Effects of drought on grassland forb reproduction

by

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INYUVESI YAKWAZULU-NATALI

PREFACE

The research contained in this thesis was completed by the candidate while based in the Discipline of Grassland Science, School of Life Science, College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Pietermaritzburg, South Africa. The research was financially supported by the National Research Foundation.

The contents of this work have not been submitted in any form to another university and, except where the work of others is acknowledged in the text, the results reported are due to investigations by the candidate.

[redacted]

Signed: Miss Sibonokuhle Thandwayo Mbambo

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DECLARATION 1: PLAGIARISM

I, Sibonokuhle Thandwayo Mbambo, declare that:

1. The research reported in this dissertation, except where otherwise indicated or acknowledged, is my original work.

2. this dissertation has not been submitted in full or in part for any degree or examination to any other university.

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a) their words have been re-written, but the general information attributed to them has been referenced

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5. this dissertation does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the dissertation and in the References sections.

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Date: 6 February 2024

DEDICATION

To my late mother, Gcotyiswa Nelisa Mbambo and sister Elihleikamva Mbambo.

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And lastly for my friends and family, thank you for your patience and encouragement.

Romans 11:36

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1. INTRODUCTION

In South Africa, the grassland biome is the second largest biome, covering 28% of the country's land area, following the savanna biome which covers 32.8% (Rutherford et al., 2006). It is a valuable resource for livestock farming (Kotzé et al., 2013), particularly in rural areas where agricultural and pastoral practices are the primary source of food and income for inhabitants, making the grassland biome a crucial asset to these communities (Suttie et al., 2005). Moreover, the grassland biome in South Africa is well-known for providing a range of ecosystem services. These include the provision of water, carbon sequestration, reduction of soil erosion, climate change alleviation, pollination, and cultural services (Bengtsson et al., 2019; Zaloumis, 2013).

While grasslands are dominated by Poaceae, forbs or herbaceous flowering plants play a major role in contributing to biodiversity and species richness in the Grassland biome, while also providing a valuable source of forage for some ungulate species (Hanberry et al., 2021; Pieper and Beck, 1980; Zaloumis, 2013). Despite the ecological significance of grasslands, the South African National Biodiversity Institute (SANBI) reported that these ecosystems face various threats. The grassland biome has a considerable number of threatened ecosystem types and comprises one of the highest numbers of under-protected ecosystem types in South Africa (SANBI, 2011). This highlighted the urgent need for effective conservation strategies to preserve the rich biodiversity and ecosystem services provided by these important ecosystems.

South Africa experiences frequent droughts (Rouault and Richard, 2003) and numerous studies have shown that drought can significantly impact the functioning and biodiversity of grassland ecosystems (Carter et al., 2012; De Boeck et al., 2018). For example, drought has been shown to reduce the species richness, gross primary productivity, bud densities, abundance, and specific leaf area of forbs and grasses (Carter et al., 2012; De Boeck et al., 2018). Despite the significant impact of drought on forbs, little is currently known about its effect on the reproduction of these plants. Therefore, further research is needed to better understand how drought affects the reproductive capacity of forbs, which will be useful in informing conservation management practices aimed at mitigating the negative impacts of drought on grassland ecosystems and grassland restoration practices for future climates.

Drought-induced changes in floral traits and rewards may result in alterations to pollinator visitation rates and pollen deposition (Descamps et al., 2018; Rering et al., 2020), impacting the success of pollination and subsequent seed production. Given that 87% of plant species worldwide rely on animals, such as insects, birds, and mammals, for pollination and reproductive success (Ollerton et al., 2011), and that the frequency and duration of drought is expected to increase because of climate change (Trenberth et al., 2014), it is important to understand how drought will affect plant-pollinator relationships. Additionally, it is vital to explore the effects of drought on seed germination and viability to determine if the progeny of drought-exposed plants can grow and reproduce successfully. This knowledge is important for predicting the long-term consequences of drought on plant populations, which can ultimately have implications for the biodiversity and functioning of ecosystems. The understanding of this is crucial for conservation and ecosystem management.

This study attempted to fill a gap in our knowledge of the effects of drought on the reproductive success of grassland forbs by studying various plant and floral traits, pollinator visitation rates, seed set, seed germination and seedling growth of the F2 generation, for three common forb species in the grasslands of KwaZulu-Natal. Mesic grasslands are expected to experience extreme precipitation due to climate change, therefore an experimental approach incorporating both precipitation increase (150% rainfall) and precipitation decrease (50% rainfall) was used (Felton et al., 2019). Individuals of *C. pungens*, *T atriplicifolia* and *S.coronatus* were subjected to three different moisture levels (50%, 100% and 150% rainfall levels) in a randomized block experimental design, as part of the global Drought-Net Research Coordination Network (RCN) protocol for studying drought effects on plants, that has been run reciprocally in many countries (Luo et al., 2021; Qian et al., 2023; Smith et al., 2024a). The 50% rain level was expected to result in reduced plant growth (e.g., number of leaves, plant height) and floral visual signals (e.g., floral display), pollinator visitation rates, reproductive output (i.e., fruit and seed set), as well as second-generation development (i.e., seed viability, seed germination, seedling height, seedling leaf length, seedling specific leaf area, and seedling dry biomass). It was also expected that the low moisture treatment would cause an advance in the onset of flowering and lengthen the flowering period, while the high moisture treatment would increase the number of flowering plants, plant height and delay the onset of flowering.

1.1.Problem Statement and Rationale

Climate change changes rainfall patterns worldwide and is regarded as a global threat to pollination services (Walter, 2020). The prevalence and intensity of droughts is expected to increase in the near future (IPCC, 2007; Lei et al., 2016). Previous research suggests that mesic grasslands are susceptible to dry conditions (Carroll et al., 2021). Ukulinga Research Farm (URF), one of such mesic grasslands constitutes the area of the present study. Current research on the impact of drought on grasslands has been focused mainly on productivity (Carroll et al., 2021; Wang et al., 2019; Wei et al., 2022). There is a lack of literature on the effects of drought on forb ecology and the effects of drought on forbs even though forbs make up 80% of the diversity in mesic grasslands (Morris and Scott-shaw, 2019) and play an important role in maintaining grassland ecosystem resilience (Siebert and Dreber, 2019). Research on forb responses to drought is crucial to maintain grassland species' abundance and diversity (Bråthen et al., 2021). The effects of climate on plant-pollinator networks especially in grassland ecosystems was also identified as a knowledge gap, therefore there is a need for additional experimental and manipulative studies in different climatic regions to understand the possible consequences of climate change (Hoiss et al., 2015). Furthermore, approximately 95% of grassland forbs in South Africa's mesic grasslands are xenogamous and rely on animal pollination (Carbutt and Kirkman, 2022), and thus understanding the effects of drought on floral rewards and floral traits is important since these traits affect pollinator behaviour and ultimately pollination and reproductive success (Descamps et al., 2021b; Ollerton et al., 2011). The relationship between changes in floral traits and pollinator visitation under drought conditions is not well understood (Kuppler et al., 2021). Changes in floral rewards and other traits are expected and have often been shown to affect pollination success, seed production, and viability. It is thus important to understand the consequences of multiple levels of soil water availability on pollination and consequently reproduction to understand the limits and responses of different plant species (Phillips et al., 2018), to develop suitable management techniques for future climates. The main aim of this study was to explore the effects of moisture level on floral traits and plant traits important for pollinator visitation and reproductive success, for three common grassland forb species – *Cephalaria pungens* Szabó, *Senecio coronatus* (Thunb.) Harv. and *Thunbergia atriplicifolia* E.Mey. ex Nees. This includes documenting the breeding system of *C. pungens.* The experimental setup is affiliated with the RCN (Drought-net, 2017), whose main goals are to investigate differences in ecosystem drought sensitivity and to determine the basic mechanisms driving these differences. This network is coordinated from Colorado State University and currently comprises of approximately 112 sites worldwide.

1.2. Outline of Dissertation

This dissertation is structured into five chapters, with the first chapter outlining the rationale, aims and objectives (see Figure 1-1 for an outline of the current study). Chapter 2 is a literature review that explores South African grasslands and drought patterns and introduces the major themes presented in Chapters 3 and 4. The third chapter investigated the effects of moisture level on plant traits and floral visitation of three grassland forb species, helping us understand how floral and plant traits may change in response to different moisture levels, and the response of pollinator visitation to these changes. The fourth chapter investigated the effects of moisture levels on reproductive success of three forb species and the breeding system of *C. pungens,* which is unknown in the current literature. Understanding the breeding system of *C. pungens* can assist in management and restoration efforts of this species. The final chapter focuses on the limitations of the study and future research recommendations.

1.3. Aims and Objectives

This study aimed to investigate the impact of drought on the pollination and reproductive success of three species of forbs found in grassland ecosystems in the summer rainfall regions of South Africa. To accomplish this, the study aimed to address the following research objectives:

i. Evaluate the effects of drought on pollination ecology by measuring the onset and duration of flowering phenology; plant morphology (plant height and number of leaves); floral display; plant density; floral density; and pollinator visitation rate.
ii. Investigate the impacts of drought on the reproductive ecology by measuring fruit and seed set; seed mass; seed germination; seed viability; seedling height; seedling leaf number; seedling leaf length; seedling specific leaf area; and seedling dry biomass.

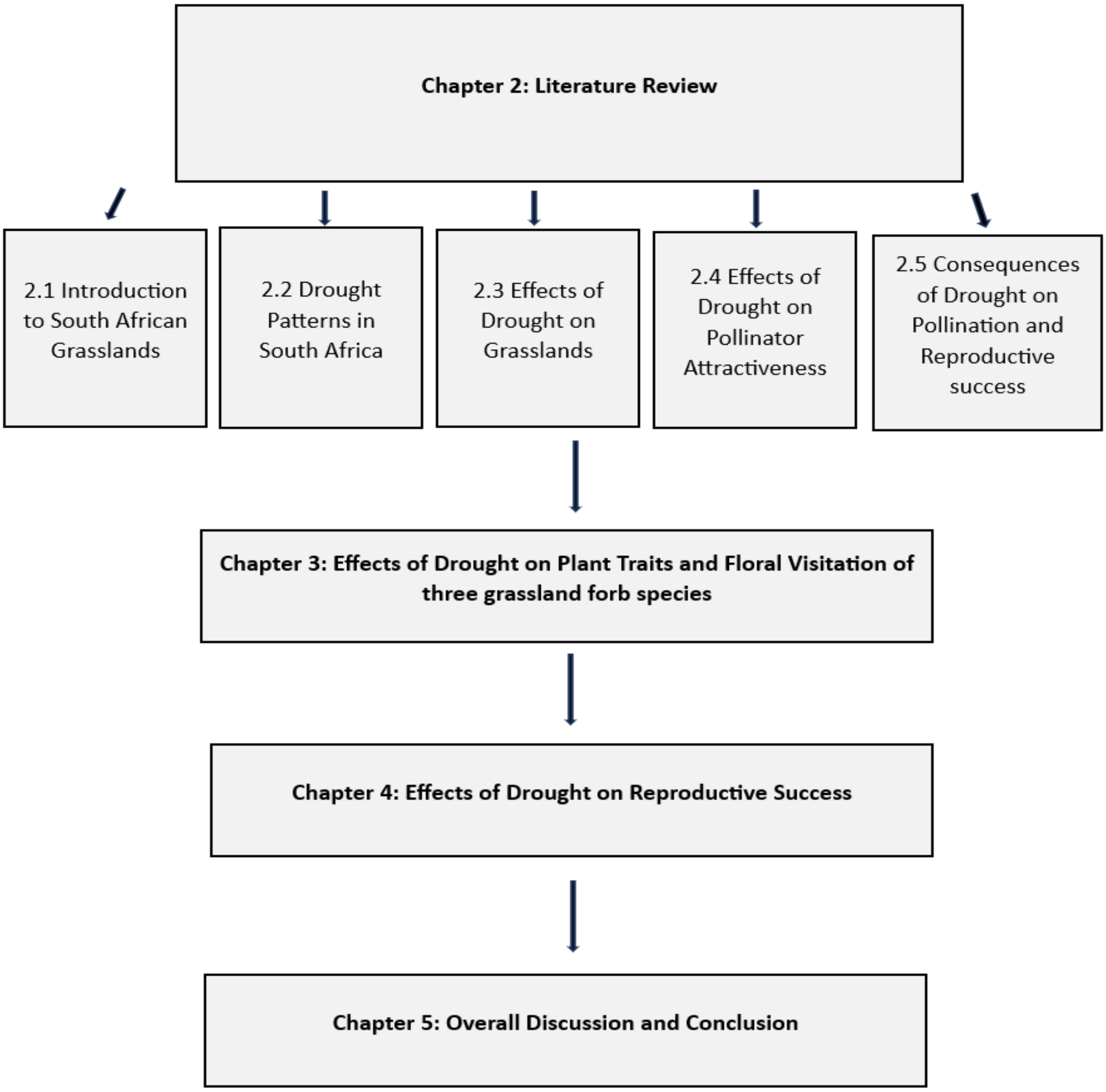

Figure 1-1: Flow diagram illustrating the different chapters of this dissertation.

2. LITERATURE REVIEW

2.1. Introduction to South African Grasslands

The grassland biome is dominated by grasses with scattered forbs instead of the trees and shrubs seen in the savanna biome (Carpenter, 1940; Chamane et al., 2019). Globally, the grassland and savanna biomes cover about 40% of the total land surface (Lei et al., 2016) and can store around 28 – 37 % of the global soil organic carbon. In South Africa the grassland biome is found mainly on the high central plateau, the inland areas of KwaZulu-Natal, and the mountainous areas of the Eastern Cape (Henderson, 1991; Mucina and Rutherford, 2006). Covering an area of more than 360,000 km^2, it is the second-largest biome in the country after the savanna (Gibson, 2009; Zaloumis, 2013). The grassland biome consists of many rare plants, threatened and endemic bird species as well as threatened mammal species (Neke and Du Plessis, 2004). South African grasslands range from arid to humid with a rainfall range of 400 – 2,500 mm per year (Mucina and Rutherford, 2006). Vegetation activity is largely seasonal, with most activity occurring during summer and little to none in the winter months (Mucina and Rutherford, 2006). In terms of suitability for grazing, there are two broad classifications of grassland in South Africa: sourveld and sweetveld, with mixed veld regions being a transition between the two. These are classified according to their moisture availability and winter nutrient retention (Mucina and Rutherford, 2006). Sourveld, which usually occurs in higher altitudes, is moist, while sweetveld is drier and mainly found in lower altitudes (Ellery et al., 1995; Mucina and Rutherford, 2006; Wang et al., 2015). Sweetveld maintains a higher nutritional content in winter, whereas sourveld loses its nutritional value (Mucina and Rutherford, 2006). The winter loss of nutrients in sourveld has been attributed to the decreased availability of phosphorus and the loss of nitrogen through leaching and denitrification in wet soils (Burke et al., 2002; Ellery et al., 1995). Other factors that affect grassland communities are fire and grazing (Koerner and Collins, 2014; Neke and Du Plessis, 2004). Regular burning increases long-term grassland productivity (Everson and Everson, 2016). Grazing increases plant diversity, but also decreases grass cover and production (Koerner and Collins, 2014). In addition to this, an increase in grazing intensity also increases nutrient depletion (Dlamini et al., 2014).

Grassland ecosystems worldwide are known for their rich biodiversity, contribution to food security, climate change mitigation, and provision of ecosystem services (Bengtsson et al., 2019; O’Mara, 2012; Zaloumis, 2013). However, these ecosystems are degrading due to overgrazing, invasion by exotic plants, encroachment by indigenous woody plants, uncontrolled burning, land-use transformation, and poor agricultural practices (Neke and Du Plessis, 2004; Török et al., 2007). In the past twenty years, more and more research has revealed the effects of climate change on grasslands around the world (White et al., 2012; Yu et al., 2012). Forbs make up most of the species richness in mesic grasslands (Scott-Shaw and Morris, 2015) and future climate conditions may threaten the composition, species richness and abundance of these species.

Forbs can be described as non-graminoid, herbaceous, angiosperm species (Bond and Parr, 2010), in this study they are herbaceous aboveground, although they may have lignified stems that are produced seasonally from a resprouting belowground storage organ. They are an important part of grassland communities and although they have a lower cover than graminoids, they are more abundant (Bråthen et al., 2021), they provide habitat for various invertebrates and help maintain the nutrient cycle and reduce alien invasion. They also stabilise grassland communities (Morris and Scott-shaw, 2019). Until recently most studies have focussed on the impacts of grazing, mowing, burning, and grass competition on forbs (Chamane et al., 2019; Fynn et al., 2004; Hayes and Holl, 2003; Little et al., 2015; O'Connor et al., 2011). However, only a few studies have investigated the responses of forbs to climate change (Buhrmann et al., 2016; De Boeck et al., 2018; Fry et al., 2018). The following climatic changes are expected as a result of global warming: increased average air and ocean temperatures; melting of ice leading to rising sea levels; changes in precipitation and ocean salinity; increased wind patterns; and increased frequency and duration of droughts and heavy precipitation (IPCC, 2007). Increased temperature may result in a decrease in forb aboveground productivity (Buhrmann et al., 2016). Forbs may reach their thermal physiological limits resulting in a decrease in photosynthetic efficiency and therefore a decrease in aboveground production (Buhrmann et al., 2016). Drought has also been shown to decrease forb species richness, biomass, photosynthetic efficiency, bud densities, abundance, primary productivity, and future fitness if there is a high initial reproductive investment (Carroll et al., 2021; Carter et al., 2012; De Boeck et al., 2018; Fry et al., 2018; Hamann et al., 2021; O'Connor, 1995; Wang et al., 2019; Williams et al., 2001).

2.2.Drought Patterns in South Africa

South Africa, a water-scare country, receives an average rainfall of 450 mm, and is classified as semi-arid (Hirooka et al., 2019; Suttie et al., 2005). The province of KwaZulu-Natal gets its rainfall in summer, while the south and south-west regions have winter rainfall (Suttie et al., 2005). There is no single definition of drought (Van Loon, 2015). In this study, drought is defined as a period of below-average precipitation compared with the long-term average climate of a given region (Meza et al., 2021). Drought is a common occurrence in South Africa and drought cycles usually last for approximately two years (FAO, 2019; Mahlalela et al., 2020; Walz et al., 2020). The frequencies of drought and flooding are projected to increase as a result of climate change (Kephe et al., 2021). These drought episodes are usually associated with El Nino events (Baudoin et al., 2017; Edossa et al., 2014; Thomson et al., 2003). However, the more recent droughts (2015 - 2016) are associated with climate change (Botai et al., 2016; Dube et al., 2022; Malherbe et al., 2016). Throughout history, South Africa has experienced many droughts with some provinces more severely affected than others (Botai et al., 2017, 2016; Ngaka, 2012). The Eastern Cape, Western Cape, Northern Cape, North West, and Free State provinces often experience droughts which have devastating effects on the agricultural sector, water resources, as well as job security (Botai et al., 2017, 2016; Mahlalela et al., 2020). The major droughts recorded in South African history were; 1965-66, 1972-73, 1982-84, 1991-92, 1994-95, 1997-98, 2001-02, 2004-05, 2007-09, 2015-2016 and 2018-2020 (Ngaka, 2012; Mahlalela et al., 2020; Tyson, 1996; FAO, 2019). Since 1970, there has been an increase in the spatial extent of droughts (Edossa et al., 2014). Between 1952 and 1999, the most severe short-term drought was in 1973 and then another followed in 1995 (Edossa et al., 2014).

2.3.Effects of Drought on Grasslands

Grasslands that are on nutrient-poor soils and are dry are most prone to climatic variability and extreme events (Van den Hoof et al., 2018). While grasslands may be resistant to heat waves, they may not be resistant to extreme drought events (Hoover et al., 2014). The effects of drought on primary production (Fu et al., 2020; Wang et al., 2019), stability of plant communities (Barros et al., 2018), forage (Emadodin et al., 2021), water provision (Leitinger et al., 2015), carbon cycling (Lei et al., 2020), species richness and cover-abundance (Copeland et al., 2016; Li et al., 2021), and biomass (Lozano and Rillig, 2020), have been recognized.

Dry conditions may affect primary production, cover, and carbon exchange in grasslands. In grasslands, grasses may respond differently to forbs. Low soil moisture decreased net primary production by increasing photoinhibition of photosynthesis (Wang et al., 2019). Such negative impacts may be more pronounced in forbs than in grasses if drought events deplete the deeper soil water reserves that are needed for the forbs (Hoover et al., 2014). Another study found that drought decreased the cover of grasses in three different vegetation types (shrublands, grasslands, and mixed vegetation). But in terms of forbs, drought decreased forb cover in shrublands but increased it in grasslands and mixed vegetation (Báez et al., 2013). These different responses may be caused by differences in plant morphological, physiological, and life history traits (Báez et al., 2013). Drought may also affect the net ecosystem carbon exchange in grasslands, by changing them to carbon sources instead of sinks (Hoover and Rogers, 2016; Jongen et al., 2011). This could be because, during drought conditions, the total photosynthetic rate of grass decreases because of the diminished activity of ribulose diphosphate carboxylase and the efficiency of the quantum yield of photosynthesis. Together these elements result in a reduction in the photosynthetic capacity of mesophyll cells. The lowered rate of total photosynthesis under drought stress is influenced by the ongoing closure of leaf stomata. During extreme drought conditions, this closure of leaf stomata reduces photosynthetic rates and production of sugars, reduces transpiration and CO_2 absorption, and increases photorespiration, which reduces plant productivity, delays flowering, and reduces flower number and fruit set (Pang et al., 2017; Seleiman et al., 2021).

While drought has been shown to be associated with undesirable changes such as a decrease in species richness and cover, particularly in annual forbs and exotic annual grasses (Copeland et al., 2016; Li et al., 2021), and reduction in plant shoot biomass and reproductive mass (Eziz and Fang, 2017; Lozano and Rillig, 2020), it can also be associated with desirable changes such as an increase in root biomass. Root biomass has been shown to increase under dry conditions (Li et al., 2021) because plants are better able to reach water in deep soils when the root length is increased (Eziz and Fang, 2017). However, in some cases, the root mass may decrease in dry conditions depending on the timing of the drought. For example, during the flowering or seed maturation stage, there may be more biomass allocated to reproductive organs than the roots causing a decrease in root mass (Greco and Cavagnaro, 2002; Lozano and Rillig, 2020; Zhou et al., 2018).

2.4. Effects of Drought on Pollinator Attractiveness

2.4.1. Floral Rewards

Although almost 90% of flowering plants require animal pollinators for pollen transfer (Hanberry et al., 2021; Ollerton et al., 2011), little is known about the impacts of drought on plant-pollinator interactions (Burkle and Runyon, 2016; Opedal et al., 2016; Phillips et al., 2018; Rering et al., 2020; Walter, 2020). The most common floral rewards are nectar and pollen (Farré-Armengol et al., 2015). Plants use nectar to attract pollinators to promote reproductive success (Pyke, 2016). An increase in nectar has been shown to increase the number of flowers probed and the time spent on an inflorescence (Johnson et al., 2004). The plant reproductive stage is sensitive to climatic changes (Zinn et al., 2010; Sage et al., 2015; Hatfield & Prueger, 2015; Descamps et al., 2018). Drought has been shown to affect nectar volume (Carroll et al., 2001; Descamps et al., 2021a), pollen production (Al-Ghzawi et al., 2009), flower number (Rering et al., 2020), flower size (Burkle and Runyon, 2016; Carroll et al., 2001), plant height (Al-Ghzawi et al., 2009), flower colour (Descamps et al., 2021b), and floral scent (Burkle and Runyon, 2016); but not nectar sugar concentration (Carroll et al., 2001; Descamps et al., 2021a; Rering et al., 2020), all of which affect the overall attractiveness of plants to pollinators (Villarreal and Freeman, 1990).

An increase in nectar production may increase insect diversity and visitation rate (de Brito et al., 2017). This infers that a decrease in nectar volume would have a negative effect on pollinator visitation and therefore reproductive success of the plant. This is particularly true for pollination systems involving nectar-feeding birds. Another study has shown that floral visitors collecting both nectar and pollen changed from being pollen collectors in the morning to nectar collectors after pollen production decreased (de Brito et al., 2017). An increase in visitation may result in an increase in pollen loads on stigmas causing an increase in seed set and an improved performance of resulting progeny in areas with good nectar production (Dogterom et al., 2000; Sáez et al., 2014).

Generally, a reduction in water availability results in a decrease in nectar volume per flower (Arroyo et al., 2020; Descamps et al., 2018; Gallagher and Campbell, 2021; Rering et al., 2020). The reduction in nectar volume could be a result of a decrease in the overall number of flowers therefore decreasing the overall nectar production (Descamps et al., 2021b, 2018; Villarreal and Freeman, 1990; Waser and Price, 2016; Wilson Rankin et al., 2020), but also due to a decrease in soil moisture and increase in plant evapotranspiration reducing the amount of nectar produced per flower, since nectar production is controlled by physiological processes (e.g. growth, photosynthesis etc.) (Descamps et al., 2021; Borghi et al., 2019). By limiting the number of flowers and nectar volume per flower, plants can conserve resources without reducing reproductive potential because it is less costly for pollinators to visit a smaller number of flowers than to discriminate between those that are secreting and non-secreting (Carroll et al., 2001; Descamps et al., 2018; Phillips et al., 2018; Rering et al., 2020). A study in Wiltshire, UK, found that drought did not affect nectar volume in *Lathyrus pratensis* L, *Onobrychis viciifolia* Scop. and *Prunella vulgaris* L. (Phillips et al., 2018). It was, however, noted in the study that the soil moisture deficit may not have been large enough to induce nectar volume changes and that the calcareous grasslands, where the study was conducted, are resistant to drought (Phillips et al., 2018). Some studies have found that plants can produce nectar-less flowers in response to environmental stress (Descamps et al., 2021a; Petanidou and Smets, 1996; Phillips et al., 2018). The differences in plant responses could be due to differences in plant breeding strategies. For example, some plants rely on cross-pollination, so they need to maintain some nectar in a few flowers to ensure pollinator visitation (Phillips et al., 2018). Phillips et al. (2018) also suggested that some plants that can self-pollinate would therefore not need to invest in nectar production to attract pollinators. Therefore, diverting resources into fewer flowers could allow the nectar volume to be maintained in those flowers (Phillips et al., 2018). It could also be that the methods used to calculate nectar volume are causing this inconsistency. For example, Phillips et al. (2018) only measured 24-hour accumulation of nectar (exclusion of nectar-collecting insects for 24 hours by covering flowers with bags), while other studies (Carroll et al., 2001; Descamps et al., 2018; Rering et al., 2020; Villarreal & Freeman, 1990) measured standing crop. According to Farkas et al. (2012), covering flowers with pollinator-exclusion bags affects nectar volume, where mean nectar volume in covered flowers is significantly greater than in uncovered flowers. Nectar standing crop refers to the amount of nectar in a flower at any given time. The standing crop in flowers within a single plant is often highly variable (Keasar et al., 2008) due to either the foraging activity of pollinators or the plant's response to environmental stress (Phillips et al., 2018).

Nectar sugar concentration is not generally affected by drought (Carroll et al., 2001; Descamps et al., 2021b, 2018; Gallagher and Campbell, 2021; Phillips et al., 2018; Rering et al., 2020; Villarreal and Freeman, 1990). This could be because plants can adjust the sugar concentration of their nectar in flowers so that the viscosity remains almost the same (Villarreal and Freeman, 1990). One study found a reduction of nectar sugar concentration as a result of decreased water (Wilson Rankin et al., 2020), but didn't provide the potential causes of this. This is quite surprising as one would expect that as the water decreases the concentration of sugar increases. Two studies have found that nectar sugar concentrations are affected by the time of day, for example, highest concentrations in the mid-afternoon and lowest in the early morning (Kajobe, 2007; Pleasants, 1983). This could be due to variations in temperature and relative humidity throughout the day and sugar concentrations appear negatively correlated with relative humidity (Pleasants, 1983). Another study that looked at foraging behaviour of bees found that most plants visited by the bees had nectar with increased sugar concentrations from 7h00 – 8h00 reaching a peak at 13h00, after that it showed a decrease (Kajobe, 2007). This may be driven by higher solar radiation causing evaporation resulting in more concentrated nectar (Kajobe, 2007). However, these responses appear to be species-dependent as another study found that sugar concentrations were negatively correlated with the time of day for *Palicourea demissa* Standl. and *Mezobromelia capituligera* (Griseb.) J. R. Grant but positively correlated for *Kohleria affinis* (Fritsch) Roalson & Boggan (Palmer, 2019).

With regards to responses of pollen production to drought, and according to several studies (Descamps et al., 2018; Waser & Price, 2016; Rering et al., 2020), pollen quantity is generally unaffected by water stress. One explanation is that pollen quantity is determined early in flower development (Goldberg et al., 1993), however, since pollen quantity is proportional to the number of flowers, a decrease in the total number of flowers will decrease total pollen production (Rering et al., 2020).

2.4.2. Floral Attractants

Floral traits such as floral colour, floral display, scent, and flower size, are important in pollinator attraction and pollinators are sensitive to changes in these. Pollinators prefer large (Conner & Rush, 1996) and tall flowers (Engel and Irwin, 2003; Hernández-Villa et al., 2020). This could be because taller flowers are more noticeable and easily accessible than shorter flowers (Ouvrard et al., 2018). However, this may be dependent on both the height of the surrounding vegetation as well as the type of species. One study found that the pollinator visitation rate for *Centaurea scabiosa* L. increased with inflorescence height between 0 cm to 40 cm in short vegetation and from 0 cm to 60 cm in tall vegetation (Klecka et al., 2018). However, for *Inula salicina* L, pollinator visitation decreased with increasing inflorescence height from 0 cm to 100 cm in short vegetation, and increased with inflorescence height from 0 cm to 50 cm in tall vegetation, after which it decreased slightly (Klecka et al., 2018).

Floral scent attracts or repels pollinators (Burkle and Runyon, 2019). Floral scents are made up of volatile organic compounds (VOCs) that are emitted by the flower petals or other floral parts (including sometimes the nectar). Pollinators can smell flowers from a long distance (Sheehan et al., 2012), depending on the species of pollinators and their sensitivity, even as far as several kilometres (Menzel et al., 1997). The type of pollination mode determines the amount of VOCs emitted; for example, entomophilous (insect-pollinated) flowers have higher

VOC emission rates and VOC richness, both for terpenes and benzenoid compounds, compared to anemophilous (wind-pollinated) flowers which do not need to attract pollinators (Farré-Armengol et al., 2015). Both terpenes and benzenoid compounds can attract pollinators and repel elective visitors (Farré-Armengol et al., 2013).

Several studies have shown that floral scent can be affected by drought (Burkle and Runyon, 2016; Glenny et al., 2018). The effect of drought on VOCs is species-specific (Burkle and Runyon, 2016; Glenny et al., 2018). Drought may increase or decrease VOC emissions and change the composition of the VOCs which can deter pollinators thus decreasing pollinator visitation rate (Burkle and Runyon, 2016; Glenny et al., 2018). Burkle and Runyon (2016) studied the effects of drought on floral VOCs of four forb species (*Campanula rotundifolia* L., *Heterotheca villosa* (Pursh) Shinners, *Phacelia hastata* Douglas ex Lehm and *Potentilla recta* L.). The specific compounds that contributed most to the differences in composition between the drought and control treatments were 6-methyl-5-hepten-2-one and benzaldehyde which tended to increase in the drought treatments (Burkle and Runyon, 2016; Glenny et al., 2018). These are common VOCs emitted by flowers and are thought to be important in attracting pollinators (Burkle and Runyon, 2016). They found that in two of the forb species, the drought treatment reduced pollinator visitation rate, in one forb there were no treatment effects, and in another forb drought increased pollinator visitation. The decrease in pollinator visitation rate for the two forbs is more likely due to the change in the composition of the VOCs rather than the total quantity of the VOCs (Glenny et al., 2018), but the response is species-specific, with some species showing no significant effects (Burkle and Runyon, 2016; Glenny et al., 2018; Rering et al., 2020).

Floral display or flower number (i.e. number of open flowers) has been shown to affect pollinator visitation frequency (Conner and Rush, 1996; Eckhart, 1991). Generally an increase in flower number results in an increase in pollinator visitation (Conner and Rush, 1996; Klinkhamer and de Jong, 1990). However, this relationship could vary depending on the gender of the flower. For example, Eckhart (1991) reported that hermaphrodites received significantly higher pollinator visitation than female-only flowers. Some insects prefer male reproductive structures to female ones possibly because in some species, for example, *Aquilegia buergeriana var. oxysepala* (Trautv. & C.A.Mey.) Kitam., *Echium vulgare* L and *Impatiens capensis* Meerb., flowers have a higher nectar reward during the male phase than during the female phase in some species, and pollen-collecting insects would be more attracted to flowers with pollen than without (Bell et al., 1984; Klinkhamer and de Jong, 1990; Misaki et al., 2018)

Although the advantage of having many flowers per plant may increase pollinator visitation, this advantage declines as more flowers per plant are produced. This is because pollinators visit more flowers on plants with many flowers, therefore increasing the rate of self-pollination (Klinkhamer and de Jong, 1990).

Generally, plant height is also reduced under water stress (Boutraa et al., 2010; Hussain et al., 2019; Rering et al., 2020; Sun et al., 2015; Zhao et al., 2020). However, the response of plant height to water stress is species-specific, with some species decreasing height with water stress, and others showing no significant effects (Burkle and Runyon, 2016; Glenny et al., 2018). The severity of the effects depends not only on the growth period stage in which the stress occurs but also on the duration of the stress (Sun et al., 2015). In terms of the growth period, water stress may have the largest effect on plant height in the elongation period (Sun et al., 2015). Sun et al. (2015) also found that the longer the drought is, the greater the effect on the plant height. The reduction in plant height could be a result of a few reasons, namely reduced stem cell water potential to levels lower than that required for cell elongation; a decrease in growth regulator hormones; as well as stomatal closure, reduction of leaf area and depression of photosynthesis as a result of drought stress (Nezamia et al., 2007; Zhao et al., 2020).

In most cases, drought decreases floral display (number of open flowers per plant) (Arroyo et al., 2020; Burkle and Runyon, 2016; Conner and Rush, 1996; Descamps et al., 2018; Kuppler et al., 2021; Opedal et al., 2016) and thus reduces pollinator visitation (Klinkhamer and de Jong, 1990). In extreme cases, pollinators avoid visiting plants in dry conditions completely (Walter, 2020). This could be because water stress increases flower abortion in some species, resulting in a low number of open flowers (Descamps et al., 2018). Abortion of flower buds or open flowers reduces the maintenance costs and water loss associated with flowers. If drought affects the number of open flowers then that could also affect pollinator activity since pollinator visitation frequency typically correlates with the number of flowers in anthesis (Chen and Zuo, 2019; Gallagher and Campbell, 2021). If flower number is reduced as a response to plants trying to conserve water during drought conditions, and pollinators are less attracted to the smaller displays, this will in turn lead to a decrease in pollination services, fruit production, and overall reproductive success during a drought period for that plant species (Mitchell, 1994).

Drought has also been shown to decrease flower size (Burkle & Runyon, 2016; Carroll et al., 2001; Kuppler et al., 2021). This is because under water stress, corollas have smaller petals with smaller and more densely packed cells than unstressed plants, due to a reduction in cell elongation (Galen et al., 1999). Smaller corollas need less water to maintain turgor in their cells, improving plant water status. Water loss from evaporation and transpiration is also reduced in smaller corollas compared to larger corollas (Galen et al., 1999).

2.4.3. Population Density

Pollination efficiency is defined as the proportion of conspecific pollen deposited on a stigma per visit (Adler and Irwin, 2006). An increase in visitation rate does not always correspond with a high deposition of conspecific pollen. Sometimes a low plant density can result in a low amount of conspecific pollen transported and deposited by pollinators, even at a high visitation rate (Delmas et al., 2016). However, at a high plant density and low visitation rate, pollinators can still deposit a large amount of conspecific pollen (Delmas et al., 2016). This highlights the importance of population density on pollen transfer.

The relationship between plant/flower density, pollination and reproductive success appears to be multi-faceted and species-specific. For example, Bosch and Waser (2001) reported that there was no difference in pollinator visitation rate and seed set in sparse and dense populations for *Delphinium nuttallianum* Pritzel ex Walpers. However, flowers of *D. nuttallianum* received significantly higher pollen loads in sparse populations than in dense populations. In contrast, pollinator visitation rates and seed set for *Aconitum columbianum* Nutt. increased significantly in sparse populations than in dense populations. In addition, *A. columbianum* received significantly higher pollen loads in dense populations than in sparse populations. Another study also found that plant density did not affect seed production of *Senecio integerrimus* Nutt. (Schmitt, 1983). However, a study conducted using natural and experimental populations of *Delphinium barbeyi* (Huth) Huth, looking at the effects of flowering density on seed set and pollen receipt found that, while pollen receipt per flower increased with flowering plant density in the natural environment, there was an increase in seed set in low-density populations compared to those in high-density populations (Elliott and Irwin, 2009). Although, these observed effects of experimental flowering plant density on pollination or seed production were found to be statistically non-significant. Given the results of these studies, any influence of drought on plant density would be expected to have differing effects on different flowering species' pollination and subsequent reproductive success.

2.5. Consequences of Drought on Pollination and Reproductive success

2.5.1. Pollinator Visitation

Pollinators play an important role in plant reproduction by transferring pollen from one flower's anther to the stigma of another flower, preferably on a different plant to introduce genetic variability in the offspring. The fertilised flower will then produce fruits and seeds. Generally, seed production increases with pollen load (Björkman, 1995). An increase in pollinator visitation rate has been shown to increase pollen receipt (Engel and Irwin, 2003), thereby increasing seed set (Chen and Zhao, 2019; Chen and Zuo, 2019; Gallagher and Campbell, 2021). In addition, when pollinator visitation frequency is high, pollination efficiency is improved. This is because the filaments of the stamens dry easily making the window for pollination small, and high visitation rates would therefore increase the likelihood of successful pollen deposition and transfer, particularly in dry areas (Chen and Zuo, 2019). Drought, increased temperature, increased carbon dioxide, plant height, floral display, and herbivory, have all been shown to affect pollinator visitation rate (Burkle and Runyon, 2016; Engel and Irwin, 2003; Glenny et al., 2018; Lee and Kang, 2018; Rering et al., 2020).

From the literature discussed above, the effects of drought on pollinator visitation rate appear to be species-specific (Burkle and Runyon, 2016; Glenny et al., 2018). In some species, drought stress has been shown to decrease the pollinator visitation rate per plant (Burkle and Runyon, 2016; Glenny et al., 2018; Kuppler et al., 2021). One possible explanation is that drought results in fewer and smaller flowers being produced per plant (Descamps et al., 2021c; Kuppler et al., 2021; Waser and Price, 2016) and it reduces nectar volume per flower (Descamps et al., 2021c; Rering et al., 2020), reducing the overall attractiveness of the plant to pollinators. Increases in the number of flowers per plant have been shown to increase pollinator visitation (Rering et al., 2020) and thus the opposite trend is inferred if drought reduces the number of flowers per plant. However, in some species, there has been no significant effect of drought observed on pollinator visitation rate per plant (Burkle & Runyon, 2016; Descamps et al., 2018; Glenny et al., 2018). Pollinator visitation is usually measured by observing floral visitors that touch the reproductive parts of a flower (Burkle and Runyon, 2016) during peak pollinator activity hours. The duration of the visit, number of visits, number of flowers visited, visitor behaviour, and type of visitor are typically recorded, and can be used in assessing drought effects on pollination success of plant species.

2.5.2. Pollinator Abundance

In addition to decreasing the pollinator visitation frequency, drought has been shown to decrease pollinator abundance (Rering et al., 2020). For example, Rering et al. (2020) found that significantly fewer bumble bees, honey bees, and flies were recorded on drought-stressed plants of *Fagopyrum esculentum* Moench in Illinois, USA. This is important since some pollinators like bumble bees, are effective pollinators of *F. esculentum* (Kambrekar et al., 2018) and may be more important than other floral visitors for ensuring successful seed production (Rering et al., 2020). These pollinators feed with their bodies close to the reproductive parts of a flower, and are covered in hairs, and therefore can carry large numbers of pollen grains (Anderson et al., 1982) while less effective pollinators are either small or feed with a long proboscis such that their bodies rarely encounter the reproductive parts of a flower. Therefore, a reduction in effective pollinators may reduce pollen deposition on the stigma and consequently reduce seed production. The reduction in bumble bee abundance under drought conditions may be related to changes in floral rewards such as reductions in the floral display, flowers per plant, nectar volume per flower, and percent of sucrose in nectar resulting in decreased visitation rates by bumble bees (Rering et al., 2020). In contrast, one study found that there was an increase in bee abundance in an area post-drought (Hung et al., 2021). This increase was attributed to an increase in abundance of a *Dialictus* bee species, which is small and has a generalist diet. The species was able to survive on low amounts of food resources and a variety of plant species, including weeds.

However, having a high pollinator abundance may not always be desirable as this could decrease seed set per flower (Klank et al., 2010), especially when pollinators can act as both pollinators and seed predators. In some cases, a decrease in one pollinator may not necessarily decrease the fruit-to-flower ratio (FFR), as there may be another pollinator that increases in visitation and compensates for the loss of the other, as in the case of *Sechium edule* (Jacq.) Sw. (Mukherjee et al., 2019). Mukherjee et al. (2019) conducted a study to understand which pollinators influenced FFR during a normal rainfall year. They found that pollinator diversity did not affect FFR but found that *Trigona sp.* was the main pollinator that positively affected FFR even though during the dry period, there was a significant drop in *Trigona sp.* visitation the FFR remained the same. The authors used the ecosystem stabilising mechanism to explain the process that might have resulted in this unchanged FFR. The ecosystem stabilising mechanism proposes that the abundance of one species providing a service increases during a decrease in the abundance of another species (Mukherjee et al., 2019).

2.5.3. Seed Production

A plant seed carries an embryo that will develop into a new plant. The quality and quantity of seeds produced are dependent on the climate, quantity and quality (e.g. self-pollen) of pollen received, diseases, pests, competition, flower and seed predation, physiology, soil, and fire (Bustamante-García et al., 2012; Hoffmann, 1998; McIntosh, 2002; Waser and Price, 1991). It is important to investigate the contribution of these factors in plants in natural environments so that we can understand their impact on the overall reproductive success of these plants (McIntosh, 2002). Drought can reduce seed set and seed mass (Arroyo et al., 2020; Gallagher and Campbell, 2021; Nezamia et al., 2007; Rering et al., 2020). The decrease in seed production could be due to an increase in flower and pod abortion, and pollen limitation (Pang et al., 2017). For example, pollen limitation may decrease plant reproduction due to low levels of pollen transfer and inadequate pollen receipt (Chen et al., 2019). Furthermore, a lack of resources might reduce seed output. For example, Pang et al. (2017) suggested that seed abortion may be induced by either reduced nutrient and water supply to the developing pod due to stomatal closure and a decrease in leaf photosynthesis, or by abscisic acid accumulation in the seed or pod, or both.

2.5.4. Progeny

Most previous research regarding the effects of drought conditions on pollination ecology has focused on floral signals (e.g., floral display, flower colour, size, and scent) and rewards (nectar and pollen) that influence pollinator behaviour. In terms of reproduction, most studies have looked at how drought has affected seed production and germination. Very few studies have tested the effects of drought on seed viability and second-generation development (Burkle and Runyon, 2016; Glenny et al., 2018; Jaworski et al., 2022). Subsequently, it is hard to understand the long-term consequences of drought.

The negative impacts of water stress can be transferred to the next generation. One study found that water stress on the parent plant of *Glycine max* (L.) Merr. affected the seed recruitment of the F1 generation by reducing seed germination rate and seedling performance, as well as delaying seed germination (Wijewardana et al., 2019). This could be due to osmotic stress, which reduces the absorption of water, and as a result, lowers embryo development (Wijewardana et al., 2019). Furthermore, seedlings of drought-stressed plants produced longer root systems and greater biomass (Herman et al., 2012).

2.5.5. Pollinator Diversity

Pollinator diversity can increase fruit production especially when the main pollinator abundance is low (Gómez et al., 2007; Vergara and Badano, 2009). One study showed a positive relationship between pollinator diversity and flower diversity (Fründ et al., 2010). Not all floral visitors perform similar pollination services. Some are pollinators while others are just visitors who do not transfer pollen between flowers. When comparing pollinators, some can potentially deposit more pollen than others (Adler and Irwin, 2006; Chen and Zhao, 2019). For example, previous research found that the honeybee *Apis mellifera ligustica* Spin was able to easily transport and deposit a larger portion of pollen on the stigmas per visit of *Caragana korshinskii* Kom. compared to other pollinators (Chen et al., 2019). In another study, *Bombus bimaculatus* Cresson, *Habropoda laboriosa* Fabricius, and *Osmia lignaria* Say were found to transfer more pollen per flower for *Gelsemium sempervirens* L. than *A. mellifera* (Adler and Irwin, 2006).

Studies have shown global pollinator diversity is declining (Biesmeijer et al., 2006; Potts et al., 2010). The loss in pollinator species diversity is concerning because this may reduce plant reproduction. Specialist pollinators are specifically at a greater risk of declining than generalist pollinators (Biesmeijer et al., 2006; Weiner et al., 2014), since generalist pollinators can feed on a variety of plant species, unlike specialist pollinators. Therefore, when habitat fragmentation, alien invasion, and climate change threaten plant species, specialist pollinators experience a shortage of food resources. A decline in pollinators results in a decrease in pollinator services and a decline in pollinator diversity, which ultimately lead to a decline in the plants pollinated by these species.

Several factors drive the decline of pollinators. For example, habitat loss has been documented to be an important driver of bee declines (Brown and Paxton, 2009). Butterflies have also been shown to decrease over the past 30 years possibly due to a loss of plant diversity and species richness as a result of habitat fragmentation (Wenzel et al., 2006). Drought has also been shown to decrease pollinator species richness (Hoiss et al., 2015).

2.5.6. Flower Phenology

Flowering phenology controls the time that reproductive structures interact (Rodríguez-Pérez and Traveset, 2016) through pollinator visitation resulting in reproductive success (Bishop and Schemske, 1998; Bloor et al., 2010; Gallagher and Campbell, 2021; Jagadish et al., 2016). The longer the flowers are open, the more opportunities there are for pollinators to visit and potentially pollinate (Dorji et al., 2020). Plants that flower at the same time as other plants in the same population have been shown to have a higher seed output than plants that are in an asynchronous population (Augspurger, 1981). The attractiveness of a flowering species' population and flowering community will change throughout a season, affecting the attractiveness of the population or community to pollinators (Bishop and Schemske, 1998) as the floral displays change over time (Gallagher and Campbell, 2021). Late-season plants may produce fewer flowers and therefore receive fewer visits from pollinators and produce lower seed set (Gallagher and Campbell, 2021). Abiotic factors (photoperiod, temperature, water), biotic factors, and urbanisation have all been shown to affect flowering phenology (Bloor et al., 2010; Carbognani et al., 2016; Hall et al., 2014; Neil et al., 2014; Suonan et al., 2019). For example, spring-blooming plants in different ecosystems in North America, Europe, and China have been shown to flower earlier in the city compared to the surrounding un-urbanised areas (Neil et al., 2014), and the Heat Island Effect (a condition where urban areas experience increased temperatures than outlying areas) has been recognized as a possible driver of this result. With climate change intensifying and spreading (IPCC, 2007), many studies have investigated the effects of climate change on plant phenology. Most of these have looked at the effects of increased temperature (Bloor et al., 2010; Carbognani et al., 2016; Cornelius et al., 2014; Daru et al., 2019; Hu et al., 2020; Price and Waser, 1998) and a few at the combined effects of increased temperature and water stress on flowering phenology (Hatfield and Prueger, 2015; Suonan et al., 2019). Despite this, our understanding of the way climate change affects plant phenology is insufficient (Piao et al., 2019). In most cases, temperature changes have been reported to advance flowering phenology (Bloor et al., 2010; Carbognani et al., 2016; Daru et al., 2019; Dorji et al., 2020; Hu et al., 2020; Price and Waser, 1998; Suonan et al., 2019). However, while one study showed early-flowering species advanced their flowering periods (Jiang et al., 2021), another study showed a delay in flowering for mid- to late-flowering species (Jiang et al., 2021). Even though temperature and precipitation may have individual impacts on plant phenology, one study found that these variables had no interactive effects on phenology for three grass species (*Elymus nutans* Griseb, *Stipa Alinea* L. and *Poa pratensis* L.), one sedge (*Kobresia humilis* (C.A.Mey. ex Trautv.) Serg.), two legumes (*Melilotoides archiducis-nicolai* (Širj.) Yakovlev and *Tibetia himalaica* (Baker) H.P.Tsui) and five forbs (*Aster diplostephioides (DC.) Benth. ex C.B.Clarke, Gentiana lawrencei* Burkill, *Gentiana straminea* Maxim, *Potentilla saundersiana* Royle, *Saussurea superba* J.Anthony) (Suonan et al., 2019). Early flowering plants may be at an advantage over later flowering plants in the absence of interspecific competition for pollinators, however, this may lead to a decrease in reproductive success if there is a mismatch between the timing of flowering and pollinator availability (Kalisz and Vogler, 2003; Kehrberger and Holzschuh, 2019; Pedroso et al., 2021). Although expected, changes in climatic variables have also been shown not to affect phenology and plant growth for certain species. One study found that the phenology and some morphological features (stems, leaves, and flowers) of *Cephalaria gigantea* (Ledeb.) Bobrov. showed no large differences to plants of the species growing under different climatic conditions (Chrząszcz et al., 2021).

Although drought impacts phenology to a lesser degree than temperature (Suonan et al., 2019), only a few studies have investigated phenological responses to water stress (Bloor et al., 2010; Desclaux and Roumet, 1996; Jentsch et al., 2009). There does not seem to be a consensus on the effects of drought on phenology. Some studies claim it has similar effects to temperature by advancing flower phenology (Kazan and Lyons, 2016), others reported no significant response (Bloor et al., 2010; Neil et al., 2014), and still others found that it was delayed (Suonan et al., 2019).

2.5.7. Breeding Systems

There are two main modes of reproduction in plants, i.e. vegetatively and by seed (Kelly, 2013). Most flowering plants reproduce sexually through seed production (Rajjou et al., 2012). Some plants reproduce vegetatively, while other plants, (especially herbaceous perennials) may reproduce using one or both forms of reproduction (Berge et al., 1998; Fenner and Thompson, 2005). Having a mixed mating system of vegetative reproduction, and self and cross pollination is also particularly important if there is a shortage of outcross pollen (Kalisz et al., 2004). The ability of a plant to reproduce both vegetatively and sexually may increase fitness since it will be able to take advantage of the favourable traits of both forms of reproduction (Fenner and Thompson, 2005; Hernández-Villa et al., 2020). Within sexual reproduction, self-pollination can result in a new population from just one individual, whereas cross-pollination is when pollen from one plant is transferred to another plant and more than one individual is needed. Both types of breeding systems have their advantages and disadvantages. For example, selfing guarantees reproduction, particularly in the absence of pollinators (Becker et al., 2011) and other plants of that species, which is favourable during colonisation of new habitats. Selfing can preserve high local adaptation and increase the spread of favourable recessive alleles (Wyatt, 1986). However, this form of breeding system is often considered to be an undesirable condition (Wells, 1979), because it increases the chances that recessive disadvantageous alleles will be made homozygous and lead to inbreeding depression (Charlesworth, 2006). For example, one study found that self-pollination resulted in very few seeds in two species despite the plants not being pollen-limited (McIntosh, 2002). Autonomous self-pollination is a type of self-pollination where anthers come into contact with a stigma or when the pollen falls on the stigma within a flower unaided by external vectors (Peter and Johnson, 2009). Some plants only make fruit through autonomous self-pollination (Kalisz and Vogler, 2003). But in general, outcrossed individuals typically have a higher fitness than self-fertilized individuals (Wells, 1979). One study found that flowers of *Collinsia verna* Nutt. that were hand-crossed produced more fruits than flowers that only received pollen through autonomous selfing (Kalisz and Vogler, 2003).

Climate change may cause shifts in mating systems for mixed-mating plants (Jones et al., 2013). For example, an increase in temperature has been shown to increase the number of plants that set fruit through selfing in cleistogamous flowers, increase the number of fruit per plant through selfing in cleistogamous flowers, and an increase in the percentage of viable chasmogamous seeds that were in the pollinator exclusion treatment for *Viola praemorsa* (Nuttallii) (Jones et al., 2013).

It is said that drought promotes higher rates of selfing, but evidence to support this is lacking (Kay and Picklum, 2013). One study suggested that the type of environment, species and timing of drought may influence the mating system (Kay and Picklum, 2013). They found that *Clarkia concinna* (Fisch. & C. A. Mey.) Greene which grows in a mesic environment and was subjected to a later onset of summer drought, increased per-flower selfing rates in the drought treatment, but *Clarkia breweri* (A. Gray) Greene which grows in a drier environment and which had an early onset of drought decreased its per-flower selfing rate in the drought treatment (Kay and Picklum, 2013). The increase in selfing could be due to a reduction in herkogamy (the spatial separation of anthers and stigmas within flowers) because of limited water availability (Kay and Picklum, 2013) or a reduction in dichogamy (a condition where male and female reproductive parts of a flower mature at different times to avoid automatic self-pollination) (Opedal et al., 2016). The decrease in selfing could be because the species was already near the limit of autogamous seed production and extra stress led to a lowered seed set due to resource limitation (Kay and Picklum, 2013). Increased selfing in the face of drought could promote reproductive assurance (Kay and Picklum, 2013). If drought also leads to smaller floral displays, low plant density and plant height, fewer floral rewards, and less attraction of pollinators, selfing will act as reproductive assurance when pollinators may be scarce as they may not be attracted to those plants and pollinator visitation may be low.

2.6. Conclusion

This review focused on the state and importance of South African grasslands, drought patterns in South Africa and the current research topics on grassland ecosystems. The importance of forb research and the potential impacts of drought on plant-pollinator interactions has been highlighted and to address this, the aim of this study is to determine the effects of moisture level on three forb species on floral and plant traits and to understand the effects of moisture levels on reproductive success.

2.7.References

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3. THE EFFECTS OF DROUGHT ON PLANT TRAITS AND FLORAL VISITATION OF THREE GRASSLAND FORB SPECIES

3.1. Abstract

Grasslands are one of the world's largest biomes and provide a myriad of ecosystem services. These systems are sensitive to changes in abiotic factors. Seasonal rainfall is likely to decrease because of climate change and understanding how this will influence plant-pollinator relationships is important for maintaining ecosystem functioning and food security. Forbs are recognised for their role in grassland ecosystem resilience and biodiversity. Although there has been research on the effects of drought on forb biomass productivity, there is still a knowledge gap on the effects of drought on forb reproduction. This study aimed to investigate the effects of experimental drought on the pollination success of three grassland forbs (*Cephalaria pungens, Senecio coronatus, Thunbergia atriplicifolia* at Ukulinga Research Farm (URF) in Pietermaritzburg, South Africa. This experiment was conducted from 2021-2023. Plant height, number of leaves, floral display and flower phenology were measured weekly the 29th of October until the 3rd of March 2022 on four individuals per plot per treatment of each species (50% rain, 100% rain and 150% rain), therefore twelve individuals per treatment. Population size, flower density, pollinator visitation frequency and flowering plant density were measured biweekly from the 19th of October 2021 until the 4th of March 2022. for each species in each plot. Plant height was lower in the 50% rain treatment for all three species. The 50% rain treatment reduced the number of leaves for *T. atriplicifolia* but did not affect the number of leaves for *C. pungens*. Unexpectedly, the 50% rain treatment increased the number of leaves for *S. coronatus*. The 50% rain treatment had no effect on the floral display, onset and duration of flowering and number of flowers per plot for *C. pungens* and *T. atriplicifolia*. *Senecio coronatus* did not flower during the duration of the sampling period. The plant density (number of plants per m^2) was lower in the 50% rain treatment for *C. pungens* and *S. coronatus* with no difference in plant density across treatments for *T. atriplicifolia*. No floral visitors were observed for *T. atriplicifolia* in all treatments. The dominant potential pollinator to *C. pungens* was from the order Hymenoptera. Moisture level did not affect the pollinator visitation rate to plants (number of visits per minute per plant), bee visitation (the number of individuals of bees per minute), the overall visitor rate (total number of visitors per minute) and the duration of visits for bees visiting *C. pungens*. The results of this study indicated that a decrease in moisture level may negatively impact plant-pollinator interactions by affecting plant height and plant densities, and thus flower abundance, which is important for pollinator attraction, but responses are species-specific and the effects of drought on floral rewards needs further investigation to fully interpret the findings of studies of this kind.

Keywords: floral display, plant height, drought, flowering phenology

3.2.Introduction

Many flowering plants need insects or other animals for pollination (Ollerton et al., 2011; Ratto et al., 2018) including common crops (Rering et al., 2020; Stoner, 2020). Some studies have shown that excluding pollinators in animal-pollinated plants may reduce fruit and seed production (Ratto et al., 2018; Zou et al., 2017) since pollen deposition is reduced (Sakamoto et al., 2012), and this could lead to low plant fitness and possibly local extinction. In South Africa, approximately 95% of grassland forbs need pollinators for reproductive success (Carbutt and Kirkman, 2022), therefore understanding changes in plant-pollinator relationships is important for South African grassland biodiversity conservation.

Pollinator behaviour is influenced by plant and floral traits (Barber and Gorden, 2013; Grindeland et al., 2005). For example, bees prefer larger floral displays for *Mimulus ringens* L. (Mitchell et al., 2004). Plants in low densities are thus likely to receive lower pollinator visitation rates (Kunin, 1993), and reciprocally an increase in the number of flowers per plant is found to increase pollinator visitation (Rering et al., 2020). Other floral traits that increase pollinator visitation frequency include increased floral height, nectar sugar concentration and pollen production (Fornoff et al., 2017). Plant height has been shown to have a direct positive effect on pollinator visitation rate and an indirect positive effect on pollen receipt (Engel and Irwin, 2003) which could be why taller plants may also have a higher genetic diversity since they experience higher rates of outcrossing (Carromero and Hamrick, 2005). The timing of flowering can also influence pollinator behaviour with early or late flowering resulting in a phenological mismatch, particularly for spring ephemerals (Gallagher and Campbell, 2020; Kudo and Ida, 2013). For perennial plants, species that flower earlier than usual may receive more visits from pollinators while those that flower at the usual time may receive fewer visits (Rafferty and Ives, 2011). Therefore, investigating how climate change will influence floral and vegetative traits that determine pollinator attraction is important for understanding how climate change will affect pollination services (Glenny et al., 2018).

Generally, drought reduces plant height, nectar volume, flower number and proportion of flowers producing nectar (Descamps et al., 2021c; Jaworski et al., 2022; Phillips et al., 2018) but its effects on flowering time seem to be varied. In some cases drought advances flowering (Cho et al., 2017), while in other instances it delays flowering (Kang and Futakuchi, 2019). The impacts of drought on plant and floral traits may be more important during the growing season since it may cause a reduction in plant size and inflorescence production (Thomson et al., 2018). Even though climate change models have predicted that there will be a decrease in seasonal precipitation in southern Africa (Arnell, 2007), little is known about the effects of varying levels of drought on grassland forb floral and plant traits, and the consequences of this on pollinator visitation which will affect seed production and ultimately biodiversity.

In this chapter, I investigated the impacts of experimental drought on the pollination success of three common grassland forbs. Specifically, I tested: how varying moisture level affects 1) plant and floral traits by measuring its effect on plant height, number of leaves, floral display (number of open flowers), and plant and flower density; (2) flowering phenology over time by measuring the duration and onset of flowering and the number of flowers produced per plants; and (3) pollinator visitation in terms of number and length of insect visits per flower/inflorescence.

3.3.Methods and Materials

3.3.1. Study Site

The study was conducted at Ukulinga Research Farm (URF), University of KwaZulu-Natal, Pietermaritzburg, South Africa (30°24’S, 29°24’E), during the 2021/2022 and 2022/2023 summer flowering seasons (October – March each season). Ukulinga is classified as a mesic grassland and has a humid subtropical climate with a mean annual precipitation of 694 mm, falling mostly as convective storms during summer (September to April) (Fynn et al., 2004). The total rainfall from October 2021 – March 2022 is shown in Fig. 3-1. It is situated at an altitude of 721 m asl and experiences warm to hot summers, with a mean maximum of 26.4 °C in February, and mild winters, with a mean maximum of 13.2 °C in July with occasional frost (Kirkman et al., 2014). The soils are acidic and are classified as Westleigh and Mispah forms derived mostly from Ecca group shale of the Karoo sedimentary sequence (Chamane et al., 2017).

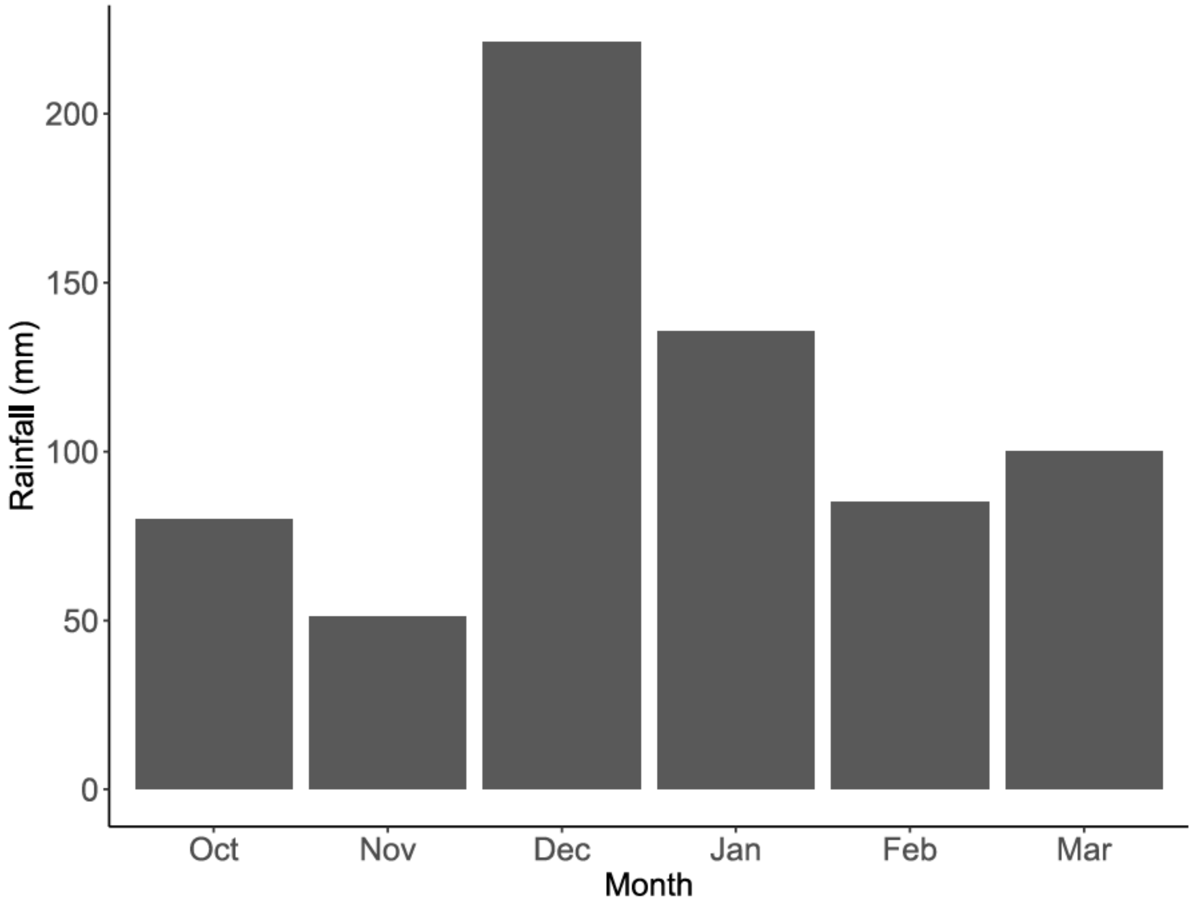

Figure 3-1*:* Total monthly rainfall during the October 2021 - March 2022 study period, obtained from the Ukulinga weather station.

The vegetation is described as Southern Tall Grassveld (Acocks, 1988) or KwaZulu-Natal Hinterland Thornveld of the sub-escarpment savanna (Mucina and Rutherford, 2006), which at Ukulinga is a tall grassland consisting of the grasses *Hyparrhenia hirta* (L.) Stapf, *Panicum maximum* Jacq., *Themeda triandra* Forssk. and *Tristachya leucothrix* Trin. ex Nees with scattered *Vachellia nilotica* (L.) P.J.H.Hurter & Mabb. subsp. kraussiana (Benth.) Kyal. & Boatwr. and *Vachellia sieberiana* (DC.) Kyal. & Boatwr. var. woodii (Burtt Davy) Kyal. & Boatwr. trees. Some common forb species are *Cephalaria pungens* Szabó*, Eriosema* sp, *Helichrysum nudifolium* (L.) Less. var. *pilosellum* (L.f.) Beentje, *Ipomoea oenotheroides* (L.f.)

Raf. ex Hallier f., *Scabiosa* L. sp, *Senecio coronatus* (Thunb.) Harv., and *Thunbergia atriplicifolia* E.Mey. ex Nees.

3.3.2. Experimental Design

An experimental rainfall trial (Drought-Net, (Smith et al., 2024b)) was arranged in a randomised complete block design made up of three moisture levels (50%, 100% and 150%) replicated three times giving a total of nine plots. Each plot covered an area of 3 x 5 m (Fig. 3-2). Each drought plot had a large rainfall exclusion shelter installed, made from clear corrugated plastic sheeting, which intercepted 50% of incoming rainfall, this was for the 50% moisture level. The rainfall captured from the drought treatments was channelled to run-on treatments through gutters and PVC pipes and increased the annual precipitation by ~50% relative to ambient amounts, this was for the 150% moisture level. The boundary of each plot was trenched to a depth of 0.5 m and lined with 1 mm thick plastic to hydrologically isolate the plots. This trial has been running since the winter of 2019 and the rainfall treatments were applied from October 2019.

Figure 3-2: Drought-Net rain manipulation trial at Ukulinga Research Farm, Pietermaritzburg, South Africa (30°24’S, 29°24’E).

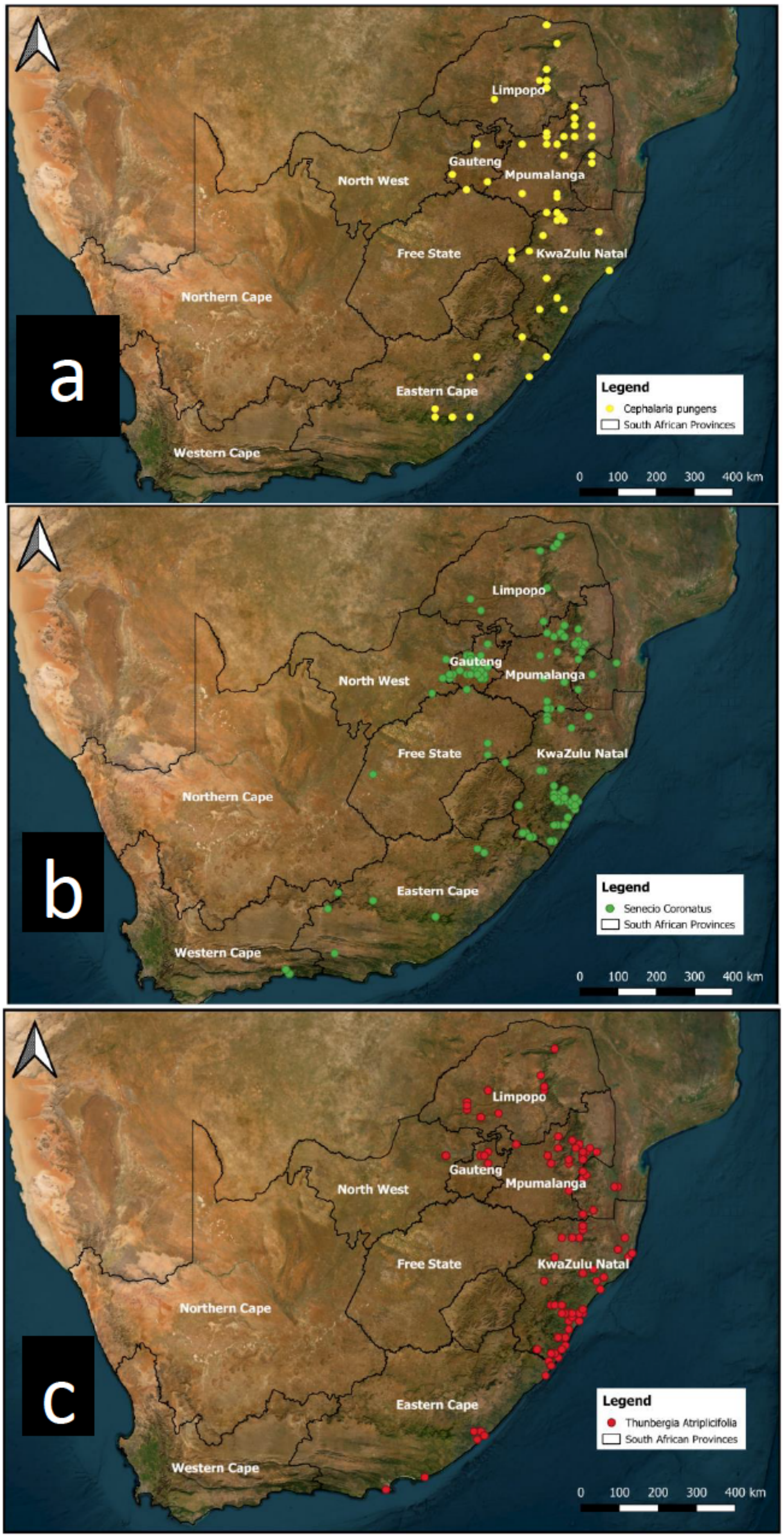

Figure 3-3: South African distribution of: (a) *Cephalaria pungens* (yellow dots), (b) *Senecio coronatus* (green dots) and (c) *Thunbergia atriplicifolia* (red dots) (GBIF, 2023).

3.3.3. Study Species

Three forb species were selected for this study due to their common occurrence in each plot over the three moisture levels and flower size facilitating regular counting. These were *Cephalaria pungens* Szabó, *Senecio coronatus* (Thunb.) Harv., and *Thunbergia atriplicifolia* E. Mey. ex Nees. All three of the species chosen are native to South Africa and are entomophilous (pollinated by insects). Flowering for all focal species is not reliant on fire, however, *T. atriplicifolia* flowers abundantly after fire (Tshitereke, 2024a) and *S. coronatus* is frequent in recently burnt areas (Molele and Koekemoer, 2023). Smoke-water has been shown to promote seed germination in *C. pungens*, with the total number of seedlings being higher in smoke-water treated seeds compared to untreated seeds (Ghebrehiwot, 2010). They are also perennial forbs with a strong dependence on resprouting from a belowground bud-bank.

Cephalaria pungens (family Dipsacaceae), commonly known as Common Mock Scabious, is a perennial herb that occurs in damp grasslands and on forest margins. The flowerheads are between 20 – 40 mm in diameter. This species flowers between January and March (Pooley, 2005a). In South Africa, it is found in the Eastern Cape, Free State, Gauteng, KwaZulu-Natal, Limpopo, and Mpumalanga (Fig. 3-3a). Outside South Africa it occurs in Kenya, Tanzania, Zambia, Zimbabwe, Mozambique, Malawi, and Swaziland. The flowering stem is between 1 – 1.5 m high. The leaves are opposite and have a very conspicuous middle vein. The inflorescences have numerous, densely arranged, tubular flowers that are cream, white, or pale yellow (Ngwenya, 2017a). The flowers have four anthers and are short-styled. Each flower contains one ovule. It is one of the most dominant forb species at URF (Zama et al., 2022). The fruits are small, with fine hairs, and 4 mm long (Ngwenya, 2017b).

Senecio coronatus (family Asteraceae), commonly known as Woolly Grassland Senecio, grows in grasslands and on ultramafic outcrops of the Barberton Greenstone Belt (Mesjasz-Przybyłowicz & Przybyłowicz, 2020). It is native to Southern Africa (Fig. 3-3b), starting from the Western Cape and occurring north to Tanzania, Zambia and Angola (Hillard, 1977). It flowers from August to December (Pooley, 2005b) and is a self-compatible, herbaceous perennial with a shortened, upright, subterranean stem that produces long (up to 20 cm), broad, simple and fleshy leaves aboveground and relatively fleshy roots belowground (Boyd et al., 2008). *Senecio coronatus* has a capitulum-type inflorescence composed of 10 - 12 ray florets and disc florets that are yellow (Beentjie et al., 2005). The ray florets are hermaphroditic. The pollen grains are yellow, and the fruits are cylindrical and narrow with a dense pappus.

Thunbergia atriplicifolia (family Acanthaceae), commonly known as Natal primrose, is a grassland species native to South Africa and Swaziland (Fig. 3-3c). It is a shrublet with pubescent stems and leaves (Balkwill & Campbell-Young, 1999) with a tendency to twine. Flowers of *T. atriplicifolia* are white to cream-white, or yellow, and open for up to five days (Msweli et al., 2018). Its flowering period is from November – early May (Msweli et al., 2018). The inner surface of the corolla tube is yellow (Retief and Reyneke, 1984). The flowers have five lobes and four stamens in pairs. *Thunbergia atriplicifolia* is hermaphroditic and self-compatible but mainly allogamous, and pollinated by generalist solitary bees that belong to the families Apidae and Halictidae (Msweli et al., 2018). The pollen grains are yellow, and the fruit is a capsule with a globose base, about 15-19 mm long, releasing greyish or reddish brown seeds with trichomes (Tshitereke, 2024b).

3.3.4. Assessment of Plant and Floral Traits

To assess the effects of drought on floral visitation and potential pollination success, several plant and floral traits were measured regularly for the three focal species in each plot in each treatment. Four individuals of each species were tagged per plot per treatment, totalling 12 individuals of each species per treatment. The individuals were tagged using 3 different colours of wires to mark the different species, and a white tag that was labelled with the number from 1-4, so that measurements were done on the same individual each week from the 29th of October 2021 until the 4th of March 2022. In total, 108 plants were used for weekly surveys (four individuals per species per plot) in which the following were measured: plant height, number of leaves, and floral display. Flowering phenology, floral density and plant density measurements were recorded for all plants of each species in each plot of each treatment.

3.3.4.1. Plant height

Plant height was measured because plant growth and development are affected by drought and thus may influence pollinator visitation rates (Engel & Irwin, 2003) through the degree of visibility to floral visitors. Plant height (in cm) was measured weekly and taken as the distance from the surface of the soil to the tallest inflorescence on each plant in each of the plots. Height was measured and averaged over tagged individuals of each species in each plot for each week from the 29th of October 2021 until the 3rd of March 2022.

3.3.4.2. Number of leaves

As with plant height, the total number of leaves per tagged plant of each focal species were counted weekly from the 29th of October 2021 until the 3rd of March 2022in each plot. This gives an indication of whether moisture level has affected growth and photosynthetic outputs of the plants.

3.3.4.3. Floral display

Floral display affects pollinator attraction (Engel and Irwin, 2003; Glenny et al., 2018) and therefore I measured floral display weekly from the 29th of October 2021 until the 3rd of March 2022, as the total number of open flowers per plant for *T. atriplicifolia* (Burkle and Runyon, 2016; Glenny et al., 2018; Thompson, 2001) and the total number of open flowerheads per plant for *C. pungens*. *Senecio coronatus* did not flower during the sampling period.

3.3.4.3. Flowering phenology

The duration of flowering was determined by noting the first day that flowers opened and the last open flower to remain for the focal plants. The following were recorded each week for each focal plant: the date, number of flower/flowerhead buds; the number of open flowers/open flowerheads; senescence. The following flowering stages were recorded: fully open (all flowers in a flowerhead with dehisced anthers); senescence; and setting fruit. If setting fruit, the fruits or infructescences were bagged with fine mesh bags to contain the fruits (for tagged and non-tagged plants). All the fruits were bagged per plot for *T. atriplicifolia*, and five flowerheads per plant per plot were bagged for *C. pungens* because *C. pungens* plants consist of several shoots with dense inflorescences with ca. 50 flowers clumped at the end of the branches. Flowering and fruiting were not measured for *S. coronatus* as its flowering period fell outside the weekly sampling surveys, and resources and capacity for starting the sampling period from August were limited and it's possible that *S. coronatus* flowers after a fire event. Fruits of *S. coronatus* were collected in the second season from plants in each treatment for germination trials (see Chapter 4). Mature seeds were harvested when they lost their green colour (Efisue et al., 2008). Once the fruits had released their seeds in the mesh bags, they were transferred to paper envelopes, a separate envelope for each plant stored in a fridge at 5°C until seeds were used for determining seed set, viability, and seed germination measurements (see Chapter 4). The onset of flowering (when the first flower opened on each focal plant) was calculated as the number of weeks from the first day of sampling (19th of October 2021).

3.3.4.4. Floral and plant density

Since both floral and plant density affect pollinator visitation (Essenberg, 2012; Grindeland et al., 2005), additional biweekly measurements were conducted on each plot for all individuals of each focal species. For the focal species, the following were determined during each survey: the total number of plants (population size) and the total number of flowers/flowering inflorescences per plot (i.e. floral density). Plant density was measured as the number of individuals per square metre within each plot of 3x5 m. (Disclaimer:The differences in density may reflect natural variation in density in the plots from the start of the experiment and not necessarily the treatment effects). To determine the effect of drought on floral density of each species, the total number of flowers/flowering inflorescences per 3x5 m plot was counted every two weeks from November 2021 – March 2022.

3.3.5. Pollinator Visitation Frequency

To examine the effects of drought stress on pollinator attraction, visitation frequency of potential pollinators (i.e., number of visits a flower received during a set observation period) was observed. A visit was recorded as any instance in which an insect encountered the reproductive parts of the flower (stamen and/or stigma). Plot-based flower visitor observations in units of 30 min were conducted. The length of each visit within the observation periods was also noted. Visits were recorded during sunny days at peak pollinator activity times (between 10:00 – 16:00 hr) (Johnson et al., 2012; van der Niet et al., 2020) for a month. During a 30-minute observation period, the observer stayed stationary at the edge of the plot and recorded the number of visits to each of the focal flowering plant species within their view (Benadi and Pauw, 2018). For each insect visiting flowers, the number of flowers probed, and the time spent on each flower in each plot was recorded. The visitation duration, number of visits per plant per minute, total number of visits per plant per observation period, and number of visits per flower per plant was measured for each focal plant species in each plot and treatment.

3.3.6. Statistical analysis

All statistical analyses were performed using R Studio, version 4.2.2 (R Core Team, 2022). Before statistical analyses were conducted, the conditions of normality were tested by using a histogram of the residuals. Data that were not normal were transformed as necessary and this is indicated below.

To investigate the relationship between moisture level and plant height for each plant species, linear mixed models (LMM) were used. These models were used because there was more than one source of variability and the dependent variables involved repeated measures taken over time in different plots and ‘weeks’ (added as a random effect). The models were run using the lme4 R package (Bates et al., 2014) for *S. coronatus* and *T. atriplicifolia*. *Cephalaria pungens* plant height data was log transformed. Transformation of the data did not lead to normality but the sample size was too small to run any other more appropriate model. Thus an LMM was retained. A posthoc test to examine differences between 50% rain, 100% and 150% rain within each plant species was done using the emmeans package (Lenth, 2023).

A generalised linear mixed model (GLMM) with negative binomial error distribution and a log link function for *C. pungens* and *T. atriplicifolia* was used to analyse the effect of moisture level on number of leaves due to overdispersion of count data. A GLMM with a Poisson distribution was used for *S. coronatus*. The number of weeks was added as a random effect. Plot number and plant number nested within plot number were added as random effects but were removed from the final analyses since models were over-fitting.

The effect of moisture level on floral display was analysed using a GLMM with a negative binomial error distribution and a log link function for *T. atriplicifolia*, due to overdispersion of count data. The day of sampling was included as a random effect. For *C. pungens* I ran a zero-inflated model because the number of observed zeros was greater than the number of predicted zeros for the GLMM and the day of sampling was included as a random effect. The effects of moisture level on onset and duration of flowering were analysed using an ANOVA.

For floral density (i.e., number of flowers per plot for *T. atriplicifolia* and number of open flowerheads per plot for *C. pungens*), a GLMM with a Poisson distribution was first run, and then the model was checked for zero inflations. It was found that the model was underfitting the zeros, so a negative binomial was run because when the model is underfitting zeros, it is recommended to use negative binomial or zero-inflated models (Hoef and Boveng, 2007; Ismail and Jemain, 2007).

Plant density (number of plants per m^2) for *C. pungens* and *T. atriplicifolia*, was log transformed and a linear mixed model was run. Transformation of the data did not lead to normality but the sample size was too small to run any other more appropriate model. Thus an LMM was retained.

To determine the effects of moisture level on the duration of visit for bees, the data was square root transformed and then a one-way ANOVA was run after the assumption of normality and equal variance was satisfied. To determine the effects of moisture level on the number of individuals of bees per minute and the total number of visitors per minute, I ran an ANOVA. An ANOVA was also run for the number of visits per minute per plant and the number of flowerheads visited per minute. One spider was recorded for *C. pungens* but was excluded from the visitation analysis.

3.4.Results

3.4.1. Plant Height

The heights of all three focal plant species were significantly shorter in the 50% rain treatment compared to the 100% and 150% rain treatments (Table 3-2, Fig. 3-4). The plant height in the 150% rain treatment was also significantly lower (Table 3-2) compared to the 100% rain treatment for *T. atriplicifolia*.

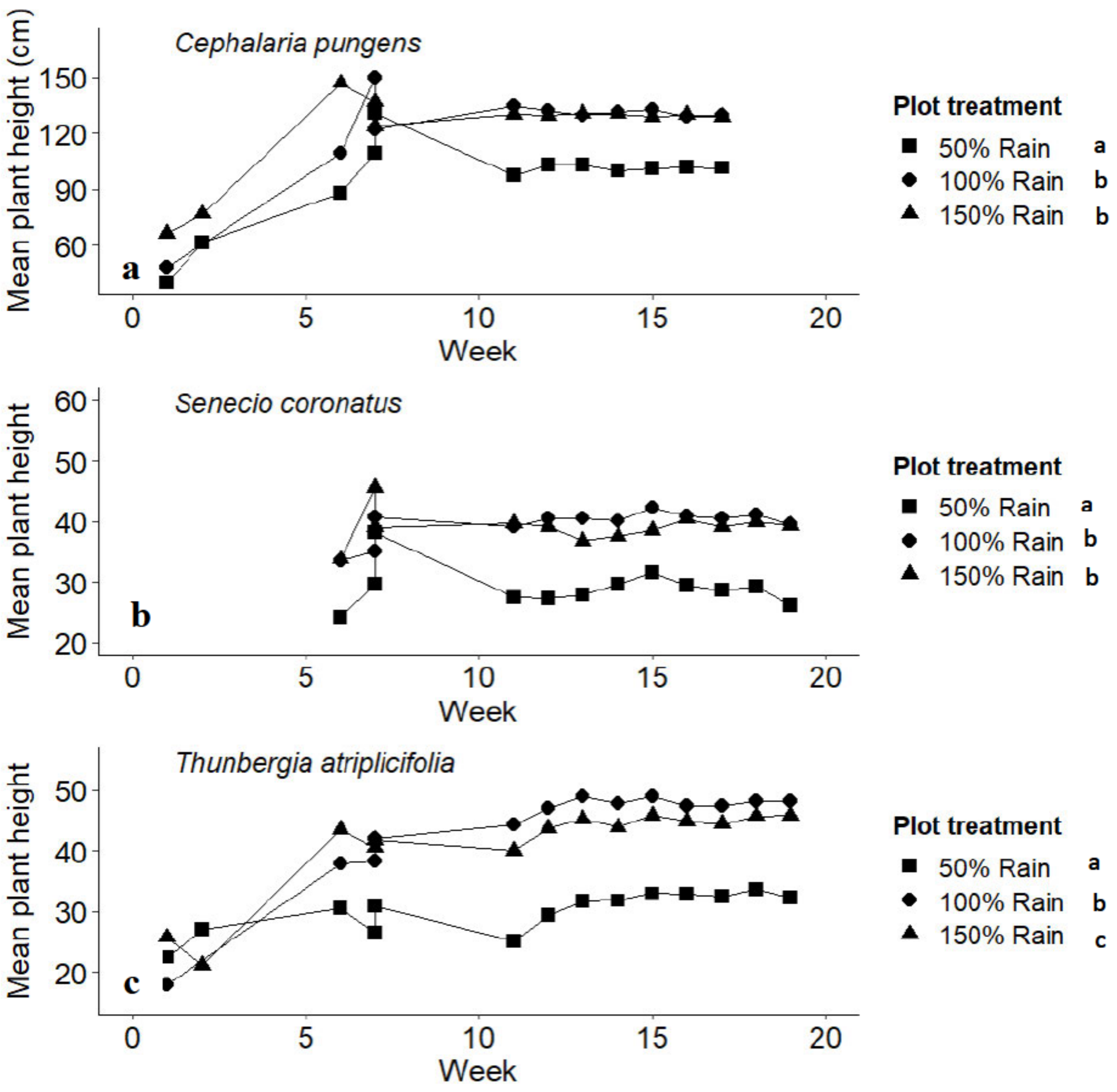

Figure 3-4: The effect of moisture level (50% rain, 100% rain and 150% rain) on plant height for each focal species a) Cephalaria pungens*, (B)* Senecio coronatus*, and (C)* Thunbergia atriplicifolia *at Ukulinga Research Farm from the 29th of October 2021 until the 3rd of March 2022. Different lowercase letters next to the key indicate significant differences between treatments according to GLMM analyses.*

3.4.2. Number of Leaves

Plants in the 50% rain treatment had significantly fewer leaves than plants in the 100% and 150% rain treatments for *T. atriplicifolia* (Table 3-2, Fig.3-5a). There were no significant differences in the number of leaves in 150% rain treatment compared to the 100% rain treatment (Table 3-2). In contrast, plants in the 50% rain treatment had significantly more leaves for *S. coronatus* compared to the 100% rain and the 150% rain treatment (Table 3-2; Fig.3-5c). Moisture level had no effect on the number of leaves for *C. pungens* (Table 3-2; Fig.3-5b).

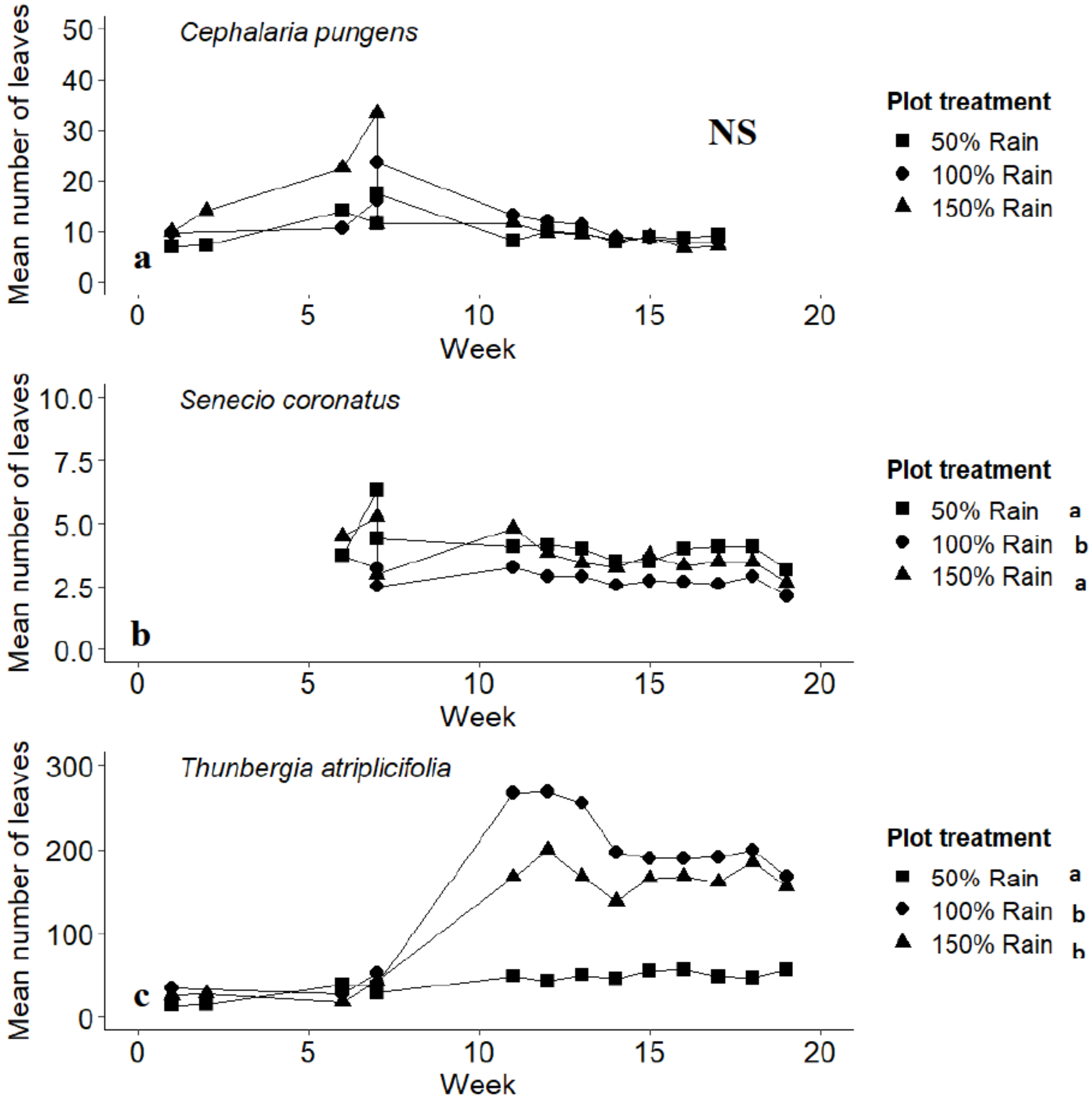

Figure 3-5: Effect of moisture level (50% rain, 100% rain and 150% rain) on the number of leaves for each focal species a) Cephalaria pungens, *b)* Senecio coronatus *and C)* Thunbergia atriplicifolia *at Ukulinga Research Farm from the 29th of October 2021 until the 3rd of March 2022. Different lowercase letters next to the key indicate significant differences between treatments according to GLMM analyses.*

3.4.3. Flowering Phenology

Moisture level did not affect the duration ($F_{2,6} = 0.333$, p = 0.729) and onset of flowering for *C. pungens* ($F_{2,6} = 0.135$, p = 0.877). From the start of the sampling period, *C. pungens* started to flower after a mean ± SE of 8 ± 1 weeks in the 50% rain treatment, 7 ± 1 weeks in the 100% rain and 7 ± 1 weeks in the 150% rain. The duration of flowering for *C. pungens* was a mean ± SE of 4.7 ± 0.88 weeks in the 50% rain, 5 ± 1 weeks in the 100% rain and 4 ± 2 weeks in the 150% rain. In the 50% rain, *C. pungens* flowered from 29 November 2021 (week 7) until 26 January 2022 (week 13), with a peak at 22 December 2021 (week 10; Fig. 3-6). In the 100% and 150% rain, the species flowered from 29 November 2021 until 11 January 2022, and flowering also peaked at 22 December 2021. *Cephalaria pungens* set fruit over a month from 5 January until 2 February 2022 for all three moisture levels. Plants of *C. pungens* started to senesce from 6 December 2021 (week 8) until 29 January 2022 (week 15) in the 50% rain, from 11 December 2021 (week 8) until 14 January 2022 (week 13) in the 100% rain, and from 5 January (week 12) until 14 January 2022 (week 13) in the 150% rain.

Moisture level did not affect the duration of flowering ($F_{2,5} = 1.502$, p = 0.308) and onset of flowering for *T. atriplicifolia* ($F_{2,5} =1.324$, p = 0.346). From the start of the sampling period, *T. atriplicifolia* took a mean ± SE of 8 ± 7 weeks in the 50% rain, 1 ± 0 weeks in the 100% rain and 3.5 ± 2.5 weeks in the 150% rain to start flowering. Mean (±SE) duration of flowering for *T. atriplicifolia* was 6 ± 5 weeks in the 50% rain, 5.3 ± 3.8 weeks in the 100% rain and 6 ± 2 weeks in the 150% rain (Fig. 3-7). *Thunbergia atriplicifolia* started to set fruit on 29 January until 15 February 2022 in the 50% rain, 14 January until 15 February 2022 in the 100% rain, and from 14 January until 29 January 2022 in the 150% rain. Only the flowering and fruiting were recorded for *T. atriplicifolia.*

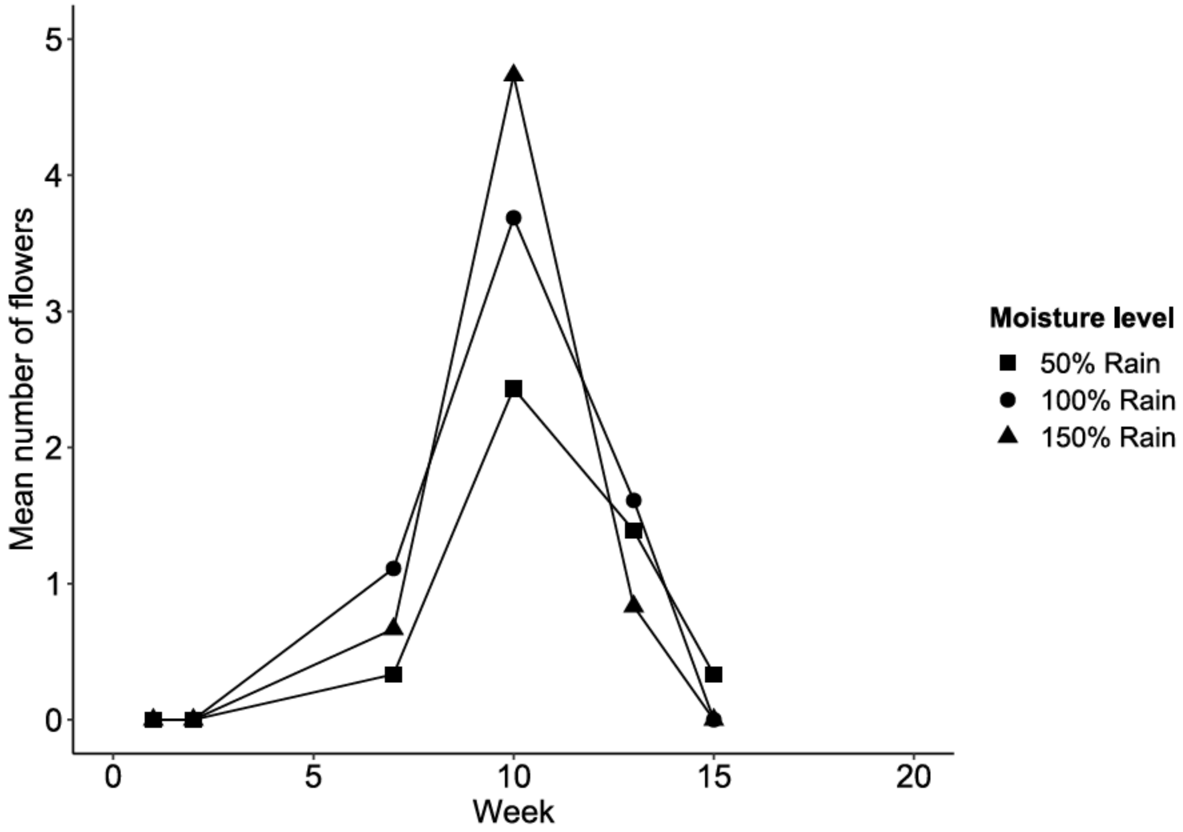

Figure 3-6: Mean number of flowers for Cephalaria pungens *at each moisture level at Ukulinga Research Farm from the 29th of October 2021 until the 3rd of March 2022.*

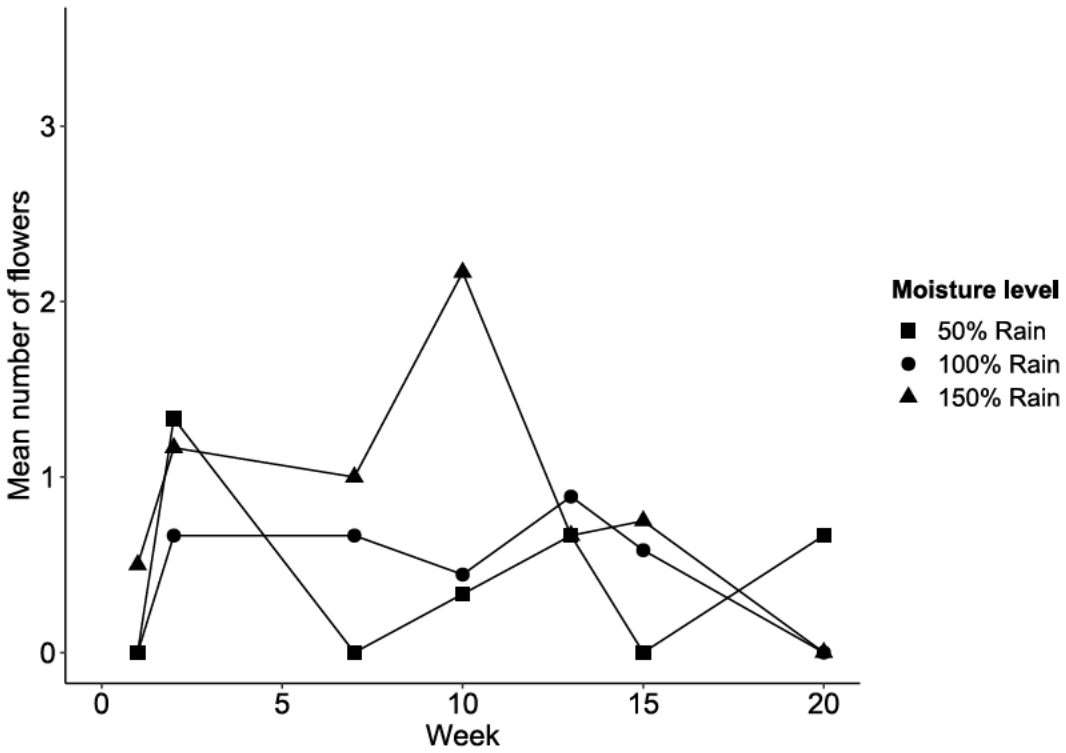

Figure 3-7: Mean number of open flowers for Thunbergia atriplicifolia *in each moisture level at Ukulinga Research Farm from the 29th of October 2021 until the 3rd of March 2022.*

3.4.4. Floral and Plant Density

Plant density (number of plants per m^2 per plot) was significantly lower in the 50% rain treatment relative to the 100% rain for *C. pungens* (Table 3-2, Fig.3-8), however, no significant changes were observed for *S. coronatus* or *T. atriplicifolia* (Table 3-2, Fig 3-8). Plant density in the 150% rain was not significantly different to the 100% rain for all three focal species. Moisture level did not affect floral display (number of open flowers per plant), nor floral density, for both *T. atriplicifolia* and *C. pungens* (Table 3-1 & 3-2). Floral density in the 150% rain was not significantly different to the 100% rain for *C. pungens* and *T. atriplicifolia.*

Table 3-1: Mean and SE of Thunbergia atriplicifolia *and* Cephalaria pungens *for floral display and floral density.*

		Cephalaria pungens	*Thunbergia atriplicifolia*
Response variable	**Moisture level**	**Mean ± SE**	**Mean ± SE**
Floral display	50% Rain	0.96±0.22	0.87±0.18
	100% Rain	1.06±0.36	0.83±0.15
	150% Rain	1.05±0.22	0.89±0.14
Floral density	50% Rain	0.45±0.15	0.3±0.0.09
	100% Rain	0.59±0.22	0.27±0.08
	150% Rain	0.64±0.37	0.38±0.12

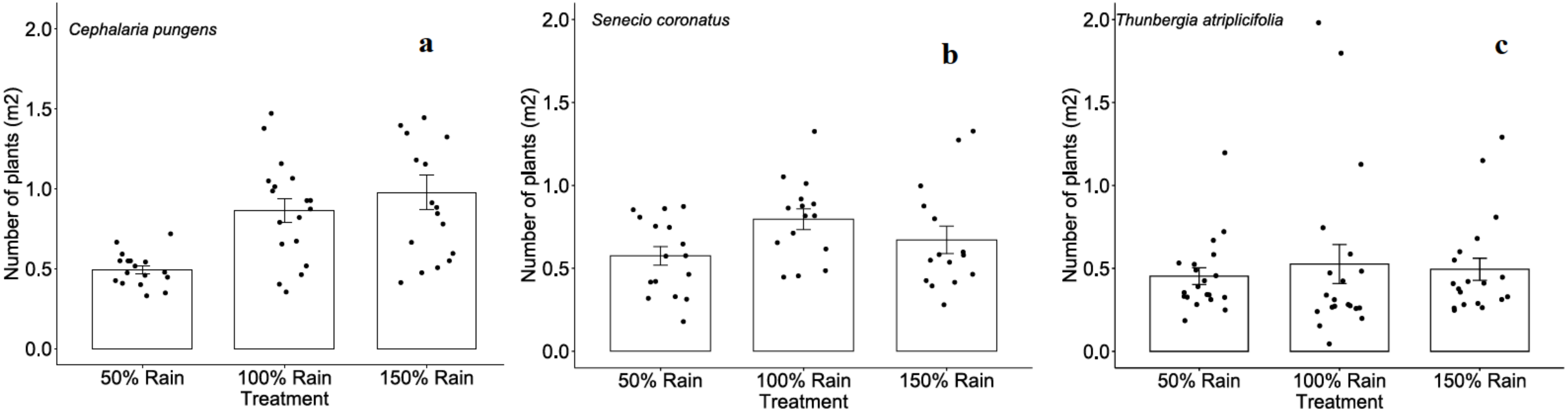

Figure 3-8: Effects of moisture level (50% rain, 100% rain and 150% rain) on plant density of (a) Cephalaria pungens*, (b)* Senecio coronatus *and (c)* Thunbergia atriplicifolia *at Ukulinga Research Farm.*

Table 3-2: Statistical model estimates and SE showing the effects of moisture level (50% rain, 100% rain and 150% rain) on plant height, number of leaves, floral display (number of open flowers/number of flowering plants), floral density (number of open flowers/plot area), and plant density (number of focal plants/plot) for three grassland forb species. Bold denotes statistical significance.

		Thunbergia atriplicifolia				***Cephalaria pungens***				***Senecio coronatus***			
Response variable	**Explanatory variable**	**Estimate**	**±SE**	**Z**	**P**	**Estimate**	**±SE**	**Z**	**P**	**Estimate**	**±SE**	**Z**	**P**
Height	50% Rain – 100% Rain	-14.47	0.82	-17.54	**<0.0001**	-28.45	2.78	-10.25	**<0.0001**	-11.94	0.99	-12.08	**<0.0001**
	150% rain – 100% Rain	-2.36	0.82	-2.87	**<0.01**	-1.68	2.76	-0.61	0.544	-1.21	1	-1.2	0.23
	150% Rain – 50% Rain	12.1	0.82	14.79	**<0.0001**	26.77	2.73	9.79	**<0.0001**	10.73	0.99	10.83	**<0.0001**
Leaves	50% Rain – 100% Rain	-0.82	0.11	-7.45	**<0.0001**	-0.13	0.07	-1.74	0.25	0.33	0.07	4.62	**<0.0001**
	150% rain – 100% Rain	-0.21	0.11	-1.94	0.053	-0.06	0.07	-0.87	0.75	0.27	0.07	3.66	**<0.0001**
	150% Rain – 50% Rain	0.61	0.11	5.59	**<0.0001**	0.06	0.07	0.88	0.75	-0.06	0.07	-0.94	0.35
Floral density	50% Rain – 100% Rain	-0.38	0.9	-0.42	0.68	0.09	1	0.087	0.931				
	150% rain – 100% Rain	1.30	0.65	1.99	0.09	1.50	0.78	1.92	0.164				
	150% Rain – 50% Rain	1.68	0.77	2.19	0.08	1.41	0.78	1.807	0.164				
Floral display	50% Rain – 100% Rain	-0.16	0.358	-0.45	0.65	-0.33	0.3	-1.1	0.813				
	150% rain – 100% Rain	0.57	0.358	1.59	0.22	-0.02	0.3	-0.07	0.942				
	150% Rain – 50% Rain	0.73	0.358	2.05	0.12	0.31	0.3	1.01	0.813				
Plant density	50% Rain – 100% Rain	0.16	0.15	1.03	0.91	-0.52	0.112	-4.69	**<0.0001**	-0.23	0.09	-2.73	**0.019**
	150% rain – 100% Rain	0.10	0.148	0.68	0.98	0.079	0.112	0.77	0.48	-0.12	0.09	-1.44	0.29
	150% Rain – 50% Rain	-0.05	0.15	-0.36	0.98	0.60	0.113	-5.33	**<0.0001**	-0.11	0.09	-1.26	0.29

3.4.5. Pollinator Visitation Frequency

No visitors were observed for *T. atriplicifolia* in all moisture levels. All pollinator visitation measures described below are thus only for *C. pungens*. Moisture level did not affect the number of visits per minute plant ($F_{2,24}$=1.083, p-value =0.354) or the total number of visitors per minute ($F_{2,24}$=1.083, p-value =0.354; disclaimer: F and p-values are similar for these tests despite different within and between sum of squares values for each test).

A total of 38 visitors were recorded for *C. pungens* belonging to 4 families (Apidae, Thomisidae, Bombyliidae and Theridiidae). The dominant potential pollinator was from the order Hymenoptera (n=34) compared to flies (n=3) and spiders (n=1). The total number of visitors to flowerheads per minute was highest in the 100% rain and lowest in the 50% rain (Table 3-3). Similarly, the number of individuals of flies and bees per minute was highest in the 100% rain and lowest in the 150% rain, there were no flies in the 50% rain. Statistically, however, the number of individuals of bees per minute did not significantly differ between moisture levels ($F_{2,24}$ = 0.484, p-value = 0.622; Table 3-3). The number of visits by flies and spiders was not analysed due to small sample sizes. Although not statistically significant but, the number of flowerheads visited per minute was highest in the 150% rain and lowest in the 50% rain ($F_{2,24}$=1.083, p-value =0.354, Table 3-3). The number of visits per minute per plant was highest in the 100% rain and lowest in the 50% rain (Table 3-3). The duration of visits for bees was highest in the 150% rain and lowest in the 100% rain ($F_{2,8}$=2.08, p-value =0.188, Table 3-3).

Table 3-3: Visitation rates and duration of insects visitors to flowerheads of Cephalaria pungens plants growing under the 50% rain, 100% rain and 150% rain treatments at Ukulinga Research Farm.

Measures	Moisture level treatment		
	50% rain (mean ± SE) (sample size)	100% rain (mean ± SE) (sample size)	150% Rain (mean ± SE) (sample size)
Number of visits per minute per plant	0.28±0.09 (10)	0.83±0.27 (7)	0.52±0.09 (10)
Number of flowerheads visited per minute	0.44±0.13 (10)	0.57±0.13 (7)	0.66±0.18 (10)
Number of bees per minute	0.22±0.06 (10)	0.31±0.1 (11)	0.26±0.05 (13)
Number of flies per minute	0	0.06±0.04 (2)	0.02±0.02 (1)
Number of spiders per minute	0.2 (1)	0	0
Total number of visitors per minute	0.22±0.06 (11)	0.37±0.1 (13)	0.28±0.05 (14)
Duration of visits for bees	0.85±0.08(4)	0.67±0.17(2)	2.43±0.95(5)

3.5.Discussion

This study tested the response of floral traits, plant traits and pollinator visitation of three grassland forb species to varying moisture levels that they are likely to encounter as a result of climate change where drought patterns are projected to be more frequent and intense in some regions (Mukherjee et al., 2018). The 50% rain moisture level decreased plant height for all the focal species.

Fifty percent rain had the greatest reducing effect on plant height for all species, a common adaptive strategy of water stress seen in a number of species (Glenny et al., 2018; Hussain et al., 2019; Kang and Futakuchi, 2019). This is likely due to a decrease in photosynthetic rate (Al-Ghzawi et al., 2009) and stem cells' water potential to a lower level needed for cell expansion and elongation which leads to shorter internodes and stem height (Anjum et al., 2017; Nezami et al., 2008). A decrease in plant height limits a plant's evaporation area and therefore water loss is minimized (Asghari et al., 2009).

Species responded differently to moisture levels, in terms of the number of leaves. The number of leaves for *T. atriplicifolia* reduced, possibly due to leaf senescence since dead leaves were observed. Leaf senescence is promoted by the phytohormone ethylene, which is stimulated by water stress. Furthermore, water stress causes plant growth inhibitors such as abscisic acid (ABA) to increase and growth regulator hormones to decrease (Nezami et al., 2008). ABA is known to promote leaf abscission and senescence (Song et al., 2016). By reducing the number of leaves, the plant limits its transpiration surface and therefore its water requirements (Descamps et al., 2021a).

Moisture level had no effect on the number of leaves for *C. pungens.* This may have been partly due to plants getting water through root systems that go deep beyond the subplot (Phillips et al., 2018). In contrast, the 50% rain increased the number of leaves for *S. coronatus*, potentially due to the presence of an underground rootstock that allows it to withstand and recover from drought conditions by using its substantial reserves of carbohydrates stored within these storage organs (Zwicke et al., 2015), and the leaves of *S. coronatus* are leathery therefore both these elements could act together to protect the plant from excessive dehydration (Qin et al., 2019). This together with the above-average rainfall during the season may have been the reason why this species was not experiencing water stress.

Moisture level did not affect both floral density and floral display for both *C. pungens* and *T. atriplicifolia*. This is in contrast to many previous studies that have shown decreases in floral display in response to a decrease in moisture level (Burkle and Runyon, 2016; Höfer et al., 2023; Rering et al., 2020). It is possible that the reduction in moisture level in this experiment was not enough to cause changes in floral display or because some rain may have been reaching the subplots as runoff, particularly during heavy rainfall events (Phillips et al., 2018). This may be due to the above-average annual rainfall in 2021-2022, resulting in the 50% rain not being intense enough to emulate drought conditions. However, it may be due to species resistance to climatic change as one greenhouse study has shown that floral traits remain relatively stable under decreased water availability (Höfer et al., 2023).

Most studies have found that plants respond to decreased water availability by flowering earlier and for longer (Dai et al., 2022; Jentsch et al., 2009; Vorkauf et al., 2021), but in our study, the onset of flowering was delayed for *T. atriplicifolia* and *C. pungens* in the 50% rain. These results agree with the conclusions reported by Castillioni et al. (2022) regarding the effects of precipitation on grassland flowering phenology, who concluded that for most species, decreased precipitation delayed flowering. This response depends on the species, species known to flower earlier flowering even earlier and late-flowering species flowering later (Castillioni et al., 2022). Delayed flowering is likely a result of prolonged dry conditions imposed by the 50% rain treatment. When the water availability is already very low, drought may have lowered normal ranges for development of reproductive tissues (Castillioni et al., 2022). Another reason for this delay could be an extended flower bud stage. Furthermore, it has been shown that increased drought stress can delay plant growth, which results in delayed reproduction. The duration of flowering was longer in the 150% rain compared to the 100% for *T. atriplicfolia*, but there were no treatment effects on *C. pungens*. A lengthened flowering period may promote increased pollinator visitation (Vorkauf et al., 2021), however, these results are in contrast to Jentsch et al. (2009) who found that heavy rainfall reduced flowering length. Since the results revealed that the date of peak flowering was the same for all three moisture levels, it is possible that the sampling design was not fine enough to detect differences in peak flowering.

The dominant potential pollinator for *C. pungens* was *A. mellifera* followed by flies and a spider. All the visitors touched the stigma and anthers except the spider, which touched the petals only. This finding suggests that bees could be the main potential pollinators for *C. pungens* which agrees with Ngwenya (2017) who also found that this species is pollinated by bees. For future studies, the measuring of pollen load on visitors may be useful for testing if these bees are just visitors or potential pollinators. Moisture level had no effect on the number of visits per minute per plant, the number of visits of individuals of bees per minute, the total number of visitors per minute, the number of flowerheads visited per minute, and the duration of visits by bees to *C. pungens*. These results were unexpected since many studies have shown that the visitation rate to flowers tends to be greater in high moisture levels than lower ones (Burkle and Runyon, 2016; Glenny et al., 2018; Höfer et al., 2021; Kuppler et al., 2021; Rering et al., 2020). However, since there were no differences in floral display and floral densities between moisture levels, it follows that the number of visits per flower would be the same between moisture levels, since changes in floral display and density are what mainly influences pollinator attraction (Kuppler et al., 2021). Besides plant height, and number of leaves, it is also possible that the sample size was too low to detect statistical significance in the 50% rain for the floral density, floral display and plant density or the plants were not experiencing water stress since there was above average rainfall during the sampling season. No visitors were recorded for *T. atriplicifolia* in this study. This is surprising since previous research has shown bees such as *Lasioglossum sp.* and *Allodapes sp.* to be frequent floral visitors for this species (Msweli et al., 2018) at Vernon Crookes Nature Reserve. However, it is possible that the differences in climate and plant composition between the two study sites may have contributed to the differences in pollinator abundance and pollinator diversity since areas with a high biodiversity are likely to receive an increased pollinator abundance and diversity (Macdonald et al., 2018). Another possibility is that some flowers of *T. atriplicifolia* may have a low nectar volume or sugar concentration needed to attract visitors because they are able to reproduce vegetatively. Despite the observation of pollen on anthers that could be a reward for bees, no visitors were seen and when the flowers were dissected, no nectar was visible.

This study aimed to understand the effects of moisture level on plant and floral traits as well as pollinator visitation. Floral rewards (nectar concentration and pollen production) were not included in this study due to the limited sample size, and the small flowers of *C. pungens* and *S. coronatus* and fragile flowers of *T. atriplicifolia* that made it almost impossible to draw up nectar for nectar volume and sugar concentration measurements. Future studies should focus more on how different moisture levels affect nectar volume and sugar concentration and pollen production to understand how changes in moisture available may affect floral rewards and ultimately plant-pollinator relationships. Based on the findings of the study, moisture level affected plant height for all three forbs, but the response in terms of the number of leaves and pollinator visitation rates were species-specific. These findings indicate that a sustained decrease in moisture level because of increased drought events in the future may result in an alteration of plant traits and pollinator visitation, which could lead to reduced pollen deposition and lower reproductive success of grassland forbs, while an increase in moisture level may yield insignificant effects on most vegetative and floral traits, or minimal and species-specific effects on these plant traits.

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4. THE EFFECTS OF DROUGHT ON THE REPRODUCTIVE SUCCESS OF THREE GRASSLAND FORBS

4.1. Abstract

Drought in South Africa is a frequent event, and climate change predictions have shown that there will be an increase in the duration and frequency of these events. Grasslands in South Africa are particularly prone to drought in the summer, during the growing season, which may impact seed production and seedling growth. A decrease in precipitation has been shown to decrease grassland gross primary production and grass cover. However, the influence of reduced moisture on seed production and germination in these systems is less understood. This study aimed to investigate how different moisture levels affect plant reproduction in three grassland forb species (*Cephalaria pungens, Senecio coronatus, and Thunbergia atriplicifolia)* at Ukulinga Research Farm, Pietermaritzburg, South Africa. Our experiment was conducted over two years, from 2021 – 2022. First, I determined the breeding system of *Cephalaria pungens*, which was unknown in the current published literature. Then I conducted a pollen limitation study, followed by an investigation on the effects of moisture levels on seed and fruit production, and seed viability. Finally, I examined the transgenerational effects of different moisture levels. *Cephalaria pungens* was found to be self-incompatible following hand-pollination with self-pollen but can set limited seed automatically in the absence of pollinators if bagged. It was found that *Cephalaria pungens* plants in the 50% rain were not pollen-limited as there was no significant difference between hand-cross-pollinated flowers in the 50% rain and naturally pollinated flowers in the 100% rain. There were no moisture level effects on average seed mass per infructescence and seed set for *C. pungens*. The average seed mass per moisture level was greatest in the 50% rain compared to the 150% rain for *T. atriplicifolia*. Moisture level did not affect the fruit and seed set for *T. atriplicifolia*. Moisture level did not affect the seed viability of *C. pungens. T. atriplicifolia* did not germinate in all the moisture levels. *C. pungens,* seed germination was highest at the 100% moisture level than at the 150% moisture level, there was no germination at the 50% moisture level. *Senecio coronatus* seed germination was highest in the 50% moisture level and lowest in the 100% moisture level. For *S. coronatus*, seedling height was significantly reduced in the 50% moisture level than in the 100% moisture level. Moisture level did not affect the number of leaves for *S. coronatus*. Biomass was highest in the 100% rain, followed by the 150% rain treatment and lowest in the 50% rain for *S. coronatus*. The results of this study show that grassland forb species have different responses to a decrease in moisture level, while some species may increase germination, others may not germinate at all indicating that some forbs may be drought tolerant. It was also found that the negative effects of a decrease in moisture level in the maternal plant may be passed on to the progeny which could decrease plant fitness of the next generation. Our results also suggest that a decrease in moisture level may have negligible influence on seed production, but important effects on seed quality and seedling development.

Keywords: seed set, seed viability, germination, pollen limitation, breeding system, seedling development, water stress

4.2.Introduction

Grasslands are known for their high plant and animal species richness (Poschlod et al., 1998) and forbs comprise more than 80% of the species richness in mesic grassland (Scott-Shaw & Morris, 2015). Climate change predictions have suggested that the whole of Africa is expected to experience an increase in drought risk between 2010 and 2100, with the central regions being more vulnerable than the northern and southern regions (Ahmadalipour et al., 2019). This is because increased temperatures in response to global warming led to more evaporation and drier conditions. If this happens more frequently and for longer periods, it can ultimately lead to an increase in the duration and intensity of drought (Trenberth, 2011). Furthermore, the resulting increase in water vapour in the atmosphere can increase the intensity and quantity of precipitation in some parts of Southern Africa (Tabari, 2020; Trenberth, 2011). While there are studies that have investigated how drought affects the photosynthetic efficiency of forbs (Fry et al., 2018), and the response and recovery of forb aboveground net primary productivity (ANPP) (Luo et al., 2023; Stampfli et al., 2021; Xu et al., 2021; Zhang et al., 2020), there are not many of studies that have looked at how drought affects forb reproduction. Therefore, it is important to understand how changes in precipitation may affect the reproduction of forbs to maintain current forb species richness so that grasslands can maintain optimal functionality and continue to provide ecosystem services. These services include pollination, flood attenuation, grazing and even reducing the risk of foliar fungal diseases since a decrease in plant species richness has been shown to increase pathogen load (e.g. *Septoria*, *Phyllosticta* and *Mycosphaerella* species; Mitchell et al., 2002), which may threaten the agricultural economy and future food security.

Climate change has the potential to shift plant reproductive success (Andrzejak et al., 2022) in grasslands. For example, one study showed that summer drought significantly reduced the seed bank abundance of forbs, while there was no effect of drought on grass seed abundance (Basto et al., 2018). This indicates that forb sexual reproduction is more sensitive to drought than grasses. Generally, increased temperature decreases seed yield while increased CO_2 increases seed yield (Madan et al., 2012; Prasad et al., 2006; Vara Prasad et al., 2002) but these findings were for crops and not indigenous forbs. Another study found that seeds produced from shrubs grown under a drought treatment seemed to have a higher viability but significantly lower germination rate compared to seeds produced from a control treatment of normal moisture levels (Jaworski et al., 2022). Similarly, another study found drought stress to have transgenerational effects by causing a reduction in seed germination of F2 generation crops (Wijewardana et al., 2019), although few studies have investigated at how drought stress affects F2 generation seedling growth beyond germination. In contrast, other studies have found that drought did not significantly affect the number of flowers for *Matthiola incana* L. (Jafari et al., 2019), or fruit and seed set, seed number per fruit, nor the mean seed mass, for *Cistus albidus* L and *Salvia rosmarinus* Spenn (Jaworski et al., 2022). While another study found that drought stress reduced seed set and total seed mass per plant for *Fagopyrum esculentum* Moensch (Rering et al., 2020). This shows that plant responses to drought may differ per species, growth habit and reproductive stage. For example, trees, shrubs, and some perennial forbs may persist during drought conditions since they have more extensive and deeper root systems which allow them increased water uptake from greater depths (Fan et al., 2017). Furthermore, the level of drought may also play an important role in the plant response. For example, George and Nissen, 2002, found that severe drought reduced shoot growth and increased fruit set while mild drought doubled the number of flowers per tree. Overall, fruit set was greatest under mild drought for this tree species, followed by control conditions, and then moderate and severe drought (George & Nissen, 2002). Such studies highlight the importance of investigating plant reproduction under varying levels of drought.

Although there have been studies that have assessed the impacts of drought on forb cover and productivity, there is a dearth of information on its effect on the reproduction of forbs. Understanding these impacts is necessary to ensure plant species' fitness and to maintain populations. Furthermore, studying seed production can help preserve grassland ecosystems, particularly aspects such as seed quantity, germinability and viability (Scotton, 2018). Therefore, an attempt is made in the present study to investigate the effects of drought on the reproductive success of three grassland forbs. These were *Cephalaria pungens* Szabó, *Senecio coronatus* (Thunb.) Harv., and *Thunbergia atriplicifolia* E.Mey. ex Nees. The pollinators and breeding system of *C. pungens* are unknown so this study also set out to investigate the importance of pollinators for this species and the effect of drought on seed set and viability. For those species that seed was obtained from the drought treatment (Chapter 3), the germination and growth of the F2 generation were investigated. The objectives of this study were thus:

1. To investigate the importance of pollinators for the reproduction of *C. pungens* by investigating whether the species sets seed in the absence of pollen vectors.
2. To determine if seed production of *C. pungens* is limited by water or pollen availability.
3. To investigate the effects of moisture level on fruit and seed set and seed mass and seed viability on *C. pungens* and *T. atriplicifolia*. (Seed production for *S. coronatus* was not measured because it was not flowering during the sampling period.)
4. To determine whether a reduced moisture level experienced by the parent plant influences the F2 generation in terms of; seed germination, seedling height, seedling number of leaves, seedling specific leaf area, seedling biomass and seedling leaf length for *C. pungens, S. coronatus* and *T. atriplicifolia*. Seeds for *S. coronatus*, were collected from plants that were not used in other experiments but occurred in plots to be used in determining the seed set and germinating rates following treatments.

4.3. Methods and materials

4.3.1. Study Site

The study was conducted at Ukulinga Research Farm (URF) (Fig. 4-1), University of KwaZulu-Natal, Pietermaritzburg, South Africa (29°40’S, 30°24’E) (Kirkman et al., 2014), over two years during the 2021 and 2022 flowering seasons (October – April). Ukulinga is classified as a mesic grassland and the vegetation is described as KwaZulu-Natal Hinterland Thornveld (Mucina et al., 2006). It has a humid subtropical climate with a mean annual precipitation of 790 mm, which falls mostly as convective storms during summer (October to

April) (Kirkman et al., 2014). It is situated at an altitude of 721 m asl and experiences warm to hot summers and mild winters with occasional frost. Summers are warm with a mean maximum temperature of 26.4°C in February. Winters are mild with a mean minimum temperature of 8.94°C in July (Phophi et al., 2017), with occasional frost (Kirkman et al., 2014). Some common tree species occurring scattered in the grassland include *Vachellia sieberiana* var. woodii and *Vachellia nilotica* (L.) P.J.H. Hurter & Mabb. and the dominant forbs on the site are *Berkheya umbellata* DC., *Cephalaria pungens* Szabó, *Commelina africana* L. var. africana, *Helichrysum nudifolium* (L.) Less. var. *pilosellum* (L.f.) Beentje, *Scabiosa columbaria* L, *Senecio coronatus* (Thunb.) Harv., and *Thunbergia atriplicifolia* E.Mey. ex Nees. The soils are acidic and are classified as Westleigh and Mispah forms derived mostly from Ecca group shale of the Karoo sedimentary sequence (Chamane et al., 2017).

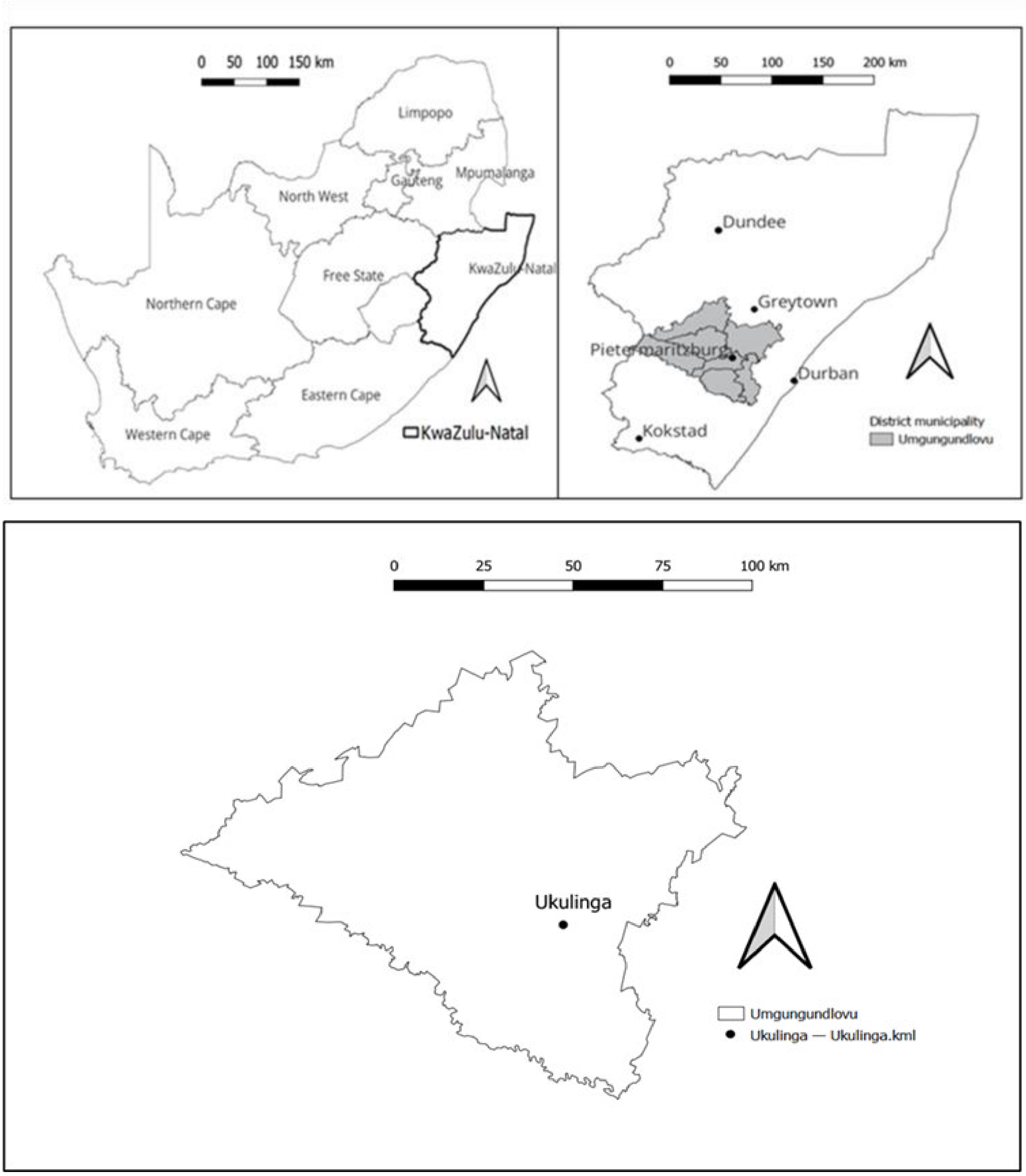


Figure 4-1: Location of Ukulinga Research Farm, University of KwaZulu-Natal, Pietermaritzburg, South Africa.

4.3.2. Study Species

Three forb species at URF were selected for this study due to their common occurrence in each plot over the three moisture levels and flower size facilitating regular counting.

Cephalaria pungens Szabó (family Dipsacaceae) is a perennial herb that occurs in damp grasslands and on forest margins (Fig. 4-2A). The flower heads are between 20 – 40mm in diameter. The flowers are short-styled and have four anthers. It flowers between January and March (Pooley, 2005a). In South Africa, it is found in the Eastern Cape, Free State, Gauteng, KwaZulu-Natal, Limpopo and Mpumalanga provinces. Outside South Africa it occurs in Kenya, Tanzania, Zambia, Zimbabwe, Mozambique, Malawi and Swaziland. The flowering stem is between 1 – 1.5 m high. The leaves are opposite and have a very conspicuous middle vein. The inflorescences have numerous, densely arranged, tubular flowers (Fig. 4-2A) that are cream, white or pale yellow (Ngwenya, 2017a). Each floret contains one ovule. It is the most dominant forb species at URF (Zama et al., 2022). This species is pollinated by bees (Ngwenya, 2017a).

Senecio coronatus (Thunb.) Harv. (family Asteraceae) grows in grasslands and on ultramafic outcrops of the Barberton Greenstone Belt (Mesjasz-Przybyłowicz & Przybyłowicz, 2020). It is native to Angola, Botswana, South Africa (Eastern Cape, Western Cape, Free State, KwaZulu-Natal, Gauteng, North West, Limpopo and Mpumalanga), Lesotho, Malawi, Swaziland, Tanzania, Zambia, Democratic of Congo and Zimbabwe (Beentjie et al., 2005). It flowers from August to December (Pooley, 2005b). It is a self-compatible, herbaceous perennial with a short, upright, subterranean stem that produces long (up to 20 cm) (Fig. 4-2C), broad, simple and fleshy leaves aboveground and relatively fleshy roots belowground (Boyd et al., 2008). There is no information available on the pollinators for this species. *Senecio coronatus* has a capitulum-type inflorescence composed of 10 - 12 ray florets that are yellow and disc florets that are yellow (Beentjie et al., 2005). The pollen grains are yellow when observed by the naked eye. It is a hermaphroditic plant. The fruits are cylindrical and narrow with a dense pappus. *Senecio coronatus* is allogamous and self-incompatible (Walter et al., 2020).

Thunbergia atriplicifolia E. Mey. ex Nees (family Acanthaceae) is a grassland species native to South Africa and Swaziland. It is a shrublet with pubescent stems and leaves (Balkwill & Campbell-Young, 1999) with a tendency to twine. Flowers of *T. atriplicifolia* are white to cream-white, or yellow (Fig. 4-2B) and open for up to five days (Msweli et al., 2018). The inner surface of the corolla tube is yellow (Retief and Reyneke, 1984). The flowers have five lobes and four stamens in pairs. *Thunbergia atriplicifolia* has hermaphroditic flowers and is self-compatible but mainly allogamous, pollinated by generalist solitary bees that belong to the families Apidae and Halictidae (Msweli et al., 2018).

Figure 4-2: Photographs of flowers, inflorescences, and fruits of three grassland forbs. A: flowerhead of Cephalaria pungens*, B: infructescence of* C. pungens*, C: flower of* Thunbergia atriplicifolia*, D: fruit of* T atriplicifolia*, E: capitulum of* Senecio coronatus *F: seedhead of* S. coronatus.

4.3.3. Experimental Design

The experimental trial used in this study is part of the global Drought-Net Research Coordination Network (RCN), which aims to investigate differences in ecosystem drought sensitivity and to determine the basic mechanisms driving these differences (Smith et al., 2024b). It was established at URF in 2019 and was made up of three treatments replicated three times: a drought (50% rain) (this reduces rainfall to the first percentile of the 100-year rainfall record for the area), a control (100% rain) and a run-on treatment (150% rain). Each plot consists of an area of 3 x 5 m. The drought plot was covered by a large rainfall exclusion shelter, made from clear corrugated plastic sheeting, which intercepted 50% of incoming rainfall. The rainfall captured from the drought treatments was channelled to the run-on treatments through gutters and PVC pipes and increased the annual precipitation by ~50% relative to ambient amounts. The boundary of each plot was trenched to a depth of 0.5 m and lined with 1 mm thick plastic to hydrologically isolate the plots. The rain shelters had a roof height of 150 cm and had open sides to avoid greenhouse effects, to allow air circulation, and allows pollinators access to the flowering plants. The trial was arranged in a randomized block design to account for any site effects.

4.3.4. Determination of Reproductive Success

4.3.4.1. Breeding system– *Cephalaria pungens*

The breeding system for *C. pungens* was investigated to determine if the species requires pollinators to set seed, as a potential measure of pollination success and to aid in interpreting results involving drought effects on seed set (Andersson, 1991). The breeding systems of *S. coronatus* and *T. atriplicifolia* are already known from the literature and therefore were not tested (Msweli et al., 2018; Walter et al., 2020). The breeding system of *C. pungens* was investigated in the field using controlled hand pollination experiments. Plants used in this experiment occurred in the surrounding vegetation and were not part of the moisture trial treatments. Before flower opening, six individuals of *C. pungens* were chosen for the experiment and all buds enclosed in organza mesh bags to exclude pollinators. Once anthesis occurred, three flowerheads from each plant were randomly allocated to each of three different pollination treatments: (1) unmanipulated bagged flowerheads were used to test for autonomous selfing; (2) self-pollinated, bagged flowerheads were pollinated with self-pollen by brushing a toothpick over the anthers and stigma of the same flowerhead or pollen between flowerheads of the same plant, to assess self-compatibility; (3) cross-pollinated, whereby bagged flowerheads were pollinated using pollen taken from flowers of a different plant and brushed onto the stigma of the flowers during their receptive stage (stigma lobes were open). The pollen that was used for cross-pollination was collected from the surrounding plants, which were not part of the experiment. The number of flowers in each flowerhead was recorded before the treatment was applied. Once fruit had formed and matured, the number of flowers that set seed in each flowerhead from each treatment was counted. The percent seed set (proportion of flowers within a flowerhead that set seed) was used as the determinant of the breeding system (Zhang & Mosjidis, 1995). Seed set percentage was calculated using the following formula:

$$\%\, Seed\ set = \frac{Total\ number\ of\ seeds\ per\ inflorescence}{Total\ number\ of\ flowers\ per\ inflorescence}\ x\ 100$$

4.3.4.2. Pollen limitation – *Cephalaria pungens*

A pollen limitation study was done on *C. pungens* only, because *S. coronatus* was not flowering during the sampling period, and there was a limited sample size for *T. atriplicifolia* and therefore other experiments were prioritised. To determine whether plants of *C. pungens* were pollen-limited or resource-limited, one inflorescence per plant for at least one plant per plot in the 50% rain treatment was cross pollinated (disclaimer: the experiment couldn't be repeated on more plants due to low sample size, and the results of this experiment must be interpreted with caution because intra-specific variation in seed viability within the population could not be fully assessed), and seed set was compared to that of naturally pollinated plants in the 50% and 100% rain treatments. Seed set of six naturally pollinated inflorescences from four plants was determined for each of the 50% rain and 100% rain treatments for comparison. Seeds were collected, weighed, and counted upon maturation. The seed set per flowerhead was calculated using the formula in the previous section for percentage seed set and compared across the three treatments.

4.3.4.3. Fruit set, seed set and seed mass

Since reproductive fitness is dependent on both the number and quality of seed progeny (Dogterom et al., 2000), the effect of drought on reproductive output of *C. pungens* and *T. atriplicifolia* was investigated at the end of the growing season by harvesting mature seeds, counting the number of seeds and weighing the seeds (Gallagher and Campbell, 2021; Tak-Cheung Lau and Stephenson, 1993).

Seed set for all flowerheads per plant for four plants per moisture level treatment was determined for *C. pungens* using the formula above. The average mass per seed for *C. pungens* was calculated by weighing all the seeds of an inflorescence and dividing them by the total number of seeds for that inflorescence. For *T atriplicifolia,* seed set was calculated as the number of seeds per fruit divided by 2 (potential ovules per fruit for *T. atriplicifolia*) x 100 for ten plants in the 150% rain, eight plants in the 100% rain, and three plants in the 50% rain treatment plots. Average seed mass per moisture level was taken as the average mass for all seeds of a moisture level for *T. atriplicifolia*. Fruit set for *T. atriplicifolia* was measured as the total number of flowers that set at least one seed divided by the total number of flowers per plant for each moisture level (expressed as a percentage).

4.3.4.4. Seed viability

Seed viability was tested for *C. pungens* only due to low seed set in all moisture levels for *S. coronatus* and *T. atriplicifolia,* all seeds of these species were reserved for testing germination success and were not used for viability testing. Twenty-five seeds collected from each moisture level in chapter 3 were assessed for viability using a standard tetrazolium staining protocol (International Seed Testing Association, 1985). The tetrazolium (TZ) tests were conducted using 25 seeds (for *C. pungens*) from each of the three moisture levels. Seeds were preconditioned for the TZ test by placing them in 40 ml of water for 24 h at 20 °C (de Souza et al., 2010). After 24 h, floating seeds were counted and discarded (regards as infertile), and seeds that sank were cut in half and each half of the seed section was immersed in 5 ml of 1% tetrazolium (2,3,5 triphenyl tetrazolium chloride) solution at 40 °C for two hours (Carvalho et al., 2013). After this, the seeds were removed, washed three times in running water, and the colour of each seed immediately assessed. Seed viability was based on tissue characteristics as described in Patil and Dadlani (2009); red and uniform staining of the embryo was taken as viable while improperly stained or unstained seeds were considered unviable.

4.3.4.5. Seed germination experiment

To determine the germination success of seeds from plants exposed to the three different rainfall treatments, the other half of the seeds collected from *C. pungens,* and all seeds collected from *S. coronatus* and *T. atriplicifolia,* were first scarified by soaking in 3.5% (v/v) sodium hypochlorite for 27 min, and then rinsed ten times with distilled water (Ndabankulu et al., 2022). Seed germination was tested by placing 20 seeds from each treatment in a petri dish, replicated three times to make a total of 60 seeds per treatment. The seeds were placed between two layers of filter paper imbibed with 10 ml of distilled water. The petri dishes were covered with a cover lid and incubated under dark conditions at room temperature (≈ 25 °C). Seeds were checked daily by removing the petri dishes from the incubator, counting and removing germinated seeds (to be planted and used in a growth trial as described below), and adding water to re-saturate the filter paper (Stump, 1984). Seeds were considered germinated when the seed coat was broken and the primary root was at least 2 mm long (Singh et al., 2018). After seven days, the remaining non-germinated seeds were removed, stored dry for two weeks and subjected to a standard tetrazolium test as described above (Stump, 1984). Final germination percentage (FGP) was calculated as:

$$FGP = \left(\frac{n}{nT}\right) x100$$

where n is the number of germinated seeds and nT is the total number of seeds (Singh et al., 2018).

4.3.4.6. Seedling growth measurements

After seed germination, the F2 generation seedlings were planted immediately in pots (15 cm top diameter; 10 cm base diameter; 12 cm height) with perforated bases, which were three-quarters filled with potting soil. Each pot contained five replicate seedlings, which were grown in the greenhouse (Ndabankulu et al., 2022). Greenhouse conditions consist of night and daytime temperatures of 12 – 14 °C and 30 – 35 °C respectively. The humidity ranged from 70 – 80 % and the irradiance was 35% of full sunlight (415.6 $\mu mol^2.s^{-1}$). Plants were watered by sprinklers for 8 seconds every 15 minutes for 16 hours a day. After four weeks, seedlings were transplanted into separate pots. After nine months, seedlings were harvested. Seedling height, number of leaves, seedling leaf length, seedling specific leaf area (SLA), and seedling dry biomass were measured for each harvested seedling (Lankinen and Madjidian, 2011). Seedling height was measured from the cotyledons to the terminal meristem of the seedling (Byrne et al., 1997). The number of leaves was calculated by counting the total number of green leaves per seedling. Seedling leaf length was measured from the base to the tip of the leaf (Byrne et al., 1997) for all leaves on the plant and an average length was calculated per plant. SLA was measured by first measuring leaf area for all leaves on a plant and taking the average using an LI 3000C leaf area meter (LICOR, Lincon, USA), then weighing the dry mass of the leaf, and finally calculating SLA as SLA = leaf area/leaf dry mass (Cornelissen et al., 2003; Wu et al., 2008). Dry mass of whole plants (including roots, stems and leaves) was determined after oven drying for 48 h at 50 °C followed by 24 h at 100 °C (Liu and Stützel, 2004).

4.4. Statistical Analysis

All statistical analyses were performed using R studio, version 4.3.1 (R Core Team, 2023). All data were checked for normal distributions (using a Shapiro test) and equal variances of the residuals (using Levene's test) between treatments before running parametric statistical tests. If either or both of these assumptions were not met, the data was transformed as noted below, or a general linear model was performed.

The percentage seed set of *C. pungens* following hand-pollination treatments in the breeding system experiment was compared using one-way ANOVA, followed by a TukeyHSD (Tukey's Honestly-Significant Difference) posthoc test. To determine whether the percentage seed set of *C. pungens* was pollen or resource-limited, an ANOVA was ran, looking at the mean differences between hand-pollinated individuals in the 50% rain, naturally pollinated individuals in the 50% rain and natural pollinated individuals in the 100% rain. To examine the effect of moisture level on average seed mass per seedhead and percentage seed set for *C. pungens*, one-way ANOVA's were used.

To determine the effects of moisture level on percentage seed set, percentage fruit set and average seed mass for *T. atriplicifolia*, ANOVA's were also used. Fisher's exact test was used to compare the number of viable seeds in the different moisture levels since one of the treatment groups had less than five observations.

To test the effect of F1 generation moisture availability on F2 generation germination, we used one-way ANOVA's for *C. pungens* and *S. coronatus*, followed by TukeyHSD post-hoc tests to compare significant differences in germination among the different moisture levels.

Since the seedling measurements were collected on seedlings of different ages, the dataset was standardised conservatively by dividing each of the measurements by the number of weeks a plant grew for, and then multiplying by the highest number of weeks before calculating: F2 seedling height, number of leaves, SLA, dry biomass, and leaf length. To determine the effect of moisture level on F2 height and leaf length for *S. coronatus*, an ANOVA was used. Data for the number of leaves, SLA, and biomass for *S. coronatus* was non-normal, and thus log transformed to meet the assumption of normality and equal variance before one-way ANOVA's were run.

To determine the effect of moisture level on F2 height, SLA, and leaf length for *C. pungens*, a two-sample t-test was used after all three of these variables were log-transformed to meet normality assumptions. To test the effects of moisture level on F2 biomass, a generalised linear model with a gaussian distribution was used with a log link function. For the number of leaves a generalized linear model with a negative binomial distribution was fitted with a log link function (due to overdispersion of the data).

4.5. Results

4.5.1. Breeding System – *Cephalaria pungens*

Self-pollinated flowers of *C. pungens* failed to produce seed, while autonomous and cross-pollinated treatments, both led to limited seed formation (Fig. 4-3). Pollination treatment significantly affected seed set percentage ($F_{2,15} = 5.148$, $p = 0.019$) with cross pollination leading to the highest seed set compared to the other treatments.

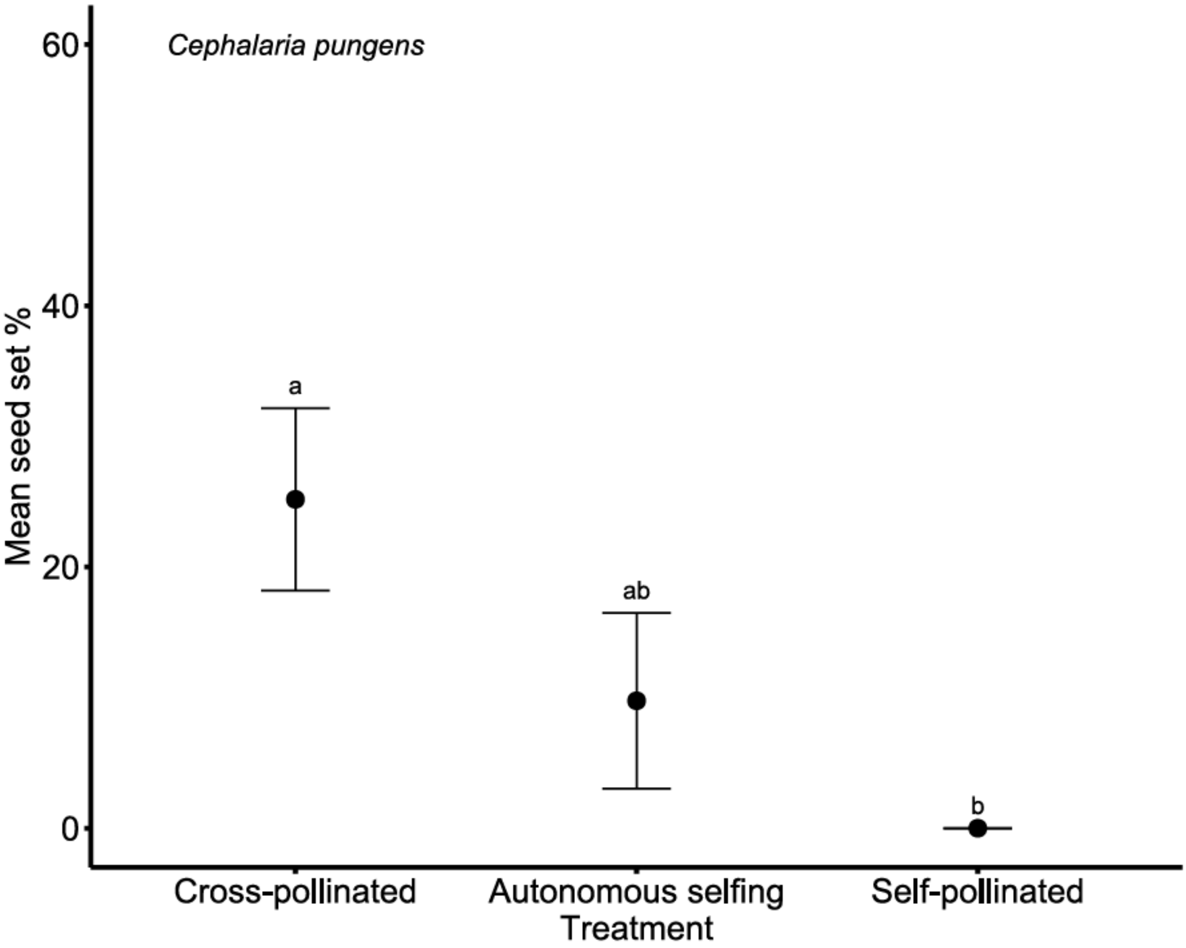


Figure 4-3: Mean ± SE Seed set of Cephalaria pungens *after being hand pollinated with self or cross pollen or left bagged and unmanipulated to test for autogamous seed production. Different letters indicate significant differences between treatments following Analysis of Variance.*

4.5.2. Pollen Limitation – *Cephalaria pungens*

Seed set did not significantly differ between hand-cross pollinated flowers in the 50% rain, naturally pollinated flowers in the 50% rain and natural pollinated flowers in the 100% rain ($F_{2,9} = 0.241$, $p = 0.791$, Fig. 4-4) for *C. pungens*.

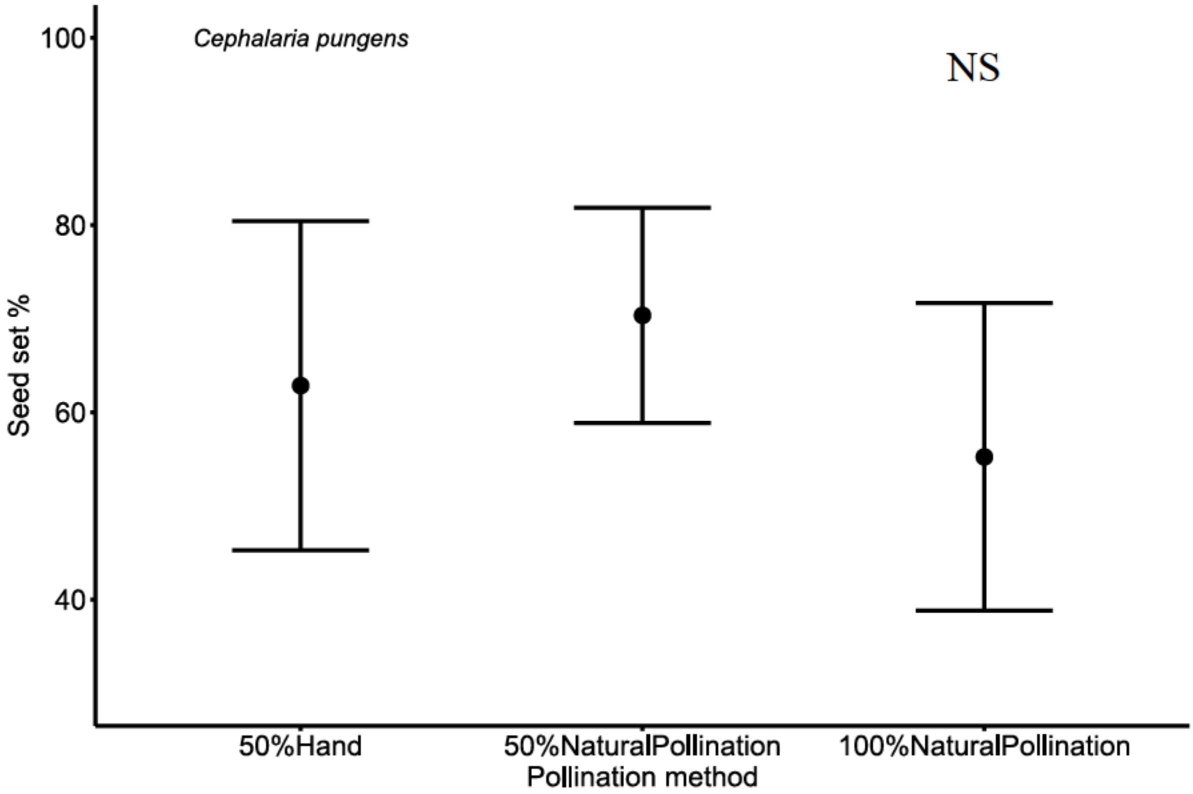


Figure 4-4: Mean ± SE Seed set of *Cephalaria pungens*, following hand-cross pollination in the 50% rain treatment, natural pollination in the 50% rain treatment and natural pollination in the 100% rain treatment. NS indicates non-significance.

4.5.3. Seed Set and Seed Mass

There was no significant effect of moisture level on the average seed set and seed mass for *C. pungens* (seed set: $F_{2,26} = 1.271$, $p = 0.297$, Fig. 4-5; seed mass: $F_{2,26} = 0.02$, $p = 0.977$, Fig. 4-6). Similarly, moisture level had no effect on seed set ($F_{2,18} = 0.351$, $p = 0.709$, Fig. 4-7) and fruit set ($F_{2,18} = 0.34$, $p = 0.716$, Fig. 4-8) of *T. atriplicifolia*. In contrast, moisture level did affect the average seed mass per moisture level of *T. atriplicifolia* ($F_{2,9} = 5.17$, $p = 0.032$) with seeds being an average of 0.024 g lighter in the 50% rain treatment compared to the 150% rain

(Fig. 4-9). Seed mass in the 150% rain was not significantly different from seed mass in the 100% rain for *T. atriplicifolia*.

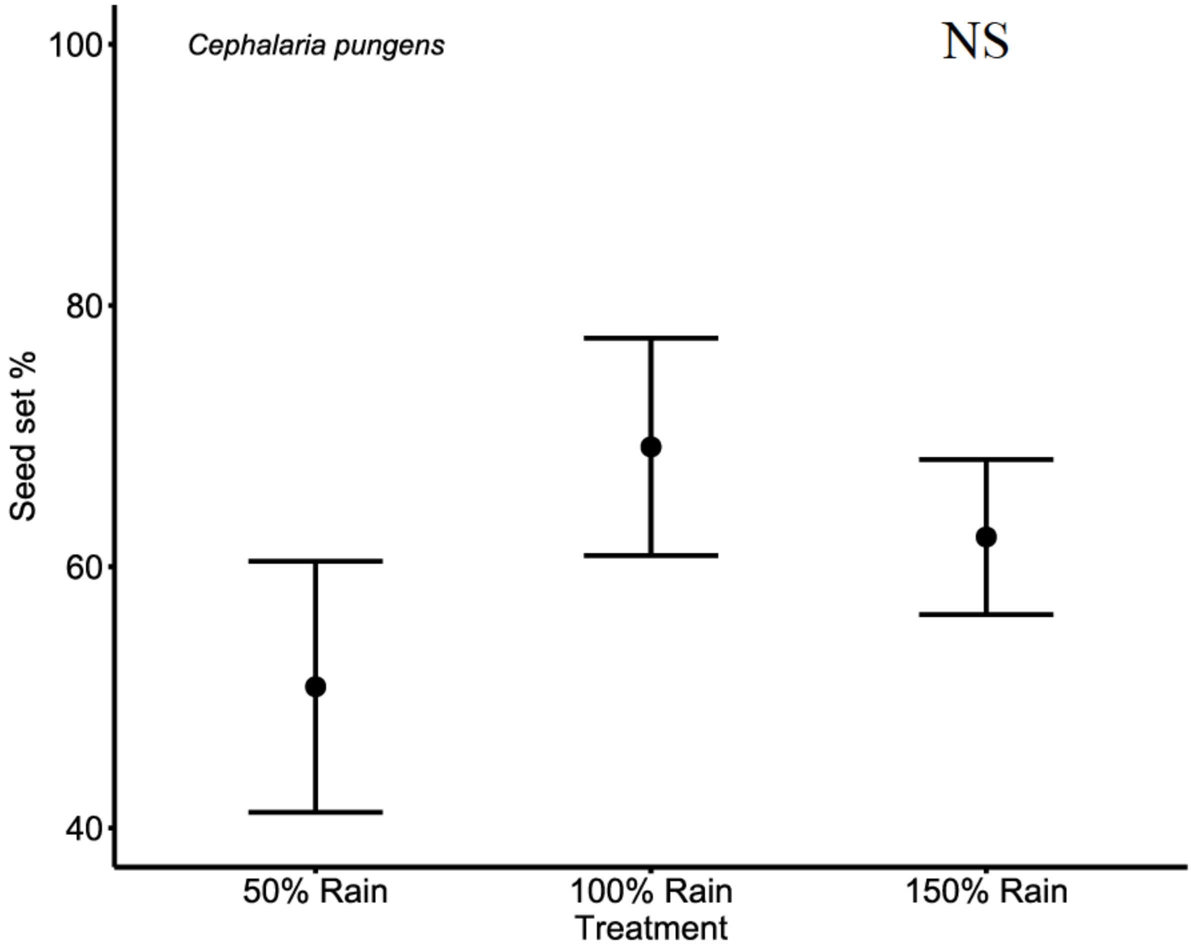


Figure 4-5: Mean ± SE percentage seed set for *Cephalaria pungens* plants grown in different moisture treatments. NS indicates non-significance.

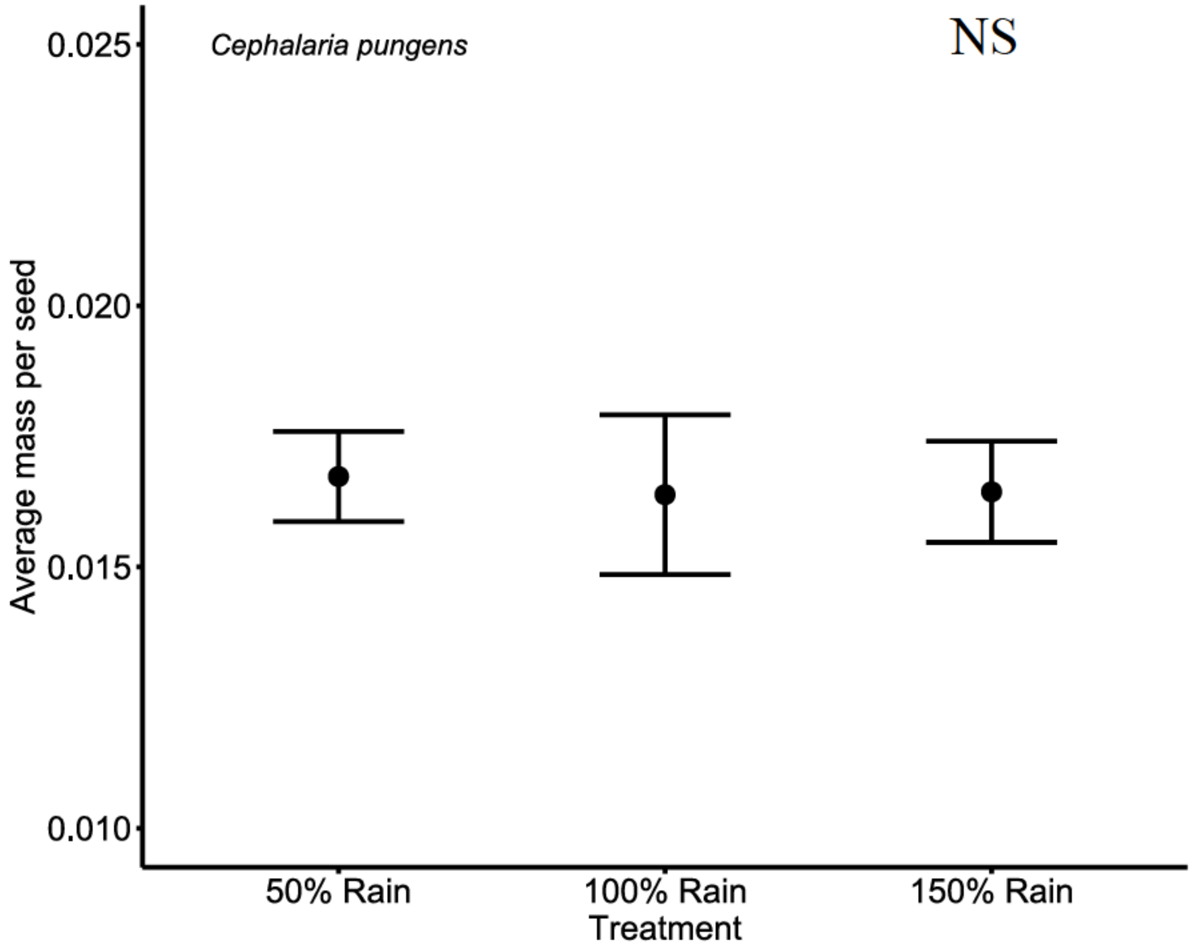


Figure 4-6: Mean ± SE average mass per seed for Cephalaria pungens *plants grown in different moisture treatments. NS indicates non-significance.*

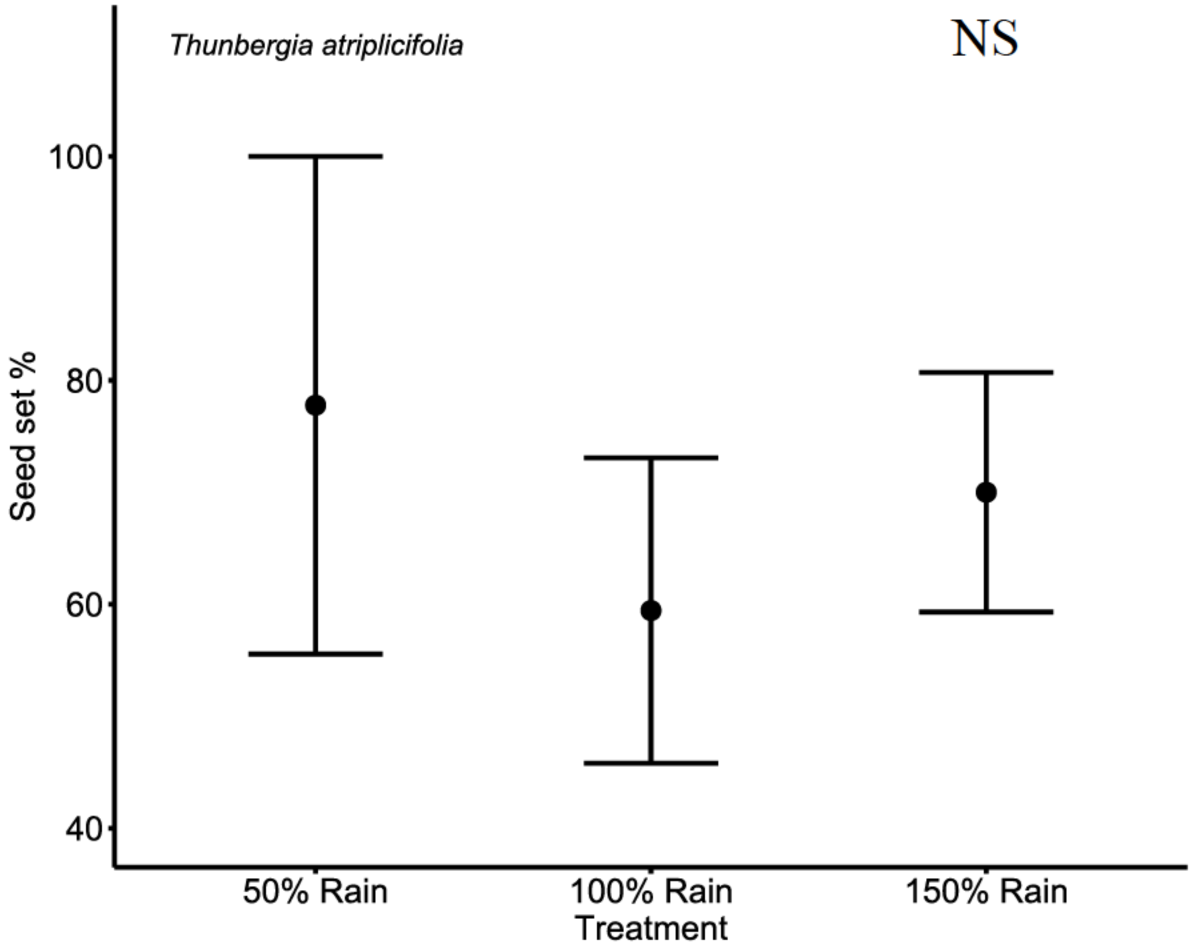


Figure 4-7: Mean ± SE percentage seed set for Thunbergia atriplicifolia *plants grown in different moisture treatments. NS indicates non-significance.*

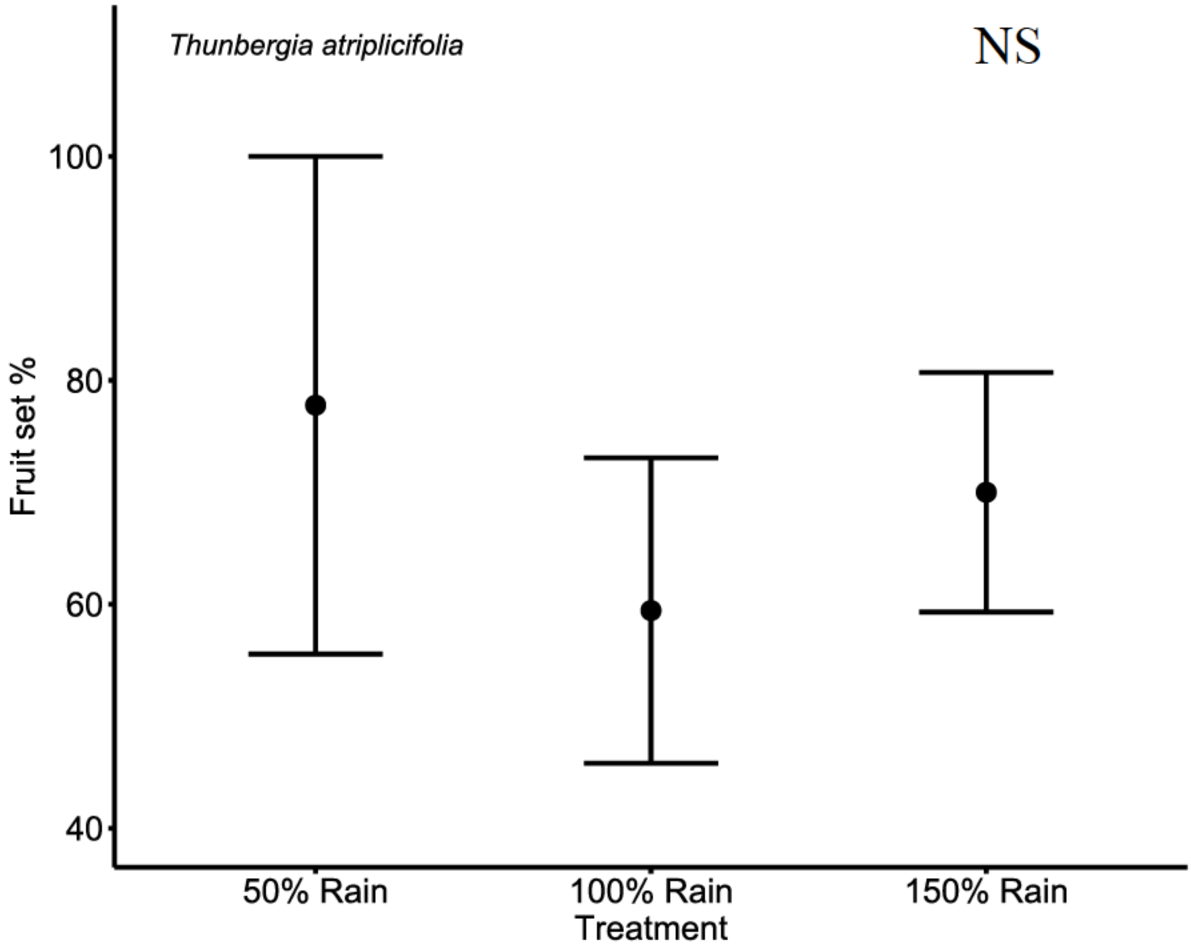


Figure 4-8: Mean ± SE percentage fruit set for Thunbergia atriplicifolia *plants grown in different moisture treatments. NS indicates non-significance.*

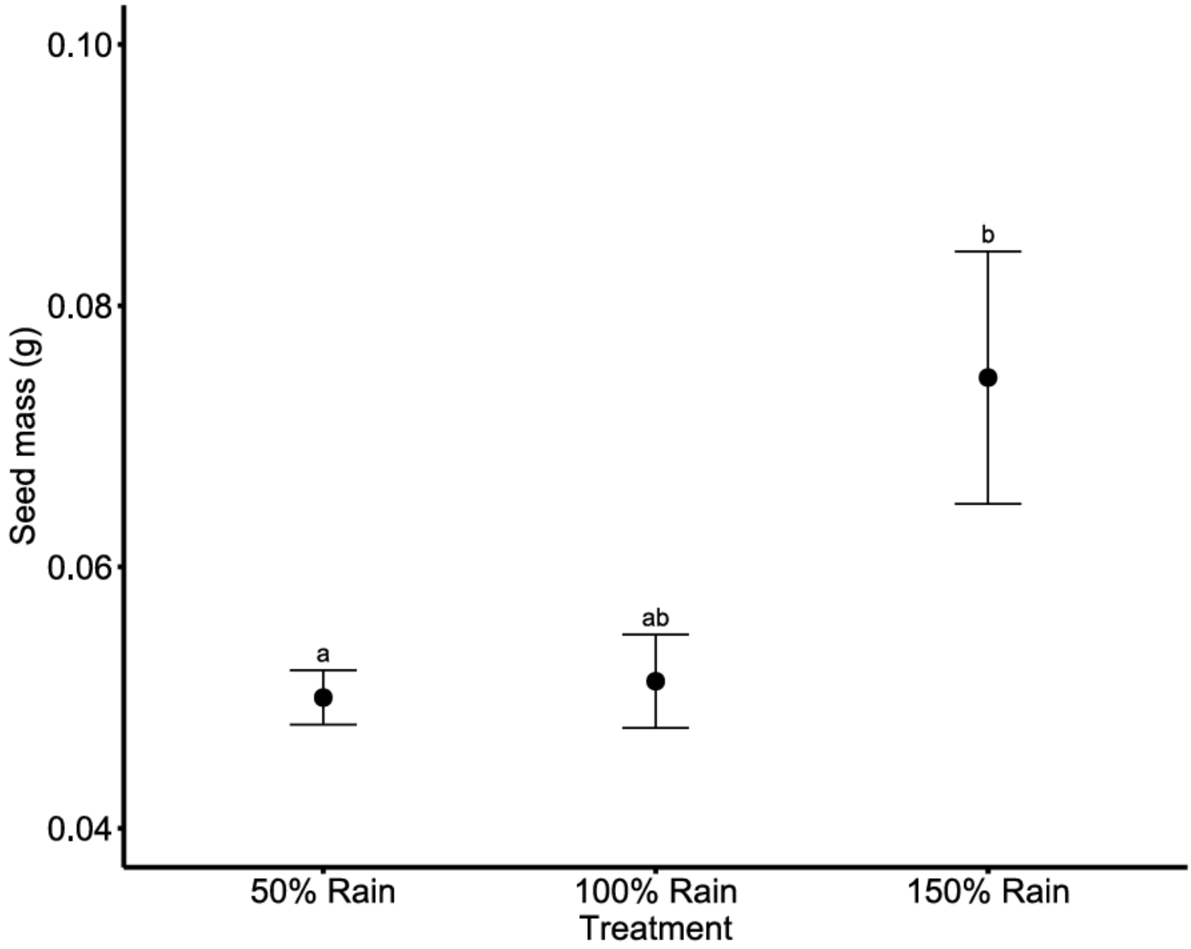


Figure 4-9: Mean ± SE seed mass for Thunbergia atriplicifolia *plants grown in different moisture treatments. Different letters indicate significant differences between treatments following Analysis of Variance.*

4.5.4. Seed Viability – *Cephalaria pungens*

Seed viability of *C. pungens* did not differ significantly among the different moisture levels (N = 75, p = 0.052; Fig. 4-10). In the 50% rain, 16% of the seeds that were tested for viability were viable; in the 100% rain, 8% were viable; and in the 150% rain, 24% were viable.

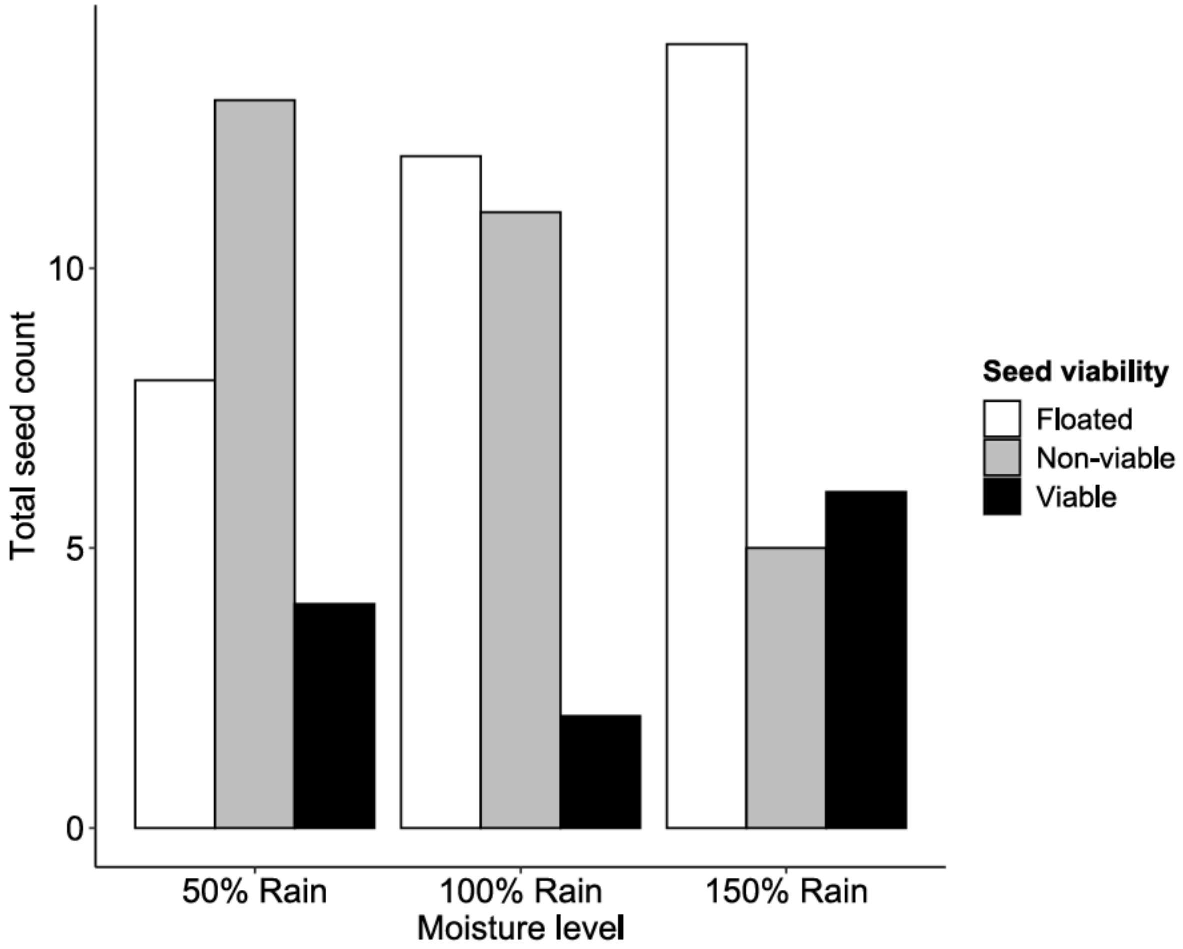


Figure 4-10: Total number of viable seeds for each moisture level for Cephalaria pungens *following Fishers exact test.*

4.5.5. Seed Germination

Mean F2 seed germination was highest from *C. pungens* parent plants growing in the 100% rain treatment (Fig. 4-11). Mean seed germination percentage for *C. pungens* was statistically different among the moisture levels ($F_{2,6} = 147.2$, $p < 0.001$). There was no F2 seed germination from the plants growing in the 50% rain treatment compared to those in 100% rain, and 9% less germination for seed from plants growing in the 150% rain compared to the 100% rain.

For *S. coronatus*, F2 seed germination was also significantly affected by moisture level ($F_{2,6} = 203.6$, $p < 0.001$), with the greatest germination percentage recorded for the 50% rain (Fig. 4-12). Germination was 71% greater in the 50% rain and 12% greater in the 150% rain, compared to the 100% rain. The 150% rain decreased germination by 59% compared to the 50% rain. F2 seeds of *T. atriplicifolia* did not germinate.

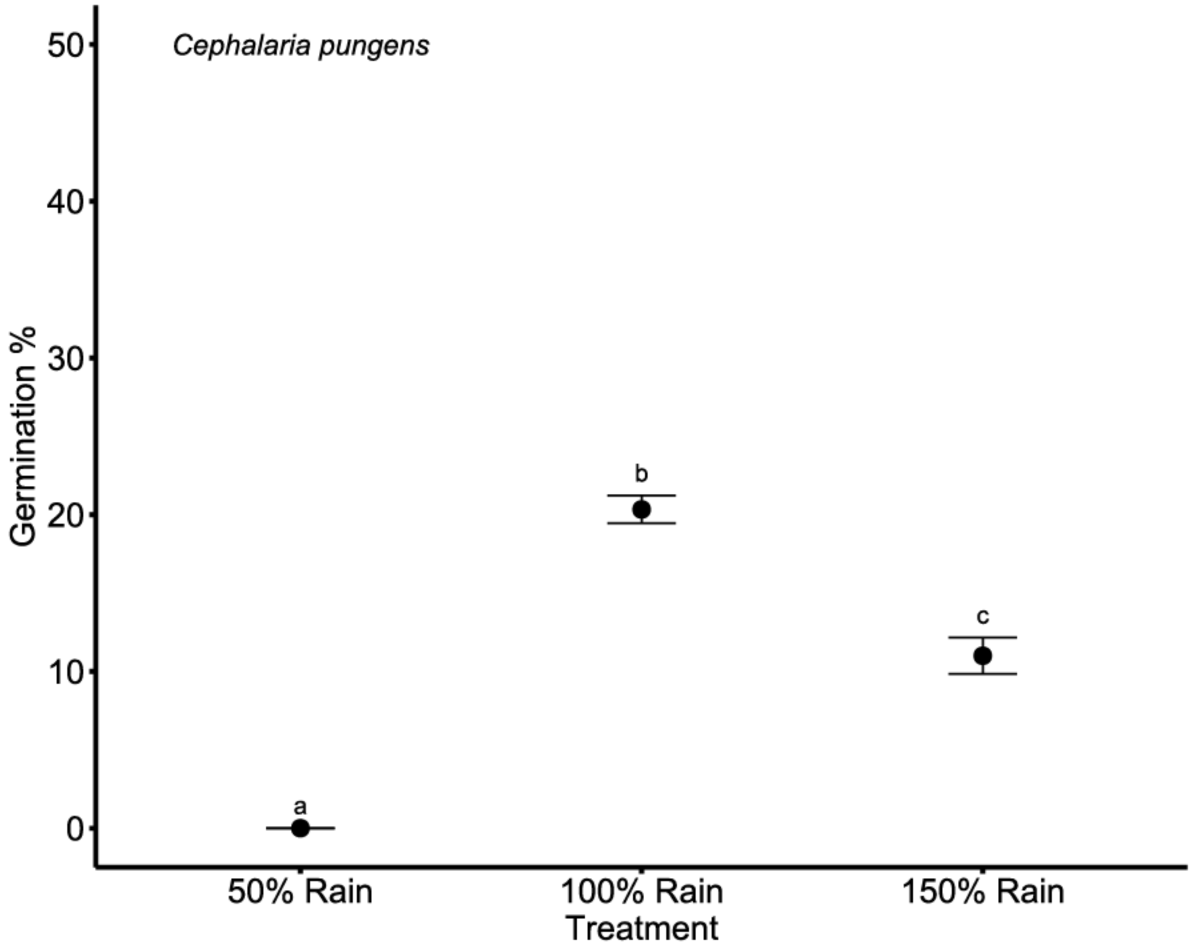


Figure 4-11: Mean ± SE germination percentage for Cephalaria pungens. *Different letters indicate significant differences between treatments following Analysis of Variance.*

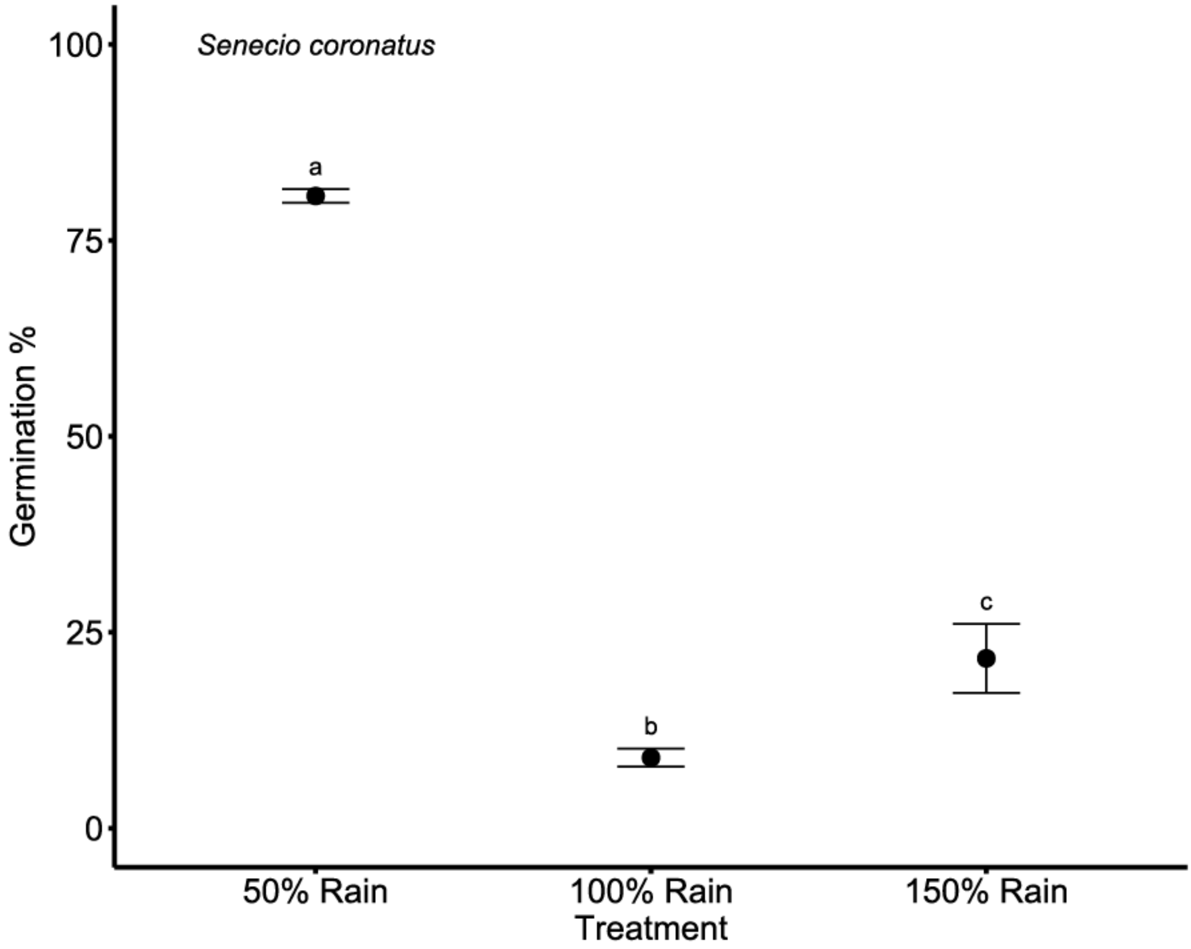


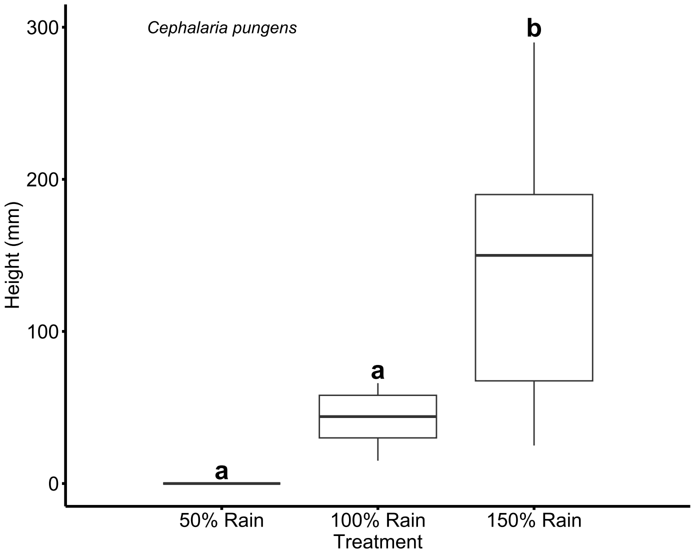
Figure 4-12: Mean ± SE seed germination percentage for Senecio coronatus*. Different letters indicate significant differences between treatments following Analysis of Variance.*

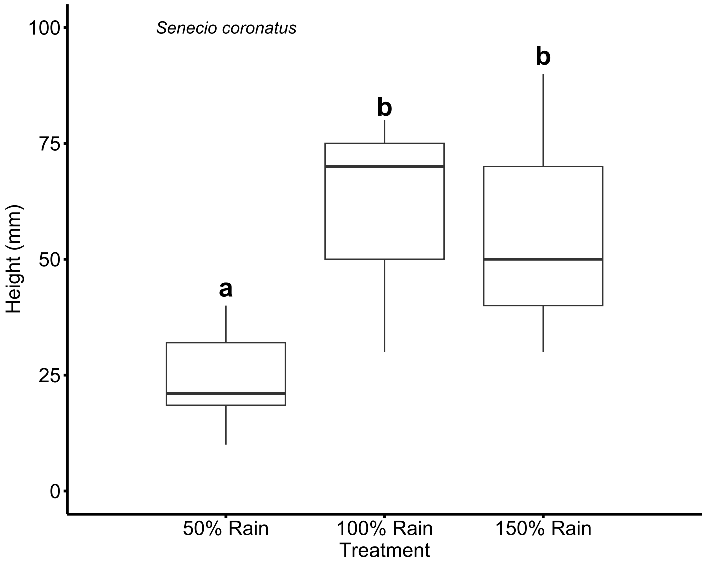
4.5.6. Seedling Growth

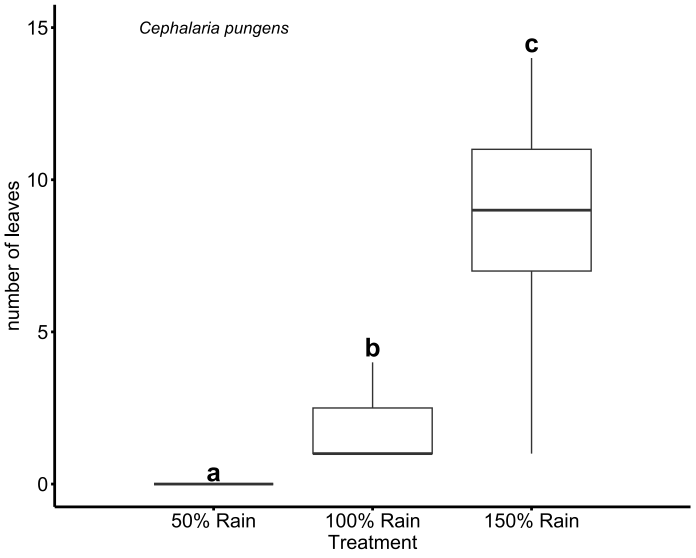
For *C. pungens*, no seed germinated from seeds produced by plants grown in the 50% rain treatment. For the remaining treatments, F2 seedling height was significantly affected by maternal moisture level treatments where seedlings were significantly taller in the 150% rain than in the 100% rain (t-value = -4.215, df = 17.587, p <0.0001; Fig. 4-13). The number of leaves for *C. pungens* F2 seedlings was significantly affected by maternal moisture level treatments (t-value=0.355, df=19, p <0.0001), where the number of leaves was greatest in the 150% rain. F2 seedling dry biomass was significantly affected by maternal moisture level treatments (t-value=3.646, df =19, p <0.01; Fig. 4-13), where biomass was greater in the 150% rain. Leaf length for the F2 generation seedlings was significantly affected by maternal moisture level conditions, where leaf length was significantly longer in the 150% rain (t-value

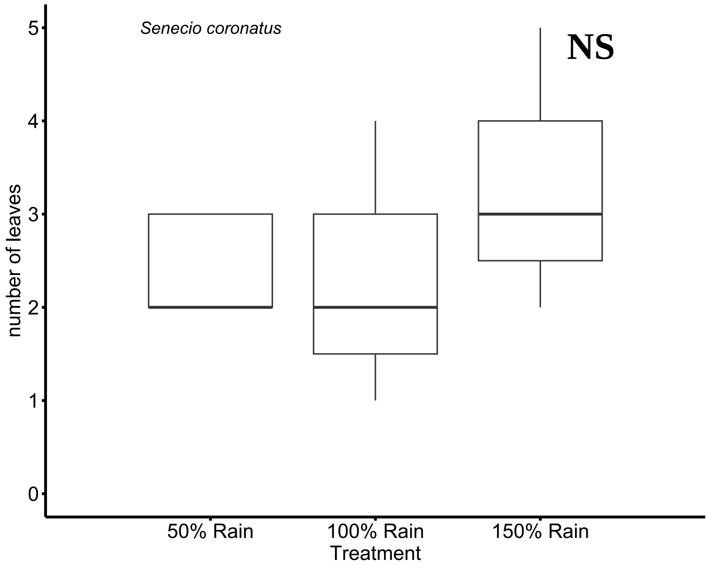
= -4.767 df =17.652, $p < 0.001$; Fig. 4-13). The F2 seedling SLA was not significantly affected by maternal moisture level treatments (t-value=0.577, df =14.25, $p < 0.572$).

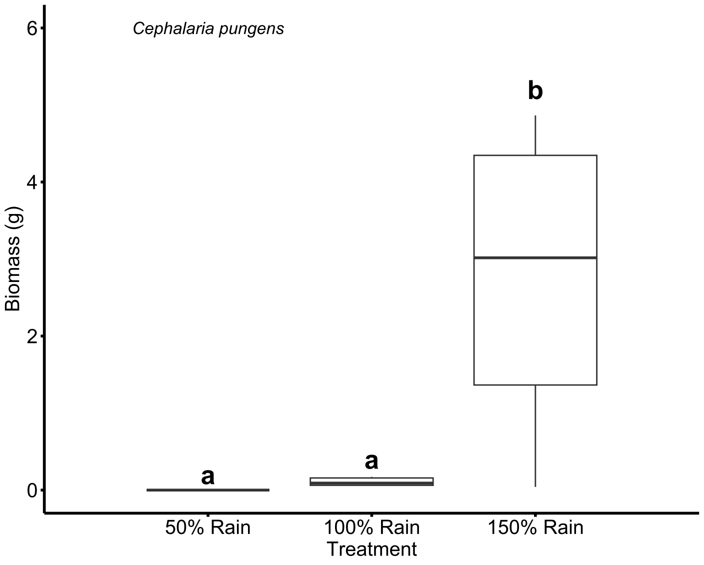
For *S. coronatus*, seedling height was significantly affected by moisture level under which the seeds were produced ($F_{2,16} = 8.925$, $p = 0.003$; Fig. 4-13). Seedling height from seeds produced under 50% rain was significantly shorter than under 100% and 150% rain, which did not differ from one another. Maternal moisture level conditions had no effect on the number of leaves for F2 seedlings of *S. coronatus* ($F_{2,16} = 1.883$, $p = 0.184$). F2 seedling dry biomass was significantly affected by maternal moisture level conditions ($F_{2,16} = 23.93$, $p < 0.001$), where biomass was greatest in the 100% rain, followed by the 150% rain treatment and least in the 50% rain (Fig. 4-13). F2 seedling SLA was significantly affected by maternal moisture level treatments ($F_{2,16}= 23.93$, $p < 0.001$), where SLA was lowest in the 150% rain and highest in the 50% rain. F2 seedling leaf length was significantly affected by maternal moisture level treatments ($F_{2,16}= 20.61$, $p < 0.001$), where under 50% rain leaf length was significantly reduced compared to 100% and 150% rain (Fig. 4-13).

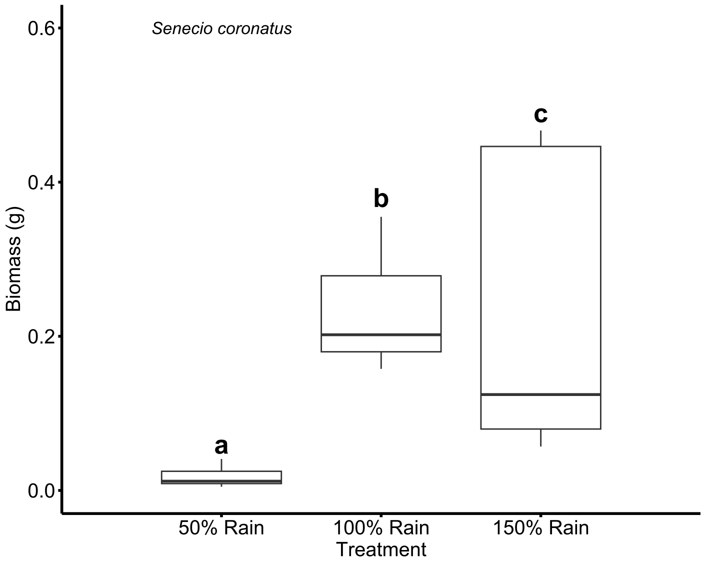

Cephalaria pungens
Height (mm)
0
100
200
300
a
a
b
50% Rain
100% Rain
150% Rain
Treatment


Senecio coronatus
Height (mm)
0
25
50
75
100
a
b
b
50% Rain
100% Rain
150% Rain
Treatment


Cephalaria pungens
number of leaves
0
5
10
15
a
b
c
50% Rain
100% Rain
150% Rain
Treatment


Senecio coronatus
number of leaves
0
1
2
3
4
5
NS
50% Rain
100% Rain
150% Rain
Treatment


Cephalaria pungens
Biomass (g)
0
2
4
6
a
a
b
50% Rain
100% Rain
150% Rain
Treatment


Senecio coronatus
Biomass (g)
0.0
0.2
0.4
0.6
a
b
c
50% Rain
100% Rain
150% Rain
Treatment

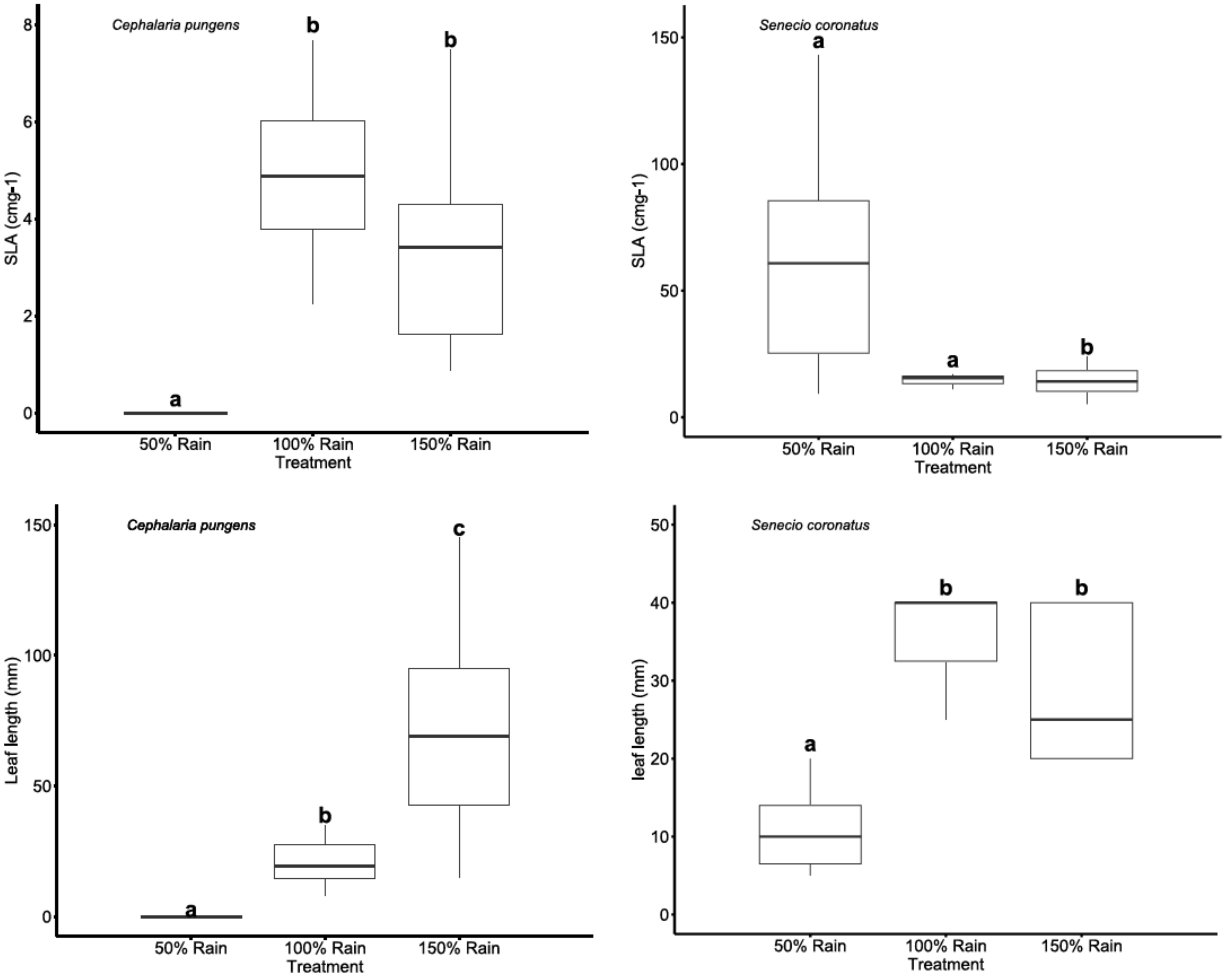


Figure 4-13: Effects of moisture level on seedling growth (seedling height, number of leaves, dry biomass, Specific Leaf Area (SLA), leaf length) of Senecio coronatus *(left panel of graphs) and* Cephalaria pungens *(right panel of graphs). NS= non-significant. Different letters indicate significant differences between treatments following Analysis of Variance and generalised linear models. Lines spanning from the centre of boxplots represent mean, SD, and minimum and maximum values.*

4.6. Discussion

Changes in seasonal rainfall resulting from climate change may lead to an increase in the frequency and duration of drought events which will increase the loss of soil moisture in mesic grasslands (Jones et al., 2016). This could affect plant-pollinator relationships, seed production, seed germination and viability. Decreased seed production can restrict plant species richness in grasslands (Zeiter et al., 2016). Furthermore, understanding the breeding system of forb species is important to understand if these species can adapt to changing pollinator visitation patterns because of drought. This study aimed to determine factors affecting seed set of three forb species under drought conditions and the effects of moisture stress on the germination and growth of the F2 generation. Furthermore, the breeding system of *C. pungens* was investigated, addressing gaps in our knowledge of this species' mating system to aid in interpreting results of the drought trials.

The breeding system of *C. pungens* was determined with field experiments in a natural population. The absence of seed set from the selfed pollination treatment and significantly low seed set in the autonomous treatment compared to the higher number of fruits set in the outcross treatment shows that *C. pungens* is strongly self-incompatible. This is because the flowers of *C. pungens* are heterostylous which encourages outcrossing. The occurrence of autogamy may be an adaptation to reproduction if pollinator activity is low or extremely unpredictable, therefore allowing for reproductive assurance (Kalisz and Vogler, 2003), facilitated by self-pollen only being accepted when contacting stigma lobes in wilted flowers.

There was no indication that plants of *C. pungens* in our experiment were pollen-limited. The seed set did not significantly differ between hand-supplemented individuals in the 50% rain treatment, non-pollinated individuals of 50% rain treatment, and non-pollinated individuals in the 100% rain even though visitation frequency per plant was significantly lower under 50% rain (see chapter 3). Possible explanations include (1) that the pollinators are efficient at cross-pollinating inflorescences, even with infrequent visits; (2) that even if sufficient pollinators were available to visit all inflorescences, many pollination events involved self (i.e. incompatible) pollen leading to low seed set in naturally pollinated plants (Copland and Whelan, 1989); or, (3) reproductive assurance mechanisms facilitated seed set in poorly visited inflorescences, as determined with the breeding system experiment above. The results of this study indicate that inadequate pollination only rarely limits seed set in *C. pungens*.

In this study, I found that moisture level had different effects on seed set and seed mass of the F2 generation, depending on the species. For example, in *C. pungens*, moisture level conditions experienced by the parents did not affect seed set and seed mass of the F2 generation. This is in contrast with previous findings for *Fagopyrum esculentum* Moensch (Rering et al., 2020) and *Carthamus tinctorius* L. cv. Kooseh (Soheili-Movahhed et al., 2019), for which researchers found that drought-stressed plants produced fewer seeds. This could be the result from different explanations: firstly, the deep roots of *C. pungens* allow it to access water in the subsoil. Secondly, some of the seed could be from autogamous selfing. Furthermore, previous grassland studies in California have also reported little effect of drought on seed mass (Valliere et al., 2019). These results support the idea that seed mass remains generally one of the most consistent plant traits (Fenner, 1985). For *T. atriplicifolia*, the 50% rain decreased seed mass. This result is in agreement with that reported by Gallagher and Campbell (2021) on *Mertensia ciliata* (James ex Torr.) G. Don., who found that plants in the high rainfall treatment produced heavier seeds than those in the low rainfall treatment.

The results show that for *C. pungens*, the maternal plants under the 50% rain produced the greatest number of non-viable seeds. This pattern was also observed for the F2 seed germination results, which showed that the F2 generation from the plants in the 50% rain had the least germination percentage of the moisture levels. This could be because the maternal plants in the 50% rain had a high concentration of ABA which was passed down to the progeny leading to reduced imbibition of water and eventually decreased embryo growth and development (Wijewardana et al., 2019). It was found that *C. pungens* had a high number of empty seeds in the 150% rain, this could be due to pollinator visitation reaching a peak at intermediate water levels (Gallagher and Campbell, 2017) resulting in a surplus of unfertilised ovules.

In this study, a decreased moisture level experienced by the F1 generation had a negative effect on the F2 seed germination of *C. pungens*. There was no germination of seeds from plants grown in the 50% rain indicating that a reduction of moisture level on the parent plant may reduce seed germination in the F2 generation, even if the F2 seeds are grown under optimum water levels. These results are consistent with those for *Glycine max* (L.) Merr. (Wijewardana et al., 2019) and *Pinus pinaster* Aiton (Cendán et al., 2013) in that germination percentage was greatest in seeds coming from well-watered plants than those from water stressed plants. In general, the germination percentage of seeds decreases as drought stress of maternal plants increases (Chen et al., 2022). This is likely related to the osmotic potential. The effect of osmotic potential on seed germination could be due to reduced imbibition of water and subsequent effects on embryo growth and development (Wijewardana et al., 2019). An alternative explanation, is the high concentration of ABA in water-stressed plants (Liu et al., 2019) compared to well-watered plants suggesting that there was a chance of ABA transfer to the seeds under the 50% rain conditions consequently preventing germination (Chibarabada et al., 2015) by interrupting embryo development just before radicle growth initiation, preventing water absorption which is associated with embryo growth (Schopfer et al., 1979). Germination was also reduced in the 150% rain compared to the 100% rain. For *S. coronatus*, different results were obtained. Seed germination was highest from plants grown in the 50% rain (germination of up to 76%). It could be that this species was not experiencing water stress in the 50% rain. This implies that *S. coronatus* may be tolerant to drought conditions. For *T. atriplicifolia*, the seeds did not germinate from seed collected from plants grown in any of the moisture levels. *T. atriplicifolia* may have a different germination strategy than the other two species. It may be that the seeds require smoke water to germinate as there is evidence that smoke solutions affect the germination of South African mesic grassland species (Ghebrehiwot et al., 2009).

This study found that the response of seedling development to maternal stress varied between *C. pungens* and *S. coronatus*. In this study, the only morphological trait that changed similarly for both species was leaf length. Seedling leaf length was significantly reduced in plants produced from seeds collected from the 50% rain compared to the 100% and 150% rain treatments. These results are in agreement with previous studies showing adaptive strategies of offspring from water-stressed individuals (Herman et al., 2012). By decreasing leaf length, this transgenerational response allows plants to minimise water loss and maintain their internal water balance (Seleiman et al., 2021). No significant response was found for the number of leaves for both species. It is possible that the moisture level experienced by the parent plant was not enough to cause significant changes in leaf production by the F2 generation, or other factors such as nutrients were more important determinants of leaf production.

Contrary to other studies (Hatzig et al., 2018; Herman et al., 2012), seedling dry biomass in this study was significantly reduced in seedlings grown from seed collected at low moisture levels for two forb species (at 50% rain for *S. coronatus* and no germination from 50% rain for *C. pungens*). Rendina González et al. (2018) also found that drought stress in the maternal generation resulted in lower offspring biomass compared to the maternal plants that did not receive water stress. This reduced biomass in the offspring might be due to the reduced growth of the maternal plants in the dry maternal environment. These findings support the results in chapter 3, where plant height was reduced in the 50% rain for both *S. coronatus* and *C. pungens.* The small size of maternal plants can be transferred into the small size of offspring plants due to limited resource availability from maternal plants (Rendina González et al., 2018).

Similarly to patterns of reduced F1 plant heights and F2 seedling biomass, seedlings of *C. pungens* and *S. coronatus* from seed collected from maternal plants grown in drought conditions had reduced plant heights compared to seedlings from the other treatments. These findings suggest that reduced soil moisture levels in the parent generation may have negative transgenerational effects on the fitness of the offspring. One possible explanation for this could be that drought stress experienced during the reproductive stages may have a heritable effect on shoot growth and developmental traits (Wijewardana et al., 2019).

Interestingly, seedlings grown from seed collected under 50% rain showed increased seedling SLA for *S. coronatus*, similar to patterns in the increased number of leaves found in Chapter 3. This species might not have been experiencing water stress under 50% rain, and that the amount of water was within its range of tolerance. For *C. pungens*, there were no treatment effects on the F2 seedling SLA. This may have been partly due to plants getting water through root systems that go deep beyond the subplot (Phillips et al., 2018).

Findings from this study suggest that varying levels of moisture experienced by maternal plants may influence forb seedling growth and germination, and that responses are species-specific. Some species have functional traits that allow them to adapt to water stress, while others experience transgenerational effects on offspring that are not always be positive, reflecting parental responses to water stress.

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5. OVERALL DISCUSSION AND CONCLUSION

This research project aimed to determine the effects of varying moisture levels, specifically drought, on the pollination and reproductive success of three grassland forbs. Pollination and reproductive success were determined by assessing floral and plant traits, flowering phenology, seed set and viability, seed germination, and F2 seedling growth. Previous studies have looked at the effects of drought on grassland forbs in South Africa, but these mainly focused on productivity, resilience and community composition (Siebert et al., 2020).

What prompted this investigation into varying moisture level effects on forb reproduction, was that there is a lack of literature on forb reproductive ecology under climate change, and because the incidence and length of drought events are projected to increase from climate change. Even though, many perennial grassland forbs can persist, even without pollinators or with a reduced seed set because of their ability to resprout from belowground storage organs, understanding how grassland forbs will respond to changes in moisture availability is important because many forbs rely on pollinators for maintaining and increasing genetic diversity through cross-pollination and drought might change floral traits and rewards necessary to attract these pollinators. Reduced pollinator visitation will ultimately affect seed production and seedling development. The persistence of forbs in natural grassland is important as they maintain grassland ecosystem resilience and species richness, and provide forage (Siebert et al., 2022). Many studies investigating the effect of reduced moisture on plant pollination and reproduction have been conducted with crop species. There are very few that have investigated forbs in natural settings, and even less looking at the reproductive biology of South African forbs, particularly the type of pollinators, breeding system, flowering phenology, and transgenerational responses to drought. Understanding the breeding system of a plant species is an essential part of conservation planning because it affects genetic diversity within and among populations (Boscaiu and Güemes, 2001). Knowledge of the types of pollinators and flowering phenology of species is also important because it influences plant-pollinator relationships and ultimately pollination and reproductive success.

Chapter two provided an overview of the current research topics in grassland forb ecology in South Africa, the importance of grassland forbs as well as effects of drought on forb pollination and reproductive ecology. In Chapter three, the effects of varying moisture levels on plant height, number of leaves, flowering phenology, floral density, floral display, plant density and pollinator visitation were investigated. Results showed that the 50% rain treatment decreased plant height for all three focal species. The number of leaves was reduced in the 50% rain only for *T. atriplicifolia*, while this treatment increased the number of leaves for *S. coronatus* and did not affect *C. pungens*. Results also indicated that moisture level had no effect on floral density and floral display for all three species. *Thunbergia atriplicifolia* plant density showed no response to moisture level, but both *S. coronatus* and *C. pungens* experienced decreased plant density under 50% rain. Reductions in plant height and number of leaves are a common response to water stress. In chapter four, the effects of moisture level on seed germination, seed viability, seed set and seedling growth were investigated. Pollen limitation and breeding system were investigated for *C. pungens.* The breeding systems of *T. atriplicifolia* and *S. coronatus* were obtained from literature. There was no of the details of the reproductive biology of *C. pungens* in the literature, and thus this study made a significant contribution to the body of knowledge through the documentation of the breeding system and fruit set of *C. pungens* in a mesic grassland of South Africa. Results showed that *C. pungens* can set limited seed through autogamy but mainly relies on cross pollination. The results for seed germination varied. While there was no germination from seeds produced under 50% rain for *C. pungens*, *S. coronatus* germination was highest from seeds produced under this moisture level. Seeds from *T. atriplicifolia* did not germinate at all, regardless of the moisture level experienced by the parent plants. Similarly, seed mass was not affected by parent plant moisture level for *C. pungens* but increased with increasing parent plant moisture level for *T. atriplicifolia*. Seedlings produced from parent plants experiencing 50% rain exhibited significantly decreased seedling height, biomass, and leaf length for both *S. coronatus* and *C. pungens*. From this chapter, it is deduced that the effects of decreased moisture level experienced by the parent plants on the germination of grassland forbs varies from species to species, and seedling growth may be diminished. The findings of this study can assist in drought mitigation in mesic grasslands of South Africa by providing knowledge on forb responses to drought.

The results show that a decrease in moisture level due to climate change may reduce plant attractiveness and pollen transfer by decreasing plant height, number of visitors per minute and number of flowerheads visited per minute. Our findings suggest that *S. coronatus* is relatively tolerant to decreased moisture levels due to its increase in both the number of leaves and germination in the 50% rain treatment of the maternal plants and may be able to persist in future climates when the soil moisture is lower. Our results also show that a reduction in soil moisture may decrease seed mass for *T. atriplicifolia*, which may decrease seed and seedling quality. It was also found for *C. pungens* that number of leaves, floral density and floral display remained unaffected by different moisture levels possibly due to its tap root system. The development of tap roots is an adaptive strategy for improving plant survival in areas of low water availability. However, the lack of F2 seed germination from plants under the 50% rain for *C. pungens* implies that reduced moisture level may decrease seed quality resulting in a loss of species abundance and possibly local extinction of that species. This result is consistent with the finding of a high number of non-viable seeds from plants in the 50% rain. Nevertheless, moisture level had no effect on seed mass and seed set for *C. pungens* which supports the hypothesis that seed quality is more sensitive to drought stress than yield levels (Hatzig et al., 2018). Furthermore, a decrease in F2 seedling biomass, SLA and leaf length produced from plants under the 50% rain, suggest that a decrease in moisture may have negative transgenerational impacts that might lower plant fitness.

5.1. Challenges/Limitations

This study has not been without its challenges. When it came to data collection, one of the biggest challenges was the low sample size of focal species in each plot. For this reason experiments could not be replicated as much as planned, and other responses such as pollen production, nectar volume and nectar sugar concentration could not be measured without destructive sampling and thus lowering the samples sizes further. The low sample size also resulted in issues during data analysis, such as multicollinearity, not being able to perform statistical tests on some variables, and possibly non-significant results (especially pollen limitation studies) caused by Type II errors, so models were compared, random effects were removed, and the best models based on AIC values were chosen. This also meant that there were not enough seeds of *T. atriplicifolia* to test for treatment effects on response variables (e.g., seed viability and pollen limitation), meaning that this species was not as well represented in this study as the others. Another challenge that was identified was the small flowers of *C. pungens* and *S. coronatus* and fragile flowers of *T. atriplicifolia* that made it almost impossible to draw up nectar for nectar volume and sugar concentration measurements.

5.2. Future Work

Key goals of future research into grassland forb ecology should be to identify 1) rapid and effective mechanisms for breaking seed dormancy in grassland forbs and the influence of climate change on breaking seed dormancy in field experiments, 2) the most important pollinators in these systems, and 3) assessing microclimatic variability on seed germination.

This information will assist with efforts to understand forb ecology. Additional studies looking at the effect of varying moisture levels on pollen production/viability, heterospecific pollen transfer and nectar production would be valuable to understand how drought may affect floral rewards and pollination efficiency and thus pollinator attraction. Future studies should consider 1) investigating how varying moisture level may affect pollinator abundance, diversity, floral scent, floral colour, and pollen transfer in grasslands, 2) looking at interaction effects on response variables (e.g., moisture level and temperature, moisture level and nutrients or moisture level and fire) since these factors may have synergistic effects (Koerner and Collins, 2014), 3) investigating the combined effects of reduced rainfall and fire exclusion on the sexual reproduction success of grassland forbs, and lastly, it would be profitable to repeat similar experiments in greenhouse and laboratory conditions under controlled conditions, with larger sample sizes, so as to not depend on natural densities in the grassland and in the experimental plots, in order to investigate more delicate measures such as pollen and nectar production and thus reducing risks related to field experiments.

5.3. References

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