompson, 2005). This strategy, known as staggered germination, allows seeds to germinate only when environmental conditions are more favorable, increasing the chances of establishment and survival (Baskin & Baskin, 2014).

The species *G. brasiliensis* and *M. splendens* exhibited the most dispersed germination over time, which is why they were grouped in the same clade. It has already been reported in the literature that the seeds of *G. brasiliensis* exhibit some dormancy, requiring temperature alternation to germinate (Carvalho et al., 2018). *M. splendens* is a species with desiccation-sensitive seeds (recalcitrant), maintaining high water content and active metabolism after dispersal (Pammenter & Berjak, 2000; Berjak & Pammenter, 2008; Carvalho, 2008). As a result, they are more sensitive to environmental conditions, which can lead to less synchronized and slower germination (Farrant et al., 1988; Berjak & Pammenter, 2008). The temporal distribution of germination is a strategy adopted by plants to increase the chances of species survival in the face of unfavorable environmental events, such as periods of drought, fires, and flooding (Ferreira et al., 2021), as in the case of Restinga ecosystems. This characteristic allows seeds to germinate in a staggered manner, reducing competition among seedlings and increasing the likelihood that they will find suitable conditions for establishment (Daws et al., 2006; Tweddle et al., 2003).

However, a prolonged T_{50} can also imply risks, such as greater exposure of seeds to predators, pathogens, or unpredictable climatic events that may reduce the soil seed bank (Dalling et al., 2011). In *Restinga*, where competition for resources is intense and the availability of suitable microsites for germination is limited, delayed germination may result in lower competitive success compared to species with faster germination (Martins et al., 2012). On the other hand, species with a longer T_{50} may benefit from the formation of microclimates under existing vegetation, where moisture, temperature, and light conditions are more stable, facilitating seedling establishment (Zaluar & Scarano, 2000).

Correlating functional traits and ecosystem service attributes can be a key tool for studying species' responses to different stresses and ecological restoration (Carlucci et al., 2020). Since the most widely used technique is direct sowing, understanding dispersal mechanisms and germination requirements is of paramount importance to minimize costs and ensure that the greatest number of seeds from all germination spectra/niches manage to germinate and become established (Souza and Engel, 2023). Pierce et al. (2014) use seed

production and other seed traits to correlate with CSR strategies, where seed mass and other seed production traits are correlated with plant size traits and the ecological CSR strategy. However, size and seed production are traits that can affect survival, regardless of CSR strategy. Meanwhile, Simpson et al. (2021) found that for species with high LDMC, large seeds were associated with faster size-standardized growth rates than small seeds. This aligns with the data found in our study, where LDMC correlated positively with germination attributes.

The relationship between leaf area and seed size can be understood as part of a resource allocation trade-off. Plants that produce larger seeds may have fewer resources available for the development of large leaves, while plants with smaller seeds may invest more in leaf area to maximize light capture (Moles et al., 2005). Studies have shown that species with larger seeds tend to have thicker leaves and higher LMA, which may be related to a resource conservation strategy in stressful environments (Wright et al., 2004).

To maintain natural populations, it is essential to understand how anthropogenic actions and climate change will impact dynamics at the individual, population, community, and ecosystem levels (Malhi et al., 2020). The integration of functional traits, both vegetative and seed-related, will provide evidence of species tolerance or susceptibility during recruitment and seedling establishment (Aubin et al., 2016). The selection of species for restoration purposes must take into account the availability of seeds and pre-sowing treatments, the species' ability to overcome dispersal and establishment limitations, and the socio-economic value or ecosystem services they provide (Gusmán et al., 2023). While relying solely on vegetative functional traits provides insight into the adaptation of adult (already established) individuals (Pierce et al., 2017), functional seed traits offer a more holistic view of the ecophysiology of seed germination (Saatkamp et al., 2019).

CONCLUSION

From the species studied here, it can be concluded that *Restinga* vegetation is stress-tolerant, a key characteristic for survival in stressful environments. Using vegetative leaf data provides a comprehensive view of the strategy adopted by adult individuals and how they respond to environmental conditions.

Seed functional traits can provide valuable information for ecological restoration. Germination requirements, dormancy, timing, speed, and synchronization of germination are fundamental attributes to consider when selecting species for the regeneration of these coastal

environments. Understanding how seeds respond to new challenges posed by pollution, as well as the increasing intensity, frequency, and duration of stressors, is crucial for maintaining the integrity of this ecosystem.

Linking reproductive traits with vegetative traits is a strategy that has revealed a correlation between these attributes. This approach can be adopted in the selection of species for restoration studies based on LDMC (when seeds are not available) and may be effective for understanding the functionality of natural ecosystems.

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AUTHOR CONTRIBUTION STATEMENT

GSD, EMB conceived and designed the research; GSD, MTO, EGP, and EMB collected the species and took biometric measurements; GSD, MTO carried out all the experimental work; GSD performed the statistical analysis; GSD, MTO, and EMB contributed to data interpretation; GSD, EMB wrote the manuscript. All the authors contributed to data discussion, and conclusions, and reviewed and approved the final manuscript.

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Table 1: Species collected in the Paulo César Vinha State Park (PEPCV) and their respective families and geographical coordinates.

Species	Family	Geographical coordinate
Aechmea lingulata (L.) Baker	Bromeliaceae	S 20 36 449 W 040 25 034
Aechmea nudicaulis (L.) Griseb.	Bromeliaceae	S 20 36 355 W 040 25 008
Bromelia antiacantha Bertol.	Bromeliaceae	S 20 36 355 W 040 25 008
Canavalia rosea (Sw.) DC.	Fabaceae	S 20 36 403 W 040 25 034
Centrosema virginianum (L.) Benth.	Fabaceae	S 20 06 913 W 043 589 82
Chaetocarpus myrsinites Baill.	Peraceae	S 20 36 372 W 040 25 020
Gaylussacia brasiliensis (Spreng.) Meisn	Ericaceae	S 20 07 065 W 043 59 208
Jacquinia armillaris Jacq.	Primulaceae	S 20 36 403 W 040 25 034
Monteverdia obtusifolia (Mart.) Biral	Celastraceae	S20 36 306 W 040 24 929
Myrcia splendens (Sw.) DC.	Myrtaceae	S 20 36 360 W 040 25 054
Pilosocereus arrabidae (Lem.) Byles & Rowley	Cactaceae	S 20 36 326 W 040 25 001
Sophora tomentosa L.	Fabaceae	S 20 36 403 W 040 25 034

Figure 1

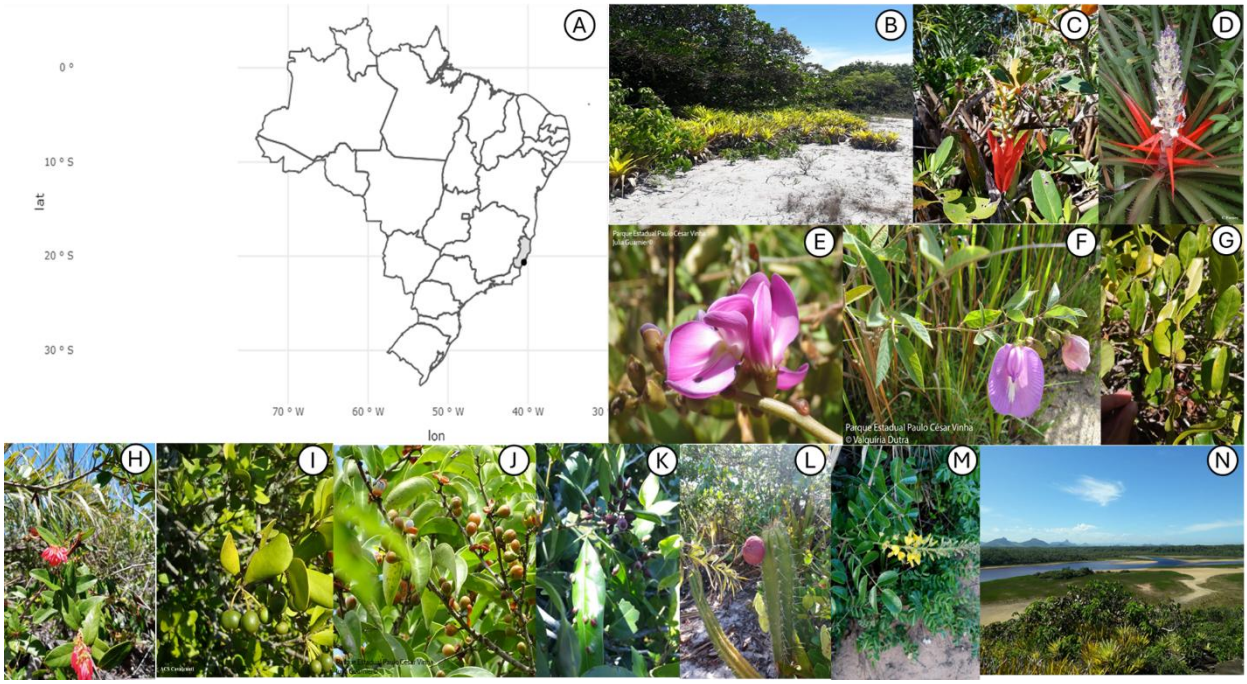
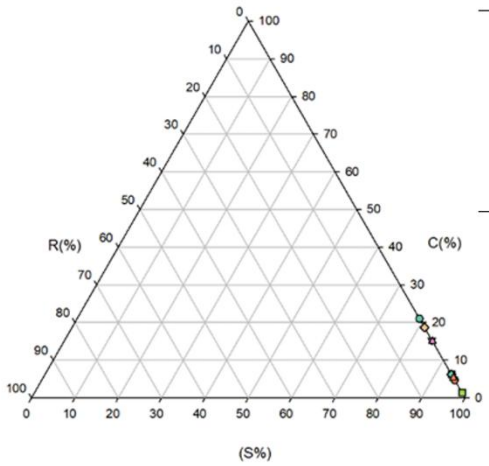


Figure 1: Study area in Restinga (A) The gray area indicates the state of Espírito Santo, and the black circle represents where the Parque Estadual Paulo César Vinha is in the municipality of Guarapari - ES. Figures to the right and below show the 12 species studied. *Aechmea lingulata* (B), *Aechmea nudicaulis* (C), *Bromelia antiacantha* (D), *Canavalia rosea* (E), *Centrosema virginianum* (F), *Chaetocarpus myrsinites* (G), *Gaylussacia brasiliensis* (H), *Jacquinia armillaris* (I), *Monteverdia obtusifolia* (J), *Myrcia splendens* (K), *Pilosocereus arrabidae* (L), *Sophora tomentosa* (M), and the Paqrque Estadual Paulo César Vinha (M). Photographers: B, C, G, H, L, and M (Geovane Dias); K, and N (Milena Oliveira); D (C Farney); E and J (Julia Guanieri); F (Valquiria Dutra); I (ACS Cavalcanti).

Figure 2



Espécie	C(%)	S(%)	R(%)
Monteverdia obtusifolia	21	79	0
Gaylussacia brasiliensis	5	95	0
Myrcia splendens	15	85	0
Chaetocarpus myrsinites	15	85	0
Sophora tomentosa	1	99	0
Canavalia rosea	6	94	0
Centrosema virginianum	18	82	0
Aechmea lingulata	6	94	0
Aechmea nudicaulis	5	95	0

Figure 2: Ecological strategies, C = competition, S = stress tolerance and R = ruderal, for each of the species studied. The table on the right shows the percentage of CSR for each species.

Figure 3

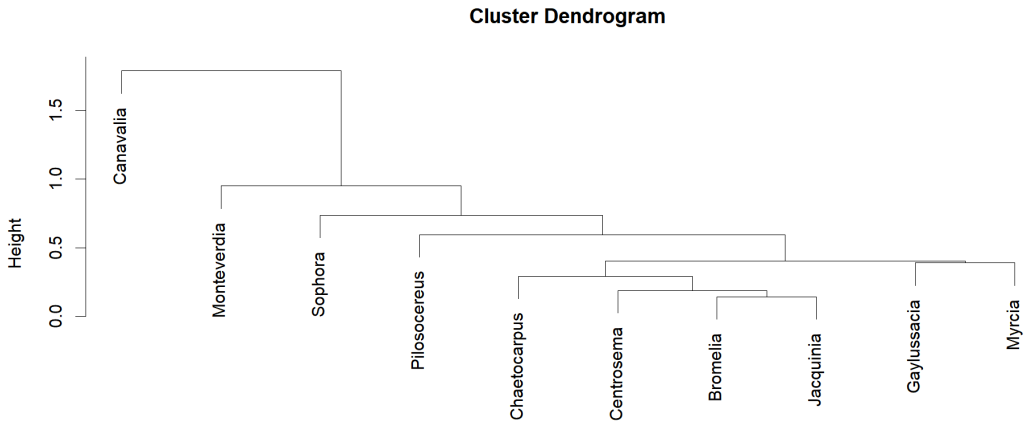


Figure 3: UPGMA clustering based on biometric functional traits and seed germination of species from the PEPCV Restinga, Guarapari-ES, Brazil.

Figure 4

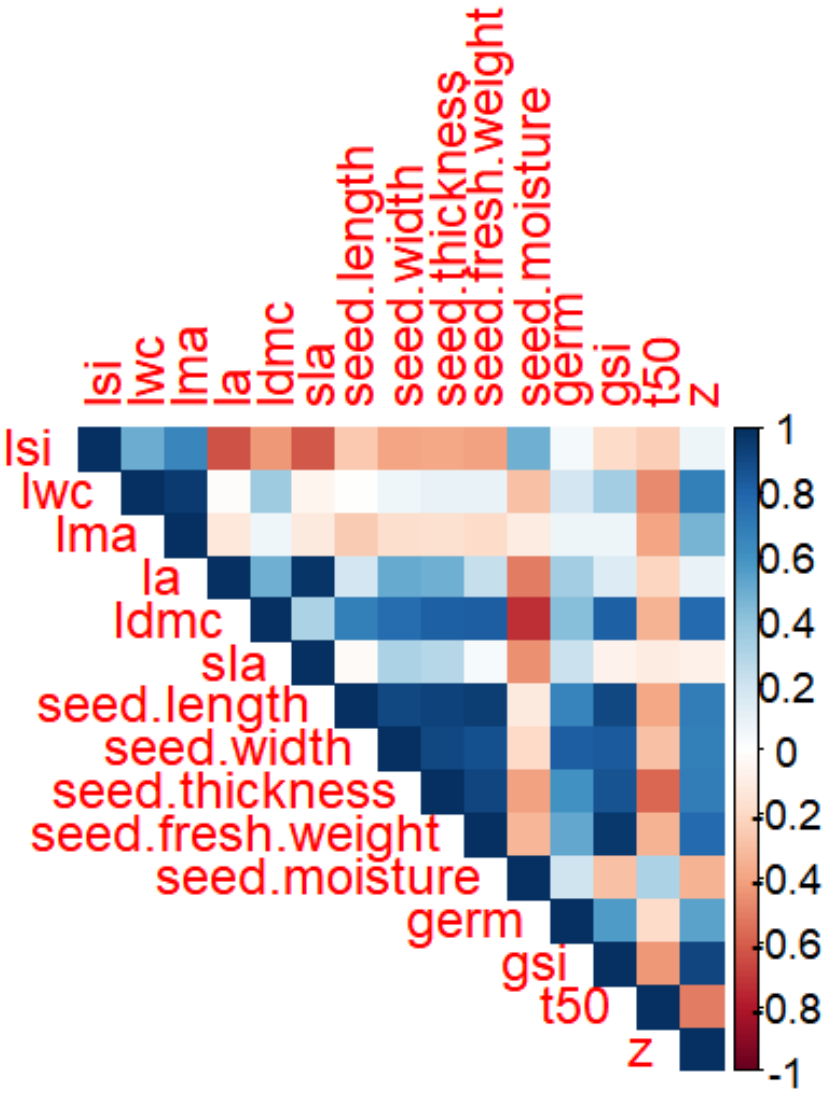


Figure 4: Pearson's correlation between vegetative and seed functional attributes of *Restinga* species from PEPCV, Espírito Santo, Brazil.

Table 2: Biometric and functional germination characteristics of species found in the PEPCV Restinga, Guarapari-Espírito Santo, Brazil. Mean values ± standard error.

Species	Length (mm)	Width (mm)	Thickness (mm)	Fresh weigth (g)	Germination (%)	Germination Speed Index	T_{50} ($days^{-1}$)	Synchronization Index	Moisture contente (%)
Bromelia antiacantha	4.3318±0.21	3.6495±0.18	2.5136±0.13	0.2993±0.01	71±2.96	0.6049±0.03	23.4666±0.39	0.0612±0.01	11.4±0.08
Pilosocereus aribadae	1.3326±0.05	0.9179±0.07	0.606±0.05	0.0488±0.00	31.5±10.5	2.3663±0.89	7.125±1.51	0.1551±0.08	4.77±1.40
Monteverdia obtusifolia	7.3145±0.31	4.0475±0.10	2.3056±0.12	0.5078±0.01	80.8±2.08	2.2175±0.07	8.909±0.51	0.0993±0.01	31.2±0.46
Gaylussacia brasiliensis	3.1366±0.06	1.4274±0.02	0.6932±0.02	0.0361±0.00	12.8±3.81	0.0931±0.02	33.25±2.20	0.0133±0.01	14.42±1.05
Canavalia rosea	15.8077±0.31	10.5396±0.22	9.1419±0.49	7.6901±0.19	93±1.65	20±0.63	1.654±0.04	0.6319±0.05	8.51±0.08
Sophora tomentosa	6.7008±0.17	6.5203±0.16	5.2863±0.16	1.1551±0.03	76±5.54	2.4447±0.19	6.265±0.47	0.1115±0.01	6.70±0.19
Centrosema virginianum	3.9602±0.13	2.3943±0.05	1.8657±0.07	0.1884±0.00	53±6.41	5.35±0.63	1.5919±0.05	0.4457±0.03	9.6±0.94
Myrcia splendens	5.9986±0.21	3.5998±0.04	1.6088±0.05	0.1685±0.00	70.4±3.85	0.6042±0.03	28.9733±0.87	0.0486±0.00	17.82±0.31
Chaetocarpus myrsinites	5.0562±0.21	4.026±0.17	2.6235±0.14	0.3121±0.01	0±0.00	0±0.00	0±0.00	0±0.00	9.59±0.14
Jacquinia armillaris	4.1814±0.07	2.9642±0.09	3.5048±0.10	0.3204±0.00	89±2.17	5.6468±0.62	8.25±1.82	0.0652±0.01	10.53±0.51

PAPER 4: THE COMBINATION OF HEAT STRESS AND DISCONTINUOS HYDRATION IN *CANAVALIA ROSEA* (SW.) DC. SEED GERMINATION: A PERSPECTIVE FROM A NATIVE SEASHORE SPECIES

Manuscript written according to the Plant, Cell & Environment guidelines

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ABSTRACT

Temperature and water availability are some of the environmental factors that influence seed germination. The plant species that inhabit the *Restinga* are adapted to environments with water and salt stress and high temperatures. For the southeastern region of Brazil, climate projections predict an increase in rainfall and temperature over the coming decades. Given the current climate scenario, our work aimed to investigate what mechanisms *Canavalia rosea* seeds exhibit when exposed to combined events of water availability and temperature increase. It was found that *C. rosea* germinated at high temperatures without affecting the percentage, speed, and viability of its seeds. This probably triggered the accumulation of soluble proteins, proline, and the synthesis of heat shock proteins (HSPs). However, when these seeds were exposed to discontinuous hydration (DH), there were losses in germination and viability parameters. In response to this stress, the seeds accumulated higher levels of MDA, H_2O_2, and ABA. It can be concluded that HD has a negative effect on germination, being more deleterious as the temperature rises. In addition, *C. rosea* seeds have high-temperature tolerance mechanisms that favor the pioneering of the species in the colonization of *Restinga* areas, once they are not hydrated.

Keywords: Heat stable proteins, *Restinga*, proline, warm temperature, water availability.

INTRODUCTION

Plants are exposed to a wide range of temperatures and water availability during their life cycle, requiring continuous adaptation. Climate change will lead to rising temperatures, and extreme rainfall events may become more frequent and intense (Perkins, Alexander & Nairn, 2012; Zhu, Lima & De Smet, 2021). The gradual increase in temperature and more intense rainfall will affect plant growth, development, and reproduction (Marshall, 1988; Hatfield & Prueger, 2015). These changes impact pollen production, viability, and germination, as well as the synchronization of flowering and pollination, ultimately

influencing seed formation and germination (Zamith, Cruz & Richers, 2012; Yi et al., 2019; Bogdziewicz et al., 2020; Descamps, Quinet & Jacquemart, 2021; Arathi & Smith, 2023; Gomes, Gomes & 2023).

The maintenance and survival of plant populations depend on the recruitment of new individuals (Eriksson & Ehrlén, 2008). After dispersal, seeds are exposed to various environmental factors that can affect their germination and viability, such as light, oxygen availability, burial depth, temperature, and precipitation (Bewley et al., 2013; Baskin & Baskin, 2014). Environmental factors like soil moisture and ambient temperature must remain favorable for a sufficiently long period to allow plant establishment (Baskin & Baskin, 2022).

Restinga ecosystems span the entire Brazilian coastline, extending inland to the west (Falkenberg, 1999). Formed during the Quaternary period through marine regressions and transgressions, *Restingas* consist of sandy deposits parallel to the coastline, created by sedimentation processes. These areas host diverse communities influenced by marine conditions (Suguio & Tessler, 1984; Fernandez et al., 2019). *Restingas* are characterized by edaphoclimatic features such as salinity, temperature fluctuations, floods, droughts, constant winds, and nutrient-poor soils (Brasil, 1996; Brasil, 2002; Scarano, 2002). Plant communities in *Restingas* play a crucial role in stabilizing sandy substrates and maintaining natural drainage, thereby helping to prevent erosion (Brasil, 2002). This vegetation also provides food resources and nesting sites for resident and migratory fauna (Brasil, 1999; Scherer et al., 2005). *Restinga* environments exhibit spatial and temporal variations in water availability (Martin et al., 1993; Cavalin & de Mattos, 2007; Braz & Mattos, 2010). Due to the physical nature of *Restinga* soil, such as its granulometry and porosity, water is not readily available to plant species, subjecting them to constant low water availability. In summer, soil surface temperatures can reach 50–70°C (Scarano, 2002; Mantovani & Iglesias, 2008).

The maintenance of seed germination and viability under various stresses may be related to tolerance mechanisms. The natural species of the *Restinga* presented different protection mechanisms to mitigate the damage caused by light, heat, water, and salt stress (Smirnoff & Stewart, 1985; Martínez et al., 1992; Garcia & Lucas, 1994; Garcia, 1999; Pinheiro & Borghetti, 2003; Mantovani & Iglesias, 2008; Mantovani & Iglesias, 2010; Del Vecchio et al., 2020; Li et al., 2021; Zhao et al., 2023). A series of protective metabolites can reduce osmotic potential, decrease cell permeability under stress, and act as thermo- and osmoregulators of proteins and membranes (Ghosh et al., 2021; Guihur, Rebeaud &

Goloubinoff, 2022; Wang et al., 2022). Metabolites that may act as (osmo-) thermoprotectants include soluble proteins, amino acids (e.g., proline), polyamines, glycine betaine, sugars, polyols, sugar alcohols, aminobutyric acid (GABA), and tertiary sulfonium compounds (Dutta et al., 2019; Pryia et al., 2019; Ozturk et al., 2021). Other mechanisms employed by seeds include the activation of the enzymatic antioxidant system, which reduces levels of reactive oxygen species (ROS). ROS are highly reactive and can oxidize and damage lipids, carbohydrates, proteins, and genetic material (Bailly, 2004; Pandhair & Sekhon, 2006).

The synthesis of heat-stable proteins (HSPs) is also activated in response to stress caused by water deprivation, salt, cold, and high temperatures (Artur et al., 2019). HSPs act as molecular chaperones, preventing protein denaturation and aggregation (Batra et al., 2023; Mondal et al., 2023). Due to their physicochemical properties, such as extreme hydrophilicity and high glycine content, they can stabilize cell structures and macromolecular complexes (Garay-Arroyo et al., 2000). Understanding the effects of elevated temperature and water stress on the germination process can help predict conditions necessary for maintaining natural populations in unique environments like *Restinga*.

Canavalia rosea is an herbaceous plant that grows along beaches and coastal dunes. It is a pioneering, colonizing, and sand-stabilizing species with large seeds that exhibit physical dormancy and can tolerate burial and temperature fluctuations (Moreno-Cassasola et al., 1994; Mendoza-González, Martínez & Lithgow, 2014). *C. rosea* is an important species in the beach and dune vegetation of the Neotropics, with unique adaptations to this environment (Mendoza-González, Martínez & Lithgow, 2014). Additionally, the presence of nitrogen-fixing nodules on its roots suggests that *C. rosea* could act as a nurse plant (Moreno-Cassasola et al., 1994; Chen et al., 2000; Thornton et al., 2002; Lourenço-Junior et al., 2013).

The current climate scenario and future projections from the Intergovernmental Panel on Climate Change (IPCC, 2021) and the *Painel Brasileiro de Mudança do Clima* (PBMC, 2013) indicate that Restinga areas in the southeast will experience a temperature increase of 2.5–3°C and a 25–35% rise in rainfall by the end of the century. However, due to the physical nature of *Restinga* soil, even with increased precipitation, water availability may not be sufficient for seeds to imbibe and complete germination (Scarano, 2002). Given the current climate scenario, there are still gaps in understanding how heat stress affects reproduction, particularly in the early stages of the plant life cycle (Jagadish et al., 2021), especially when combined with water availability. The physiological effects of the co-occurrence of increased

temperature and water availability events on coastal species remain poorly investigated. Therefore, in this study, we hypothesize that discontinuous hydration has a natural priming effect, mitigating the impacts of high temperatures on the germination physiology of *C. rosea* seeds. Our study aimed to uncover the metabolic and physiological mechanisms activated by *C. rosea* seeds when exposed to combined elevated temperature and discontinuous hydration.

METHODOLOGY

Seed Material

C. rosea seeds were collected in 2022 from the *Parque Estadual Paulo César Vinha* (PEPCV), located in *Espírito Santo*, Brazil. The seeds were stored in kraft paper bags under refrigeration until the experiments began. According to the Köppen climate classification (Alvares et al., 2013), the region has a tropical monsoon climate (type Am). Seed moisture content was determined using the oven method at 105 ± 2°C (Brasil, 2009), with values expressed on a dry basis. Five replicates of 20 seeds each were used, and the seeds were found to have a moisture content of 8.51%. To break physical dormancy, the seeds were sanded with 80-grit sandpaper on the side opposite the hilum.

Discontinuous Hydration Treatments

The fresh weight of *C. rosea* seeds was measured before treatment. The seeds were then disinfected in a 1% sodium hypochlorite (NaClO) solution with two drops of commercial detergent for 10 minutes, followed by three rinses with deionized water. After disinfection and initial weighing, the seeds were placed on moistened filter paper in gerboxes, with 20 seeds per plate. The plates were kept at room temperature, and the fresh weight of the seeds was measured at 1, 3, 6, 9, 12, 15, and 18 hours. An imbibition curve was constructed based on the fresh weight values over time. The weight stabilized after 13 hours, which marked the imbibition phases used for discontinuous hydration (Fig. 1A).

The desiccation curve was based on the imbibition curve. Seeds were hydrated for 18 hours and then dried. Five replicates of 10 seeds each were dried in germination boxes containing 70 g of silica gel (replaced every 24 hours) at 20°C in the dark. The seeds were weighed every hour for the first 12 hours, then at 24, 36, 48, 60, 72, and 84 hours, until they returned to their initial fresh weight at 84 hours (Fig. 1B). A rehydration curve was constructed to determine the timing for biochemical analyses. Five replicates of 10 seeds each were weighed every hour for 12 hours until root protrusion (Fig. 1C).

Criteria for Determining Temperatures

The temperature for the experiments was determined based on historical data. According to the *Instituto Capixaba de Pesquisa, Assistência Técnica e Extensão Rural* (Incaper) (https://meteorologia.incaper.es.gov.br/mapas-de-temperatura-anomalia-de-media), the average annual maximum temperature in *Guarapari, Espírito Santo* (where PEPCV is located) between 1984 and 2019 was 30°C. Adding the 3°C increase predicted by the IPCC and PBMC projections, a temperature of 33°C was adopted as one of the treatments. The 60°C treatment was based on studies showing that the surface temperature of Restinga soils can exceed 50°C during summer (Zaluar & Scarano, 2000; Mantovani & Iglesias, 2008).

Treatments

After breaking physical dormancy, the seeds were subjected to different treatments: control at 25°C (CH25) seeds were acclimatized at 25°C for 96 hours; Discontinuous hydration at 25°C (DH25) seeds were imbibed for 13 hours (half of phase 2), dried at 20°C for 84 hours, and acclimatized similarly to CH25; Control at 33°C (CH33) seeds followed the same treatment as CH25 but were acclimatized for 24 hours at 25°C and then at 33°C for 72 hours. Discontinuous hydration at 33°C (DH33) seeds underwent soaking and drying, with acclimatization and temperature imposition identical to CH33; Control at 60°C (CH60) seeds followed the same treatment as CH33 but were exposed to 60°C instead of 33°C; Discontinuous hydration at 60°C (DH60) seeds underwent soaking and drying, with acclimatization and temperature imposition identical to CH60 (Fig. 2).

After treatment application, the seeds were disinfected in 1% NaOCl with two drops of commercial detergent and rinsed three times with deionized water. For germination, 20 seeds per replicate (five replicates per treatment) were placed in germination boxes containing two sheets of filter paper moistened with deionized water (three times the weight of the paper). The boxes were placed in germination chambers at 25°C, with a 12-hour photoperiod and 40 μmol photons m^{-2} s^{-1}.

Biochemical Analyses

Biochemical analyses were conducted similarly to the germination tests, but with five replicates of 15 seeds each (including histolocalization). After the imbibition period (9 hours for DH seeds and 13 hours for control seeds), the seed coat was removed, and the embryos

were frozen in liquid nitrogen. The 15 seeds from each replicate were pooled, and 0.1 g of tissue was used for biochemical analysis.

Germination parameters

The criterion used to assess germination was radicle protrusion (> 2 mm), where germination (G) was calculated using the formula $G = \frac{N}{100} \cdot 100$, where: N = number of germinated seeds at the end of the test. The germination percentage was expressed as the accumulated germination over the evaluation time after the treatments were applied. The Germination Speed Index (GSI) was calculated according to Maguire (1962), $VG = \sum \frac{G1}{N1} + \frac{G2}{N2} \ldots\ldots . \frac{Gn}{Nn'}$, where G= number of seeds germinated, and N= days since the test was carried out. Seeds from each treatment were collected at the end of the germination test. The viability test was carried out by cutting the seeds lengthwise and placing them in black plastic vials containing 2-3-5 triphenyltetrazolium chloride solution (pH 7.0) at 0.1% for 3 hours at 35ºC. Viable seeds were those with a crimson-red color at the hypocotyl-radicle axis (cotyledons were not included in the assessment).

Protein content

For the quantification of total soluble proteins, extraction was carried out according to the protocol of Gomes, Soares & Garcia (2014). The embryos were macerated in liquid N_2, then 1mL of extraction buffer was added and they were centrifuged at 15000g, 4ºC for 25 min. Total soluble proteins were determined in a spectrophotometer at an absorbance of 595 nm, using a standard curve of known albumin, according to the method described by (Bradford, 1976).

Proline quantification

To obtain the supernatant, 0.1 g of fresh embryos was homogenized with 3% sulphosalicylic acid and centrifuged at 5000 rpm for 10 minutes. Endogenous proline levels were quantified using a known proline standard curve (100µg/mL) and acid ninhydrin solution prepared in 6M phosphoric acid and glacial acetic acid. After incubation at 100 ºC for 60 minutes and cooling, they were read using a spectrophotometer at 520nm (Bates, Waldren & Teare, 1973).

Lipid peroxidation estimative

The estimation of lipid peroxidation was determined by the quantification of malondialdehyde (MDA). 0.1g of fresh embryos material was homogenized in 80% ethanol. After centrifugation, a solution containing trichloroacetic acid (20%), thiobarbituric acid (0.65%), and butylated hydroxytoluene (0.01%) was added to the supernatant. It was then heated to 95ºC, cooled in an ice bath, and read at 440 nm, 532 nm, and 600 nm (using a molar extinction coefficient of $1.57x10^5$) according to the protocol described by Du & Bramlage (1992) and adapted by Bicalho et al. (2015).

Hydrogen peroxide content

Hydrogen peroxide (H_2O_2) levels were extracted by homogenizing 0.1g of fresh embryos with 0.1% trichloroacetic acid. A known standard curve of H_2O_2 was used, for the reaction the supernatant was added to 10mM potassium phosphate buffer (pH 7.0) and 1M potassium iodide and read at 390nm, according to Velikova; Yordanov & Edreva (2000).

Enzymes activity of the antioxidant system

Enzyme extraction [superoxide dismutase (EC 1.15.1.1) - SOD and glutathione reductase (EC 1.6.4.2) – GR] was carried out using 0.1g of seeds macerated in N_2 liquid and homogenized with 1 mL of phosphate buffer 100 mM; pH 7.8 (the same used for protein extraction).

SOD activity was measured by inhibiting the photoreduction of nitro blue tetrazolium (NBT). The reaction mix was prepared using 100 mM potassium phosphate buffer (pH 7.8), 70mM methionine, 10µM riboflavin, 1mM NBT, 0.2mM ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA), and water. After 15µL of the sample was added to the reaction mixture, it was exposed to white light for 5 minutes and read at 560 nm using the method proposed by Giannopolitis & Ries (1977). The enzymatic assay of GR was carried out according to Foyer and Halliwell (1976), through the decrease in absorbance at 340 nm due to the oxidation of NADPH (£= 6.2 mM^{-1} cm^{-1}). The reaction mix consisted of 100 mM potassium phosphate buffer pH 7.8, 10 mM oxidized glutathione, 5 mM NADPH, and 45 µL of the extract aliquot.

Histolocalization of superoxide anion (O_2 •)−

Seeds were collected at 9 and 13 hours (corresponding to the middle of phase II of germination of hydrated-dehydrated, and control seeds, respectively). They were then

transferred to gerboxes containing deionized water for 12 hours at 25ºC. After 12 hours, the seeds were segmented longitudinally with a scalpel and tweezers and placed in vials containing a solution of 1 mM NBT and 10 mM TRIS-NaOH buffer (pH 7.0), in the dark for 50 minutes (Oracz et al., 2007 modified by Ferreira et al., 2024) and photographed afterward.

Heat stable proteins

C. rosea embryos that had undergone all treatments were used to determine the electrophoretic pattern of total and heat-stable proteins. To determine the concentrations of total and heat-stable proteins, 0.1 g of macerated seeds were used and 1 mL of extraction buffer containing 500 mM Tris HCl pH7.5, 5 mM NaCl, 5 mM $MgCl_2$, 0.01 M phenylmethylsulfonyl fluoride, and 1 µL β-mercaptoethanol was added. The samples were then vortexed and centrifuged at 12000 rpm for 30 minutes at 4ºC (Alfenas, 2006). A second extraction (TCA protein precipitation protocol from Dr. Luis Sanchez), was then performed by adding 100% (w/v) trichloroacetic acid at a ratio of 1:4 per sample (from the first extraction). The microtubes were then incubated at 4ºC for 1 minute and centrifuged at 14000 rpm for 5 minutes, the supernatant was discarded, and the pellet was washed with 200 µL of ice-cold acetone (repeated twice). After the second wash, the pellet was dried in an oven at 95ºC for 10 minutes to completely remove the acetone.

The proteins in the extracts (heat-stable and total) were quantified using the Bradford method (1976). The samples were applied to a discontinuous acrylamide gel, 40 µg per protein sample was used, together with 20 µL of sample buffer (2.5 mL glycerol, 0.46 g SDS, 20 mg bromophenol, and completed to 20 mL with Tris HCl pH 7.5 extraction buffer). The electrophoretic run was carried out in a vertical vat with SDS buffer at 200V/60A for 6 hours. After the run, the gels were transferred to a container for fixing in a solution containing 40% methanol and 7% acetic acid for 30 minutes. The gels were then washed in deionized water, stained in a solution containing 0.08% (w/v) Comassie Blue G250, 1.6% (v/v) orthophosphoric acid, and 12% (w/v) ammonium sulfate, and kept in a shaker for 3 days. The gels were discolored in a 0.1M Tris base solution pH 6.5, adjusted with phosphoric acid. The discolored gels were kept immersed in deionized water until scanning (Martins et al., 2020). The images were scanned using a densitometer and analyzed using the GelAnalyzer software. The molecular weight marker PageRuler™ Unstained Protein Ladder protein standards were used.

Acid abscisic content

ABA was quantified using 0.1 g fresh embryos macerated in liquid N_2, followed by 2 mL of extraction buffer containing 100 mL of 80% methanol, 20 mL/L^{-1} acetic acid, and 10 mg/L^{-1} butylated hydroxytoluene (Weiler, 1982; Bicalho et al., 2015; Bicalho, Santos & Garcia, 2018). The microtubes were centrifuged at 12000g for 15 minutes at 4ºC. ABA quantification of the standard curve and samples was carried out using the Phytodetek® ABA test kits (Agdia Incorporated, Indiana, USA).

STATISTICAL ANALYSIS

The experiment was conducted in a completely randomized design with a 2 x 3 factorial arrangement [two discontinuous hydration conditions (control and hydrated-dehydrated) and three temperatures (25, 33, and 60ºC)], five replicates for treatment, 20 seeds for replicate each. Normality and homogeneity of data variances were assessed using the Shapiro-Wilk and Levene tests, respectively. Data were subjected to two-way ANOVA and, when statistically significant (F test at 5% significance), means were compared using a Tukey post-hoc test at 5% significance. Non normal data were transformed by the square root +1. Calculations were performed in R version 4.3.1 (ExpDes.pt package). The germination and biochemical data were normalized to vary between -1 and + 1 using the Normatiza function of the MultivariateAnalysis R package. Relationships between DH and temperatures events were investigated using principal components analysis (PCA).

RESULTS

C. rosea seeds that underwent discontinuous hydration (DH) followed by exposure to high temperatures exhibited impaired germination parameters. Control treatment (CH) seeds had an average germination percentage of 92% ± 1.52 ($F = 4.484$, $df = 2$, $p < 0.05$; Table 1). In contrast, DH seeds showed a decrease in germination with increasing temperature, with mean values of 76% ± 4.97 (25°C), 69% ± 5.72 (33°C), and 54% ± 3.57 (60°C) ($F = 47.815$, $df = 1$, $p < 0.01$; Figure 3A, Table 1). The Germination Speed Index (GSI) showed a significant difference only for the DH treatment ($F = 60.364$, $df = 1$, $p < 0.01$; Table 1). Seeds from the treatment CH had a mean GSI value of 8.03 ± 0.17, whereas seeds from the DH treatment had a mean value of 5.06 ± 0.59 (Figure 3B).

Seed viability showed a significant interaction between DH and temperature increase ($F = 7.2$, $df = 2$, $p < 0.001$; Table 1). The CH treatment had an average viability of 96.11% ±

3.88, while the DH treatment showed average viability values of 62.77% ± 9.24. The CH60 treatment had the lowest viability, with only 48.33% ± 3.33 of seeds remaining viable by the end of the experiment (Figures 3C and 6B). Malondialdehyde (MDA) content differed significantly due to DH ($F = 5.7884$; $df = 1$, $p < 0.05$; Table 1) and temperature ($F = 7.3339$, $df = 2$, $p < 0.01$; Table 1). Seeds that underwent DH had average MDA values of 85.125 ± 8.09 nmol g^{-1}, while non-DH seeds contained 67.003 ± 12.87 nmol g^{-1}. The temperature of 33°C induced a higher concentration of MDA in the seed tissue of the DH33 treatment, with an average of 96.178 ± 10.12 nmol g^{-1} (Figure 4A). Endogenous H_2O_2 levels showed a significant difference only for temperature ($F = 58.663$, $df = 2$, $p < 0.01$; Table 1), with mean values of 2.89 ± 0.30 nmol g^{-1} in CH33 and 2.47 ± 0.05 nmol g^{-1} in DH33 (Figure 4B).

Total protein levels were affected by both DH ($F = 7.9121$, $df = 1$, $p < 0.05$; Table 1) and temperature ($F = 12.5703$, $df = 2$, $p < 0.01$; Table 1), with a significant interaction between the two factors ($F = 18.6419$, $df = 2$, $p < 0.01$; Table 1). In the control condition, protein concentration in seed tissue increased with temperature (CH33 = 15,445.21 ± 384.42 μg g^{-1} FW; CH60 = 15,609.52 ± 2,456.41 μg g^{-1} FW). In contrast, seeds that underwent DH showed a decrease in protein content at 60°C (DH60 = 9,993.08 ± 1,165.11 μg g^{-1} FW; Figure 5A). Superoxide dismutase (SOD) activity was modulated by temperature ($F = 118.407$, $df = 2$, $p < 0.01$; Table 1) and the interaction between DH and temperature ($F = 18.360$, $df = 2$, $p < 0.01$; Table 1). The temperature of 33°C induced lower enzyme activity, with values of 1.06 ± 3.15 × 10^{-3} U min^{-1} mg^{-1} protein for CH33 and 1.05 ± 7.62 × 10^{-4} U min^{-1} mg^{-1} protein for DH33 (Figure 5B). Glutathione reductase (GR) activity showed significant differences for DH ($F = 9.299$, $df = 1$, $p < 0.05$; Table 1), temperature ($F = 185.057$, $df = 2$, $p < 0.01$; Table 1), and the interaction of the two factors ($F = 35.844$, $df = 2$, $p < 0.01$; Table 1). Seeds that were not hydrated-dehydrated at 25°C (CH25) showed higher average enzyme activity values (0.74 ± 0.06 pmol NADPH min^{-1} mg^{-1} protein) compared to other treatments. Interestingly, no enzymatic activity was detected for the CH33 and DH33 treatments (Figure 5C).

Abscisic acid (ABA) levels were influenced by DH ($F = 4764.5$, $df = 1$, $p < 0.05$; Table 1), temperature ($F = 5722.6$, $df = 2$, $p < 0.01$; Table 1), and the interaction of the two factors ($F = 6050.3$, $df = 2$, $p < 0.01$; Table 1). The highest ABA concentrations were found in DH33 (4.48 ± 0.01 pmol mg^{-1}; Figure 6A). Proline accumulation in seed tissues was significantly influenced by DH ($F = 5.4331$, $df = 1$, $p < 0.05$; Table 1) and temperature ($F = 10.3600$, $df = 2$, $p < 0.01$; Table 1). The DH treatment showed median values of 5.50 ± 0.88

nmol g^{-1}, while CH had 4.48 ± 1.18 nmol g^{-1}. The temperature of 33°C induced the highest proline production in CH33 (6.81 ± 0.93 nmol g^{-1}) and DH33 (7.23 ± 0.41 nmol g^{-1}; Figure 6B).

Regarding $O_2^{\bullet -}$ histolocalization, all treatments that underwent DH showed greater color intensity compared to control seeds. The DH33 treatment exhibited less intense staining in the embryonic axis than other treatments, while DH60 showed the darkest staining in its embryonic tissues (Figure 7A). Differential protein expression revealed a greater abundance of total protein bands compared to heat-stable proteins (HSPs), as expected (Figure 8A). Bands between 150 and 20 kDa were more intense in the DH25, DH33, and CH60 treatments than in the control (Figure 8A). HSP expression was higher in all treatments except DH60, which showed a band intensity of 584 mm^2 (Figure 8B).

Principal Component Analysis (PCA) was performed on all germination and biochemical parameters to provide a global view of the effects of DH and temperature on *C. rosea* seeds. The analysis revealed a separation between germination and biochemical responses. PC1 and PC2 separated the treatments based on temperature and DH, explaining 47.47% and 32.71% of the total variation, respectively. Germination percentage, GSI, and viability were correlated with seeds that were not hydrated-dehydrated (CH33 and CH60) and under DH25 conditions. In contrast, markers of oxidative stress (MDA and H_2O_2), abscisic acid, proline, and proteins (ABA, PROL, and PROT) were more strongly correlated with seeds exposed to the DH33 condition (Figure 9).

DISCUSSION

In this work, it was not temperature but discontinuous hydration (DH) that negatively influenced the germination of *C. rosea*. Plants are exposed to a wide range of temperatures during their life cycle, and temperature is a critical environmental factor that influences their growth and development. To survive, plants must continuously adapt to these conditions. Climate change is expected to increase global temperatures, with heatwaves becoming more frequent and intense (Perkins, Alexander & Nairn, 2012; Zhu, Lima & De Smet, 2021). Interestingly, temperature events did not negatively affect the germination parameters (percentage, speed, and viability) of *C. rosea* seeds. This resilience may be attributed to the species' high plasticity, enabling it to thrive in coastal environments where sand surface temperatures can exceed 50°C (Zaluar & Scarano, 2000; Mantovani & Iglesias, 2008). The

ability to germinate at high temperatures is typically observed in certain desert (Daws et al., 2007), savanna (Ribeiro, Pedrosa & Borghetti, 2013), and *Restinga* species, such as *Allagoptera arenaria*, which colonizes sandy areas where temperatures can reach 70°C in summer (Scarano, 2002). However, when *C. rosea* seeds undergo DH and are exposed to different temperatures, there is a gradual reduction in germination parameters (Figure 3). Drying can induce damage to germination in some species, ultimately reducing seed viability (Oliver et al., 2020). For example, in *Restinga* bromeliads, water stress and wet-dry cycles have been shown to negatively affect seed germination, reducing germination rates and increasing the time and synchronization required for germination (Mantovani & Iglesias, 2008; Mantovani & Iglesias, 2010). In future climate scenarios for the southeastern region, where increased rainfall is predicted (PBMC, 2013), the hydration and drying of seeds may occur more frequently and intensely, potentially damaging seed longevity.

The process of hydration followed by drying caused damage to *C. rosea* seeds, as evidenced by the accumulation of malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) in the tissues, which reduced germination and seed viability. H_2O_2 has strong oxidizing capabilities, enabling it to interact with most biomolecules, including nucleic acids, proteins, and lipids, resulting in oxidative stress and cellular damage. Lipid peroxidation, one of the most widely documented toxic effects of H_2O_2, affects polyunsaturated fatty acids (PUFAs) in cell membranes and reserve lipids. Nucleic acids and proteins are also vulnerable to oxidation by H_2O_2 (El-Maarouf-Bouteau & Bailly, 2008; Wotijla et al., 2016). Notably, at 33°C, both DH and non-DH seeds showed increased levels of MDA and H_2O_2, but this did not negatively impact germination or viability. This suggests that these molecules may act as signaling agents, preparing the seeds metabolically for stress (Figures 3 and 4). At 60°C, seeds that underwent DH did not exhibit high MDA and H_2O_2 levels, as more than half of the seed population was already non-viable (Figure 3C).

The combination of DH followed by exposure to 60°C (DH60) was the most damaging treatment for *C. rosea* seeds, reducing their viability to less than 50% (Figures 3B and 7B). The accumulation of abscisic acid (ABA) in response to combined stress was insufficient to activate the antioxidant system, as glutathione reductase (GR) activity is known to be suppressed at high temperatures. GR activity is inversely proportional to temperature, with inhibition occurring at elevated temperatures (Chakraborty & Pradhan, 2011). In contrast, superoxide dismutase (SOD) activity, which has been reported to be thermostable in

several studies (Yuan et al., 2011; Kumar et al., 2020), was maintained across all treatments (Figures 3 and 6). Interestingly, SOD activity was reduced only at 33°C, correlating with the more intense blue staining observed in the embryonic axis (Figure 7A). Another mechanism activated by ABA was the accumulation of proline, which peaked at 33°C and may have contributed to maintaining seed viability. Proline accumulation in seed and seedling tissues is a well-documented response to heat and water stress (Neto et al., 2004; Harsh et al., 2016; Ozturk et al., 2021).

In response to temperature and water availability events, *C. rosea* seeds exhibited significant increases in soluble protein content. Similar protein accumulation has been reported in *Calligonum mongolicum* under drought stress (Zhang et al., 2021) and in Vigna radiata seeds under heat stress (Batra et al., 2023). Our results show that the DH33 and CH60 treatments (Figure 6A) had higher ABA levels in response to the imposed thermal and water conditions. ABA also induces the expression of Late Embryogenesis Abundant (LEA) proteins (Finkelstein, Gampala & Rock, 2002). Under stress conditions, ABA acts as a signaling molecule, triggering systemic responses such as the synthesis of heat-stable proteins (HSPs), increased antioxidant enzyme activity, biosynthesis of osmoprotectants, and activation of signaling cascades (Danquah et al., 2014; Cao et al., 2020; Shrestha et al., 2021; Rai et al., 2024).

HSPs increase their expression and abundance in response to various environmental stimuli, protecting plant cells from damage and facilitating recovery and survival under stress conditions (Al-Whaibi, 2011; Chen, Feder & Kang, 2018). In *C. rosea* seeds, the highest differential expression of HSPs was observed between 150 and 20 kDa (Figure 8A). HSPs are classified into five subgroups (HSP100, HSP90, HSP70, HSP60, and small HSPs) based on their molecular weight, amino acid sequence homology, and function (Wang et al., 2004; Gupta et al., 2010). HSPs from the HSP70 and HSP90 families are particularly abundant under water and heat stress (Aghaie et al., 2020; Shafqat et al., 2021; Kou et al., 2023). Lin et al. (2021), in a study on the same species, reported the presence of HSP20 proteins in various tissues and organs, which accumulated in response to water and salt stress. The highest differential expression of HSPs was observed in the DH25, DH33, and CH60 treatments (Figure 7B), indicating that HSPs play a crucial role in protecting cellular machinery under stress. HSPs are essential for maintaining cellular homeostasis and protecting proteins from damage under both normal and stress conditions. Although HSPs are associated with stress

responses, they are constitutively expressed at basal levels in plants (Welch, 1991). Our results show that even under natural conditions (CH25), there is a baseline differential expression of HSPs, which increases at 33°C (Figure 9). This basal expression ensures that the plant is prepared to respond to potential future stresses. However, under the DH60 condition, this expression is absent, as the treatment severely compromises seed viability (Figure 7B).

C. rosea seeds respond differently to heat and water stress when these factors are combined. While the seeds are tolerant to high temperatures without significant effects on germination (Figures 3 and 7), they are susceptible to hydration and desiccation. In response to water stress, the seeds accumulate higher levels of MDA, H_2O_2, and ABA when exposed to 33°C, possibly as a signaling mechanism for metabolic adjustment to combined stresses (Figure 8). Under natural conditions at 33°C (CH33), HSPs increase their differential expression, preventing protein denaturation and aggregation after heat stress (Salomé, 2017). However, at 60°C, combined with pre-hydration, the seeds in the DH60 condition experience protein denaturation. As exalbuminous seeds, *C. rosea* relies on the remobilization of reserves from the cotyledons to the embryo, a key mechanism for protection against combined stresses (Soriano et al., 2013; Azevedo-da-Silva et al., 2023). When exposed to thermal stress, the seeds accumulate proteins, proline, and HSPs, ensuring the species' survival in the harsh conditions of the *Restinga*. This tolerance mechanism may facilitate the colonization, succession, and establishment of *C. rosea* in *Restinga* areas (Gross, 1993; Mendoza-González, Martínez & Lithgow, 2014).

CONCLUSION

Canavalia rosea is a species that occurs in *Restinga*, a phytophysiognomy characterized by several environmental stressors, including high luminosity, salinity, elevated temperatures, low water availability, and poor nutrient availability. Despite these challenges, the germination of *C. rosea* seeds can withstand high temperatures, demonstrating their high plasticity and thermotolerance. This resilience is likely due to the accumulation of soluble proteins, proline, and the presence of heat-stable proteins (HSPs) under natural conditions. However, when subjected to discontinuous hydration (DH), these seeds experience a drastic reduction in germination and viability when exposed to different temperatures, indicating that they do not benefit from the priming effect of DH. Therefore, *C. rosea* seeds may employ thermal tolerance mechanisms to mitigate the effects of water deficit, but the reverse does not

hold true. In the current climate scenario, with projections of increased temperatures and rainfall in the species' habitat, *C. rosea* populations may face a decline in seed viability and, consequently, a reduction in the recruitment of new individuals necessary to maintain its population.

AUTHOR CONTRIBUTION STATEMENT

GSD, EMB conceived and designed the research; AMOF, GSD, JTLC, and OAOT carried out all the experimental work; GSD, JTLC performed the statistical analysis; GSD, AMOF, EMB, JTLC contributed to data interpretation; GSD, EMB wrote the manuscript. All the authors contributed to data discussion, conclusions, and reviewed and approved the final manuscript.

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Figure 1

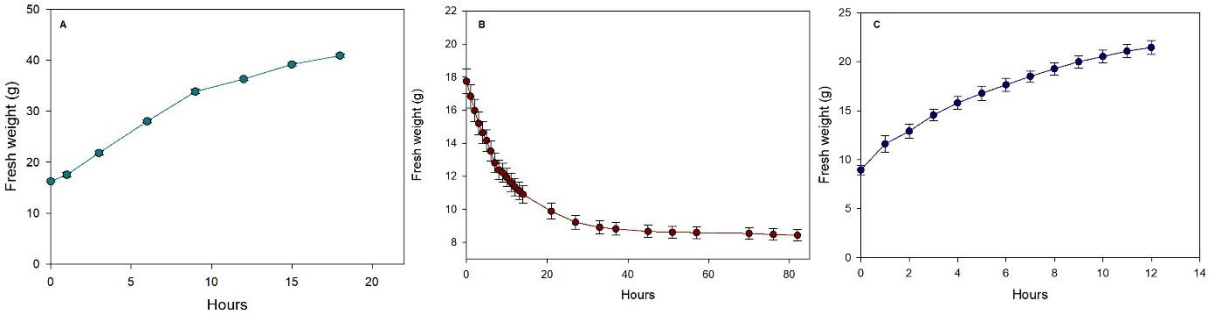

Figure 1: Imbibition (A), drying (B), and rehydration (C) curves of *Canavalia rosea* seeds.

Figure 2

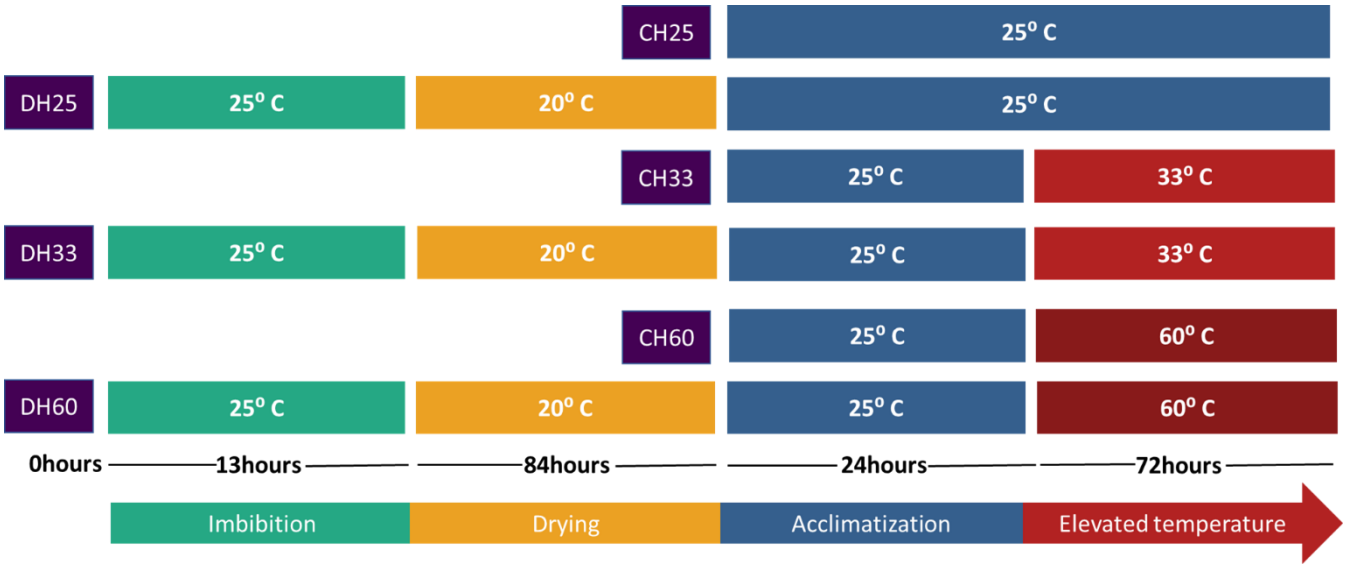

Figure 2: Experimental design of discontinuous hydration applications and temperatures events. In the diagram, each color ref how the treatments were applied to the *Canavalia rosea* seeds (imbibition at 25ºC; drying at 20ºC; acclimatization at imposition at high temperatures). CH: continuous hydration; DH: discontinuous hydration.

Figure 3

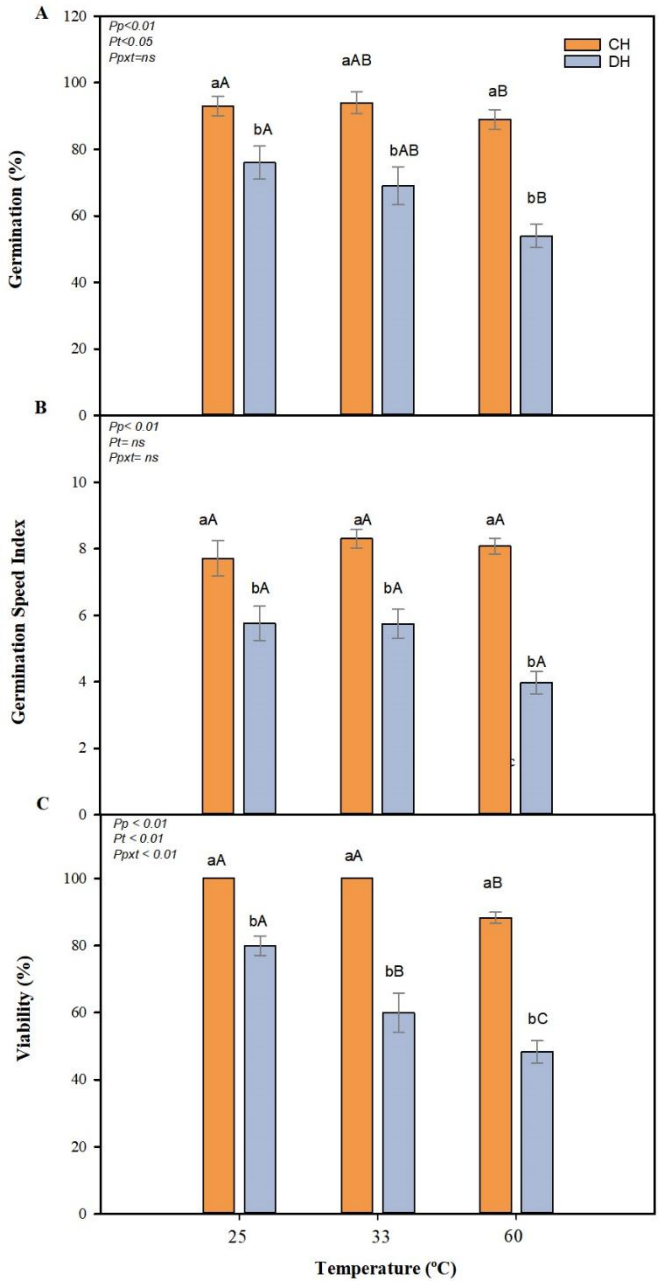

Figure 3: Germination percentage (A), germination speed index - GSI (B), and viability of remaining seeds (C) in *Canavalia rosea* seeds to discontinuous hydration (continuous hydration - CH; discontinuous hydration - DH) and temperature events (25, 33 and 60ºC). The bars are the mean ± standard error. Lowercase letters compare the statistical results for each discontinuous hydration treatment within the same temperature. Capital letters compare the same discontinuous hydration treatment at different temperatures. Means followed by the same letters do not differ significantly by Tukey's test at 5% probability, ns = not significant; pt= p-value between temperatures; pp= p-value between discontinuous hydration; ptxc= p-value of the interaction between temperature and discontinuous hydration.

Figure 4

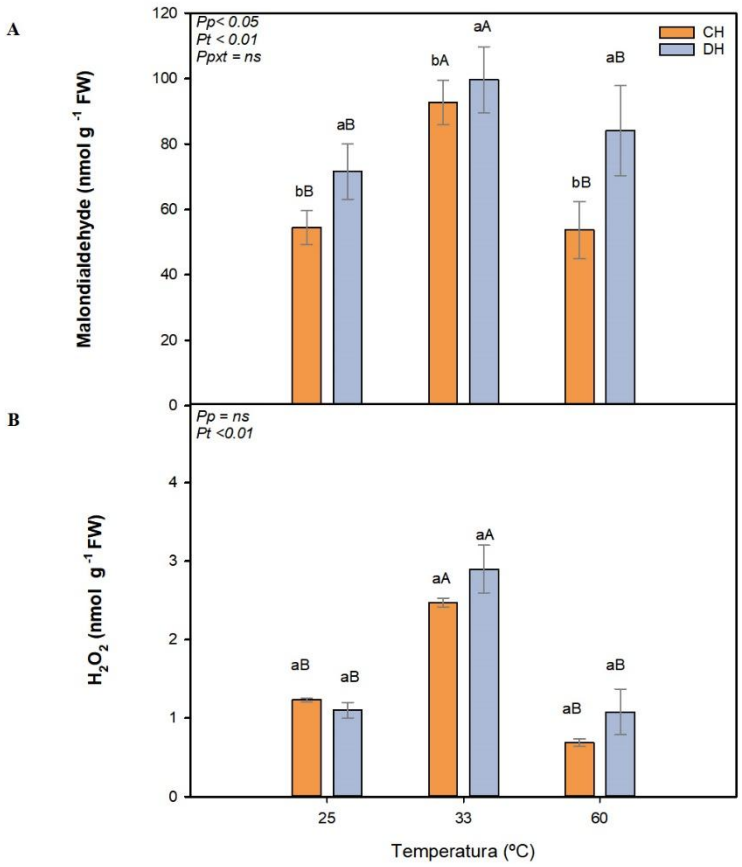

Figure 4: Malondialdehyde (A), H_2O_2 (B)in *Canavalia rosea* seeds subjected to discontinuous hydration (continuous hydration - CH; discontinuous hydration - DH) and temperature events (25, 33 and 60ºC). The bars are the mean ± standard error. Lowercase letters compare the statistical results for each discontinuous hydration treatment within the same temperature. Capital letters compare the same discontinuous hydration treatment at different temperatures. Means followed by the same letters do not differ significantly by Tukey's test at 5% probability, ns = not significant; pt= p-value between temperatures; pp= p-value between discontinuous hydration; ptxc= p-value of the interaction between temperature and discontinuous hydration.

Figure 5

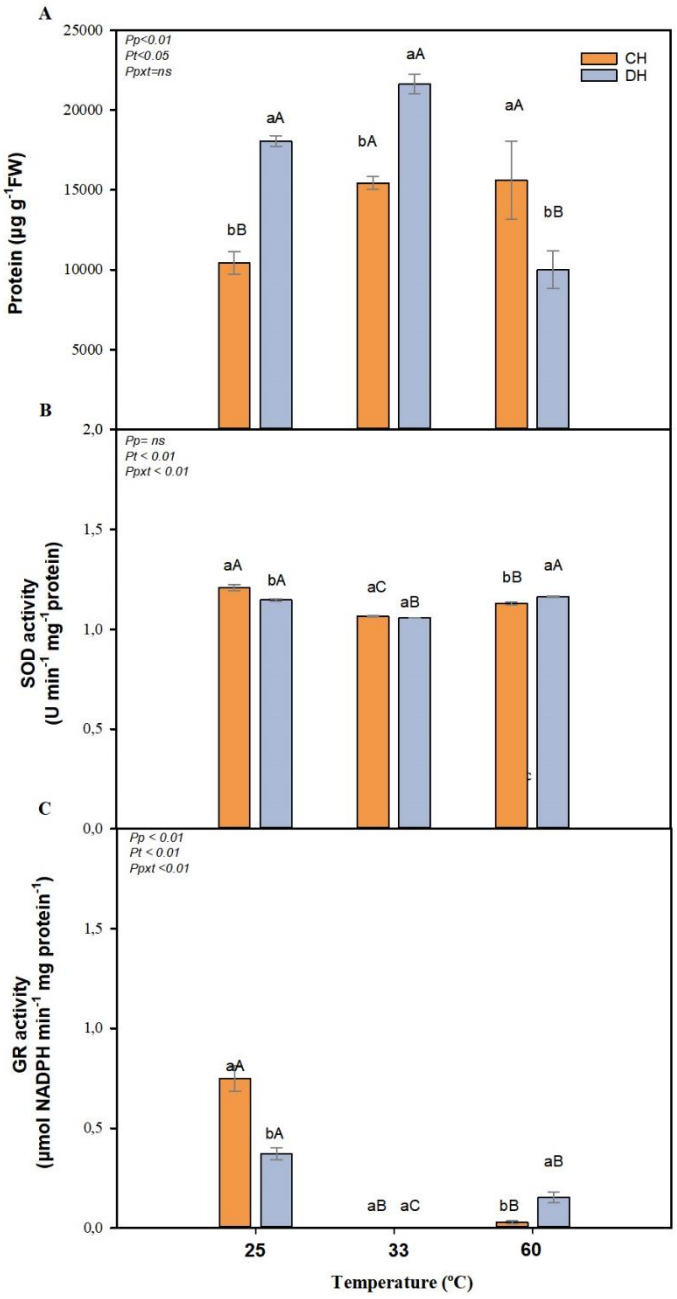

Figure 5: Protein (A), and enzyme activity of Superoxide dismutase (B) and Glutathione reductase (C) in *Canavalia rosea* seeds subjected to discontinuous hydration (continuous hydration - CH; discontinuous hydration - DH) and temperature events (25, 33 and 60ºC). The bars are the mean ± standard error. Lowercase letters compare the statistical results for each hydration treatment within the same temperature. Capital letters compare the same discontinuous hydration treatment at different temperatures. Means followed by the same letters do not differ significantly by Tukey's test at 5% probability, ns = not significant; pt= p-value between temperatures; pp= p-value between discontinuous hydration; ptxc= p-value of the interaction between temperature and discontinuous hydration.

Figure 6

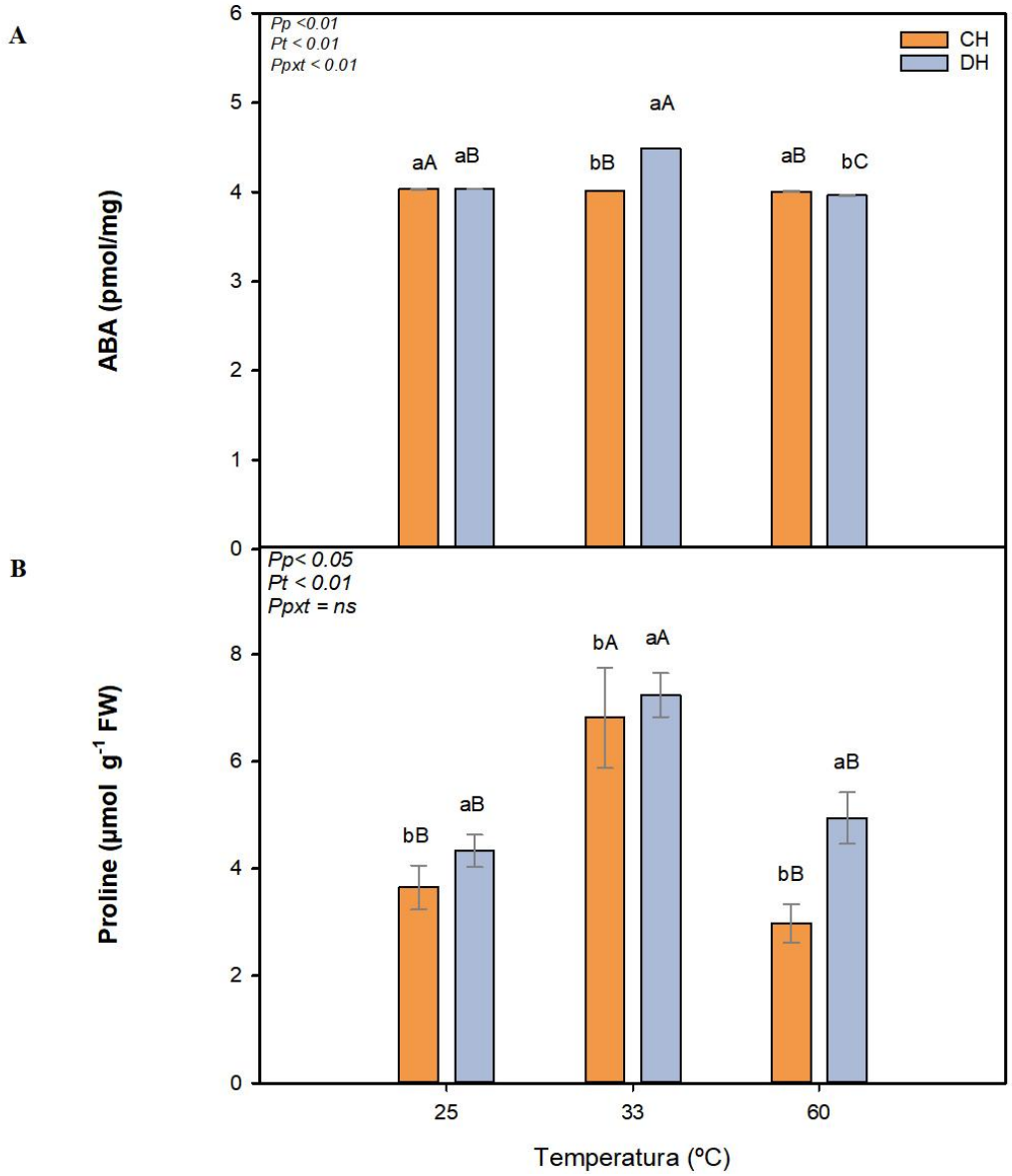

Figure 6: Abscisic acid (A), and Proline (B) in *Canavalia rosea* seeds subjected to discontinuous hydration (continuous hydration - CH; discontinuous hydration - DH) and temperature events (25, 33 and 60ºC). The bars are the mean ± standard error. Lowercase letters compare the statistical results for each hydration treatment within the same temperature. Capital letters compare the same discontinuous hydration treatment at different temperatures. Means followed by the same letters do not differ significantly by Tukey's test at 5% probability, ns = not significant; pt= p-value between temperatures; pp= p-value between discontinuous hydration; ptxc= p-value of the interaction between temperature and discontinuous hydration.

Figure 7

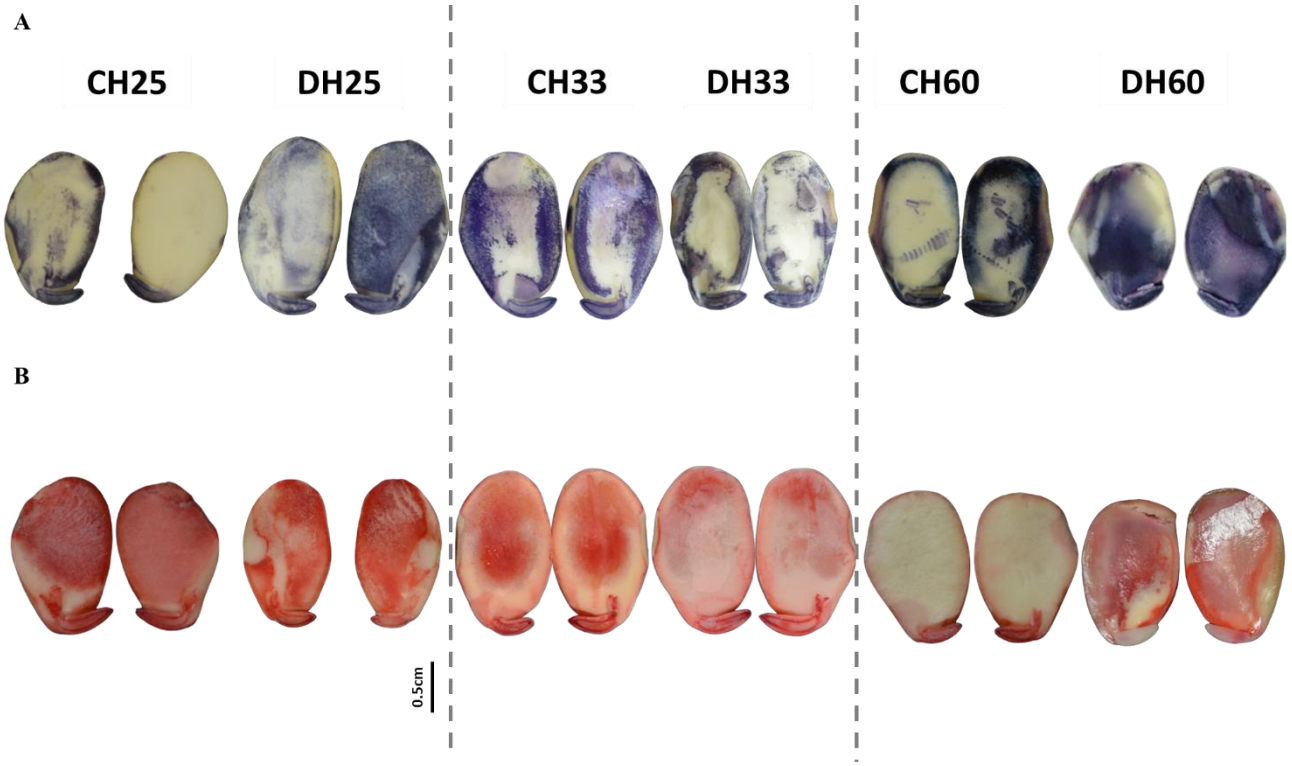

Figure 7: O_2 •− histolocalization (A) and viability (B) of *Canavalia rosea* embryos subjected to discontinuous hydr
(continuous hydration - CH; discontinuous hydration - DH) and temperature events (25, 33, and 60ºC).

Figure 8

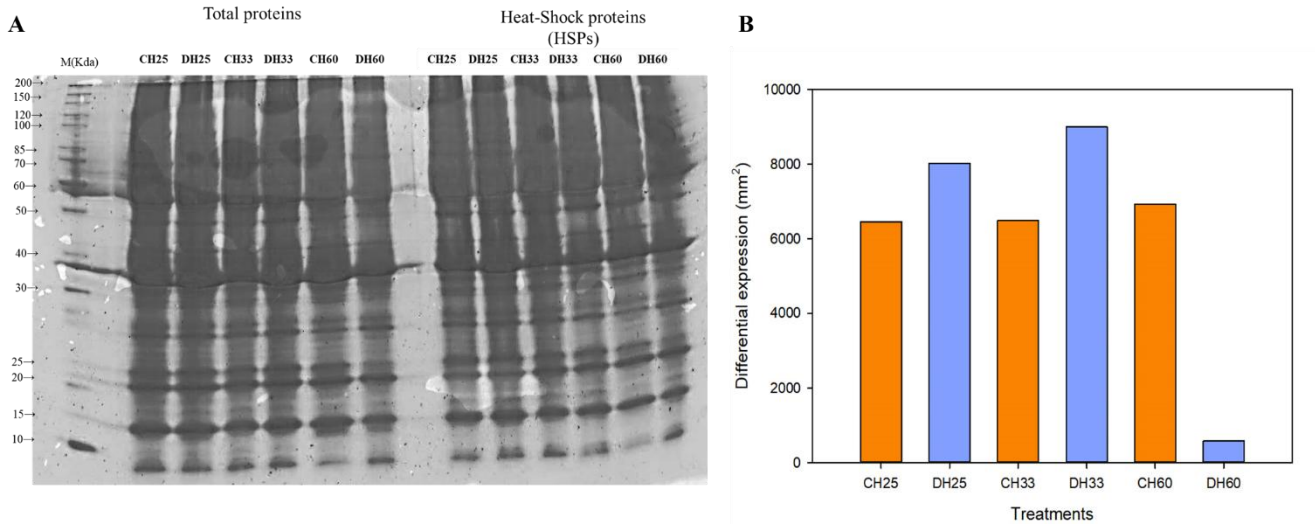

Figure 8: Electrophoresis of total proteins and heat shock proteins (A), and differential expression of heat shock proteins (B) in *Canavalia rosea* seeds subjected to temperature and discontinuous hydration events.

Figure 9

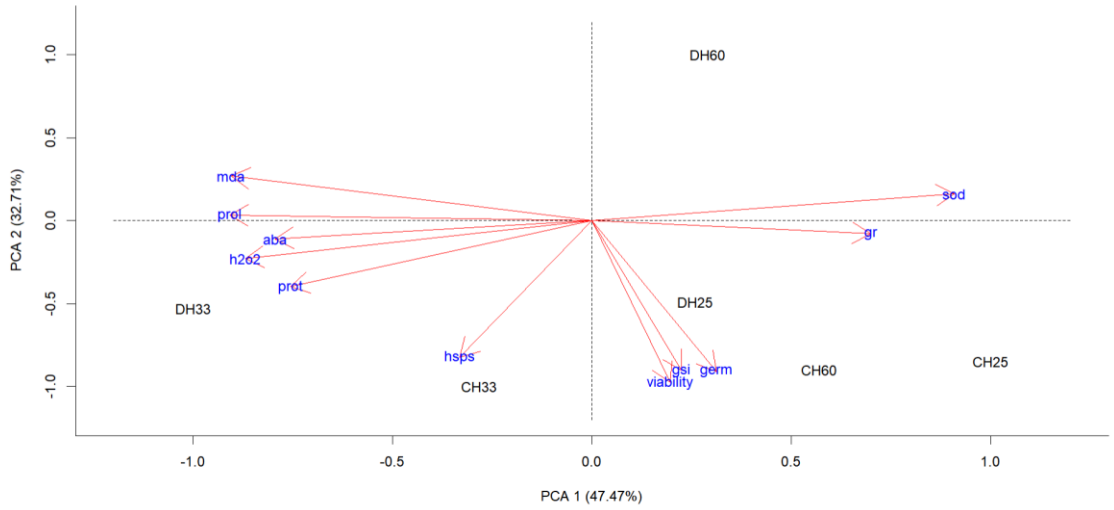

Figure 9: Principal component analysis (PCA) of *Canavalia rosea* seeds subjected to discontinuous hydration and temperature events. Abbreviations: aba- abscisic acid; ger- germination percentage; gsi- germination speed index; gr- glutathione reductase; h2o2- hydrogen peroxide; hsps- heat shock proteins; mda- lipid peroxidation; prol- proline; prot- protein; sod- superoxide dismutase; viability- viability of remaining seeds.

Table 1. Results of statistical analysis (ANOVA and Tukey test) for evaluations of germination and biochemical parameters of *Canavalia rosea* seeds exposed to temperature and discontinuous hydration event.

	F	***df***	***p***		***F***	***df***	***p***
Germination				**Proline**			
Discontinuous hydration	47.815	1	0.000000	Discontinuous hydration	5.4331	1	0.028487
Temperature	4.484	2	0.022155	Temperature	10.3600	2	0.000571
Discontinuous hydration*Temperature	1.968	2	0.161682	Discontinuous hydration*Temperature	2.4304	2	0.109354
Germination Speed Index				**Protein**			
Discontinuous hydration	60.364	1	0.000000	Discontinuous hydration	7.9121	1	0.0156665
Temperature	2.591	2	0.095717	Temperature	12.5703	2	0.0011376
Discontinuous hydration*Temperature	3.032	2	0.066986	Discontinuous hydration*Temperature	18.6419	2	0.0002084
Viability				**ABA**			
Discontinuous hydration	180.0	1	0.000000	Discontinuous hydration	4764.5	1	$5.678x10^{-17}$
Temperature	25.4	2	0.0000487	Temperature	5722.6	2	$1.320x10^{-18}$
Discontinuous hydration*Temperature	7.2	2	0.0088199	Discontinuous hydration*Temperature	6050.3	2	$9.450x10^{-19}$
MDA				**Superoxide dismutase**			
Discontinuous hydration	5.7884	1	0.02419	Discontinuous hydration	3.558	1	0.083675
Temperature	7.3339	2	0.00327	Temperature	118.407	2	0.000000
Discontinuous hydration*Temperature	0.8171	2	0.45361	Discontinuous hydration*Temperature	18.360	2	0.000223
H_2O_2				**Glutathione Reductase**			
Discontinuous hydration	2.472	1	0.141875	Discontinuous hydration	9.299	1	0.0100964
Temperature	58.663	2	0.000001	Temperature	185.057	2	0.0000000
Discontinuous hydration*Temperature	1.534	2	0.255162	Discontinuous hydration*Temperature	35.844	2	0.0000087

FINAL REMARKS

Natural ecosystems play a vital ecological role in the provision of ecosystem services. To ensure the continued availability of these resources and the services they provide, it is essential to recognize and assign value to these ecosystem services. The climate emergency scenario is evident and urgent, and it is of utmost importance to understand the dynamics of the environment in the face of the current climate scenario, which is being intensified by human actions. This is crucial for the preservation of these natural environments.

Species from the *Campo rupestre* show tolerance to their seeds undergoing hydration-dehydration cycles, increasing, accelerating, and standardizing germination. Seeds with high values of length, width, and thickness exhibit high germination rates and speed, while smaller seeds have greater thickness and proline levels. This may ensure that larger seeds are dispersed over wider areas and can make better use of available resources by germinating quickly. On the other hand, smaller seeds (with greater thickness) can persist in the seed bank for longer periods without affecting their viability when subjected to hydration-dehydration cycles. Additionally, by having or accumulating higher levels of proline after undergoing these cycles, they may also influence the residence time of these species' seeds in the soil seed bank. Hydration-dehydration cycles also confer tolerance to *Vellozia* species. When subjected to hydration-dehydration cycles followed by high temperatures, the species *V. nanuzae* benefits from increased germination, while the passage through these cycles increases endogenous proline levels in *V. compacta*, granting its seeds tolerance to fire stress. However, the species *V. caruncularis* is sensitive, showing reduced germination and increased levels of ABA and proline under the combined condition of hydration-dehydration cycles and high temperature.

In the specific case of *Restinga* species, they are classified as stress-tolerant, as they invest in structures that allow them to survive in this environment. *Restinga* seeds exhibit a wide range of germination requirements, including the absence of light, alternating temperatures, the breaking of physical dormancy, and germination itself. Due to these characteristics, the *Restinga* species studied here are distributed across different phytophysiognomies, which provide microclimates that offer favorable conditions for germination and seedling establishment. Furthermore, a positive correlation has been observed between vegetative, biometric, and germination attributes, which may become a

valuable tool in selecting species for the restoration of this ecosystem. The psammophilic species *C. rosea* is tolerant to high temperatures, but when its seeds are subjected to combined stress with discontinuous hydration, there is a drastic reduction in germination and seed viability as the temperature increases.

Our results indicate the plasticity that plant species in both environments exhibit in response to water stress and high temperature (applied individually or in combination). However, some species show success when the two stresses occur sequentially, while others are extremely sensitive. In the face of a climate emergency scenario with rising temperatures and changes in water regimes, these species will be constantly exposed to greater intensities, durations, and frequencies of these events. Our work sought to understand how the species of the *Campo rupestre* and *Restinga*, which are important environments providing essential ecosystem services, are constantly threatened by anthropogenic actions and neglected environmentally and climatically. Focusing resources, research, and funding on studies involving vegetative and seed attributes, from dispersal to germination, longevity, and seedling establishment, is crucial. Studies aimed at mitigating the impact caused or restoring degraded non-forest environments are of utmost importance for maintaining these environments at the level of individuals, populations, communities, and ecosystems.